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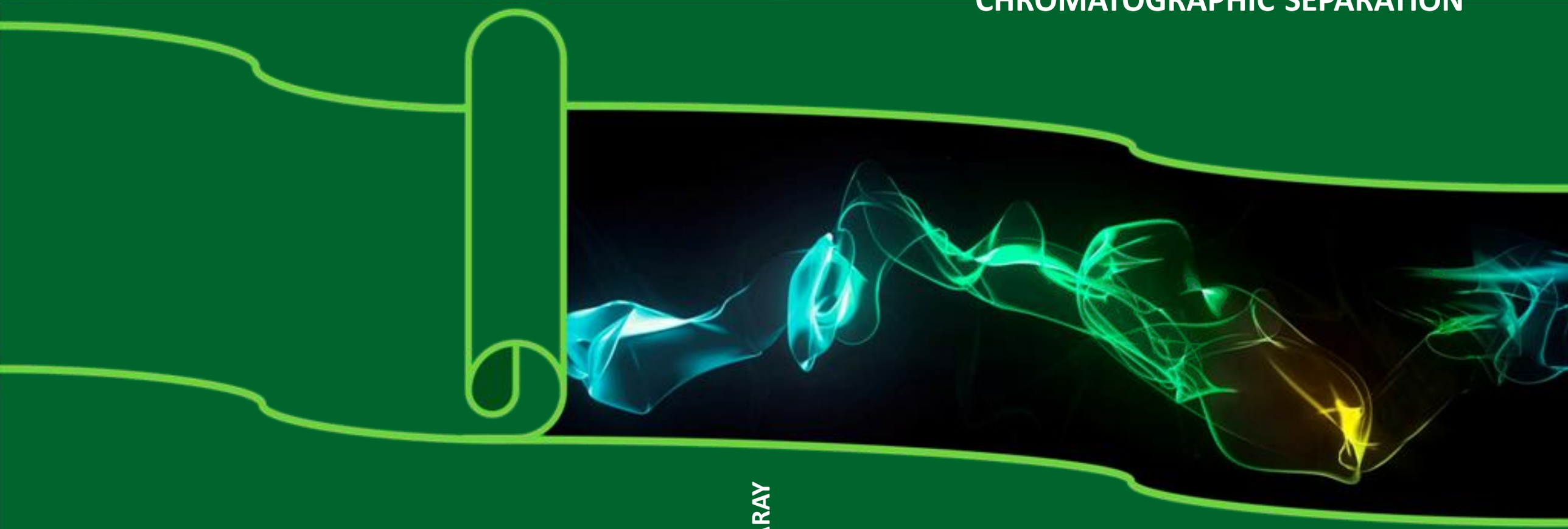
PhD Thesis

DEVELOPMENT OF PROCEDURES FOR THE TRIAZOLE FUNGICIDES DETERMINATION IN FRUITS AND LIQUID SAMPLES USING MICROEXTRACTION TECHNIQUES AND CHROMATOGRAPHIC SEPARATION



ANE BORDAGARAY
2015

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Kimika Aplikatua Saila

DOKTOREGO TESIA

Mikroerauzketa teknika eta banaketa kromatografikoan oinarritutako prozeduren garapena triazol fungiziden determinaziorako fruitu eta likido laginetan

Development of procedures for the triazole fungicides determination in fruits and liquid samples using microextraction techniques and chromatographic separation

ZUZENDARIAK: **ROSA GARCIA ARRONA**

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Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

-Albert Einstein-

Amatxori,

momentu zailenetan

beti hor egoteagatik

Esker onak

Ikerketa lan hau aurrera eramaten lagundu duten Gipuzkoako Foru Aldundia eta Euskal Herriko Unibertsitateari azaldu nahi dizkiet eskerrak, beraien laguntza ekonomikoa ezinbestekoa izan baita urte hauetan.

Nire eskerrik beroenak nire zuzendari Rosa Garcia eta Esmeralda Millán irakasleei ere. Zuen gidaritzapean ikasitakoak eta aholkuak aurrerantzean jarraituko ditudan bideetan baliagarri izango zaizkidalako. Mila esker une zailetan erakutsi didazuen pazientzia eta ulermenagatik.

I would also thank to Rasmus Bro and Jose Amigo from the University of Copenhagen. Thanks to introduce me to the huge chemometric world and to give the opportunity to meet amazing people in Copenhagen! Ainara eta Jose! Ea Master Brew batzuekin laster topa egiteko aukera dugun!

Analitikako talde osoari, lehen egunetik etxekotzat hartzeagatik: Carlos, Miren, Iñaki, Maider eta nola ez laborategiko lagun eta lankide guztioi, gaur egungoak eta hemendik pasatako denak. Mila esker zuekin pasatako une on horiengatik. Batez ere Jessica eta Itxasori, zuen laguntasunagatik eta behar izan dudanean entzuteagatik.

Bereziki nire sostengu izan den Juanillori, hasieratik edozertan laguntzeko prest egoteagatik eta momentu pertsonal gogorretan nire ondoan egoteagatik. Baina batez ere, azken txanpa honetan eman didazun indarragatik. Berriz nire burua aurkitzen laguntzeagatik eta ilusioa berreskuratzea zer den erakusteagatik.

Honaino iristeko ezinbestekoa izan dudan laguntzari: mila esker nire familiari, Aita, Ama, eta nire ahizpa Maialeni. Nahiz eta hau zer den oso ondo ez ulertu, zuen babesa izan dudalako. Eta nola ez, nire sorgintxo txikiari! Mila esker Ihintza, zure irribarreagatik!

Eskerrik asko urte hauetan nire ondoan egon zareten guztioi eta behar zaituztedanean hor zaudetela erakutsi didazuelako kuadrilla! Aupa KR!

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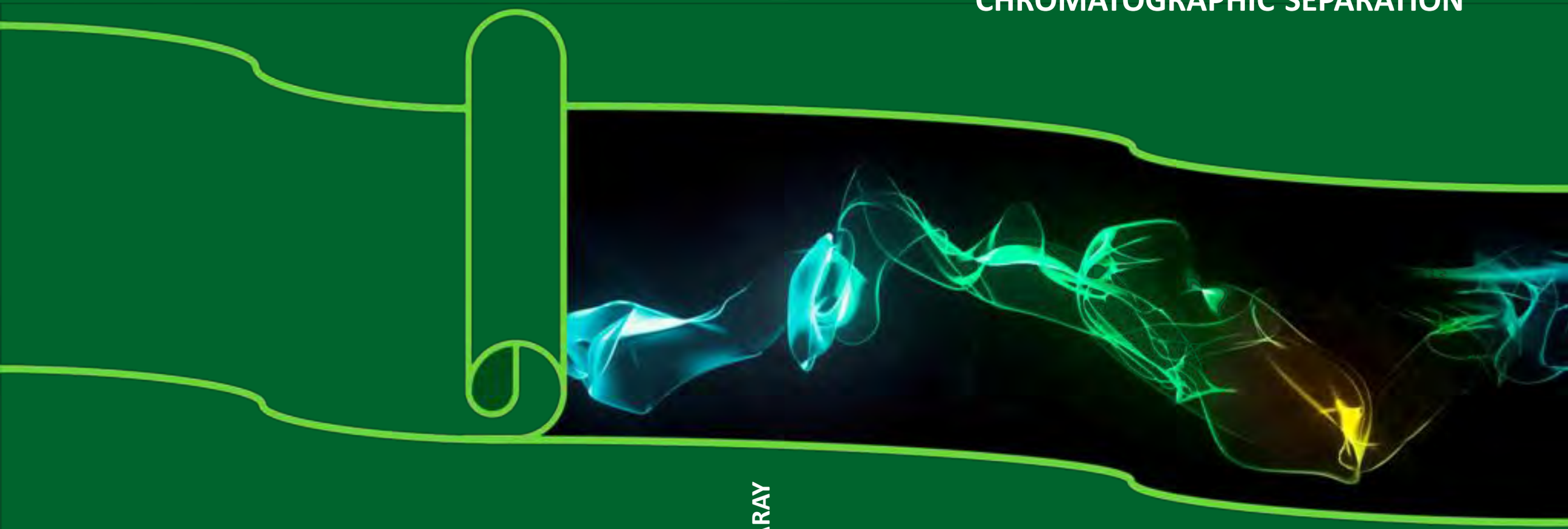
PhD Thesis



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Applied Chemistry Department

PhD THESIS

Development of procedures for the triazole fungicides determination in fruits and liquid samples using microextraction techniques and chromatographic separation

ADVISORS: **ROSA GARCIA ARRONA**

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Summary

Summary

Agricultural crops can be damaged by funguses, insects, worms and other organisms that cause diseases and decrease the yield of production. The effect of these damaging agents can be reduced using pesticides. Among them, triazole compounds are effective substances against fungus; for example, *Oidium*. Nevertheless, it has been detected that the residues of these fungicides in foods as well as in derivate products can affect the health of the consumers. Therefore, the European Union has established several regulations fixing the maximum residue of pesticide levels in a wide range of foods trying to assure the consumer safety.

Hence, it is very important to develop adequate methods to determine these pesticide compounds. In most cases, gas or liquid chromatographic (GC, LC) separations are used in the analysis of the samples. But firstly, it is necessary to use proper sample treatments in order to preconcentrate and isolate the target analytes. To reach this aim, microextraction techniques are very effective tools; because allow to do both preconcentration and extraction of the analytes in one simple step that considerably reduces the source of errors.

With these objectives, two remarkable techniques have been widely used during the last years: solid phase microextraction (SPME) and liquid phase microextraction (LPME) with its different options. Both techniques that avoid the use or reduce the amount of toxic solvents are convenient coupled to chromatographic equipments providing good quantitative results in a wide number of matrices and compounds.

In this work simple and reliable methods have been developed using SPME and ultrasound assisted emulsification microextraction (USAEME) coupled to GC or LC for triazole fungicides determination. The proposed methods allow confidently determine triazole concentrations of $\mu g\ L^{-1}$ order in different fruit samples.

Chemometric tools have been used to accomplish successful determinations. Firstly, in the selection and optimization of the variables involved in the microextraction processes; and secondly, to overcome the problems related to the overlapping peaks. Different fractional factorial designs have been used for the

screening of the experimental variables; and central composite designs have been carried out to get the best experimental conditions. Trying to solve the overlapping peak problems multivariate calibration methods have been used. Parallel Factor Analysis 2 (PARAFAC2), Multivariate Curve Resolution (MCR) and Parallel Factor Analysis with Linear Dependencies (PARALIND) have been proposed, the adequate algorithms have been used according to data characteristics, and the results have been compared.

Because its occurrence in Basque Country and its relevance in the production of cider and txakoli regional wines the grape and apple samples were selected. These crops are often treated with triazole compounds trying to solve the problems caused by the funguses. The peel and pulp from grape and apple, their juices and some commercial products such as musts, juice and cider have been analysed showing the adequacy of the developed methods for the triazole determination in this kind of fruit samples.

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List of abbreviations

Abbreviation	Definition
AALLME	Air assisted liquid liquid microextraction
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
CCD	Central composite design
CPE	Cloud point extraction
D	Diniconazole
DI	Direct immersion
DLLME	Dispersive liquid liquid microextraction
DSDME	Directly-suspended droplet microextraction
ECD	Electron capture detector
FS	Flusilazole
FQ	Fluquinconazole
GBMNE	Graphene-based magnetic nanoparticles extraction
GC	Gas chromatography
GC/ECD	Gas chromatography-electron capture detector
GC/FID	Gas chromatography-flame ionization detector
GC/FID/MS	Gas chromatography-flame ionization detector/mass spectrometry
GC/MS	Gas chromatography-mass spectrometry
GC/MS/MS	Gas chromatography-tandem mass spectrometry
GC/NPD	Gas chromatography-nitrogen phosphorous detector
HF-LPME	Hollow fiber liquid phase microextraction
HPLC	High-performance liquid chromatography
HPLC/DAD	High-performance liquid chromatography-diode array detector
HPLC/MS	High-performance liquid chromatography-mass spectrometry
HPLC/MS/MS	High-performance liquid chromatography-tandem mass spectrometry
HPLC/UV	High-performance liquid chromatography-ultraviolet detector
HS	Headspace
IL-DLLME	Ionic liquid-dispersive liquid liquid microextraction
M	Myclobutanil
MCR	Multivariate curve resolution
MRL	Maximum residue limits
MSPE	Magnetic solid phase extraction
PA	Polyacrilate
PARAFAC	Parallel factor analysis
PARALIND	Parallel factor analysis with linear dependencies
PCA	Principal component analysis
PDMS/DVB	Polydimethylsiloxane/divinylbenzene
PLE	Pressurized liquid extraction

Abbreviation	Definition
RSM	Response surface methodology
SBSE-DLLME	Stir bar sorptive extraction- dispersive liquid-liquid microextraction
SDME	Single drop microextraction
SFO	Solidification of floating organic drop
SPE	Solid-phase extraction
SPME	Solid phase microextraction
SEV-DLLME	Silylated extraction vessel-dispersive liquid-liquid microextraction
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
TB	Tebuconazole
ToF	Time of flight detector
TT	Tetraconazole
UAE	Ultrasound assisted extraction
USAEME	Ultrasound-assisted emulsification microextraction
USAEMME	Ultrasound-assisted emulsification magnetic microextraction
UPLC-MS/MS	Ultra performance liquid chromatography-tandem mass spectrometry

1. Introduction

1. Introduction

Fruits are essential foods in human diet in order to get enough vitamins and minerals. The big demand of this food leads to the growers of crops the use of different kind of pesticides in order to protect and increase their production to satisfy the needs of the society. Some studies have shown that the use of pesticides can effectively reduce the plant diseases [Scherm *et al.*, 2009] as well as the delay the oxidative process suffered by the plants delaying senescence and increasing the crop yield [Zhang *et al.*, 2010]. The treated fruits are taken by the consumers in different forms, such as the vegetable itself or processed products as wines or beverages. Consequently it is necessary to assure that the crops are safe for the consumers, because pesticide residues or its metabolites can lead to health problems. Pesticide residues or its metabolites can be found in the fruits and therefore, it is necessary a method to control the presence of the harmful agents.

There is a classification within pesticides depending on its field of action: insecticides fight against insect that can damage the crop; herbicides control the undesirable plants growing near the plant; fungicides control the fungus diseases damaging the crop; or bactericides, acaricides and algaecides that fight against specific microorganisms. Apart from other agents, such as plant growth regulators used as growth promoters [Tomlin, 2000].

1.1 Triazole pesticides

Triazole compounds are one of the most common groups of pesticides to fight against fungus diseases widely applied to crops. In this perspective, two effects can be seen regarding the use of the fungicides: the effect on health (those effects are specially studied in rats and mice) and the effect on the beverage making process.

The mode of action of these azole fungicides is the inhibition of ergosterol, an essential component in fungal cell membranes, by influencing the cytochrome P450 enzyme activity [Ekman *et al.*, 2006]. Many studies have been carried out concerning

these pesticides effects. Among others, tumorigenic effects [Wolf *et al.*, 2006], endocrine disrupting effects [Goetz *et al.*, 2007] and disturbances in reproductive systems [Taxvig *et al.*, 2007] have been shown in rats, as well as, can induce some tumors in livers of mice [Allen *et al.*, 2006]. Besides, the carryover of the analytes can hazard also environment, such as aquatic communities close to the crops that have been treated with some pesticides increasing mortality rates of *Daphnias* (tiny crustaceans), affecting also the plant growth and causing anticholigernic and oxidative stress in transplanted fishes [Echeverría-Sáenz *et al.*, 2012].

The pesticides are commonly used in vineyard and apple orchards which are used to produce fruits for beverage making. It has been demonstrated that triazole fungicides can affect the beermaking processes causing sluggish and even stuck of fermentation [Navarro *et al.*, 2011]; especially if the octanol/water partition coefficient (K_{OW}) of analyte is higher than 2, because in that case the pesticides can remain on the malt [Navarro *et al.*, 2007]. And also it had been also studied that tebuconazole can affect in the resultant aromas of wine [Noguerol-Pato *et al.*, 2011]

The analytes studied in this work exceed the mentioned limit of K_{OW} as it can be seen in table 1.1. This table shows the physico-chemical properties and the molecular structure of the analytes investigated in this thesis: diniconazole (D), fluquinconazole (FQ), flusilazole (FS), myclobutanil (M), tebuconazole (TB) and tetraconazole (TT). According to the pesticide manufacturers, these pesticides can remain in the fruit for a period and therefore the pre-harvest security period established can be up to a month for some cases. Furthermore, the superficial run-off is not able to take out easily the pesticides and those can remain strongly adsorbed to the soils. Nevertheless, soil column leaching studies and leaching models demonstrate that the pesticides will not leach to deeper soil layers and contaminate the groundwater [Tomlin, 2000].

Table 1.1. *Structures and physico-chemical properties of the studied triazole fungicides.*

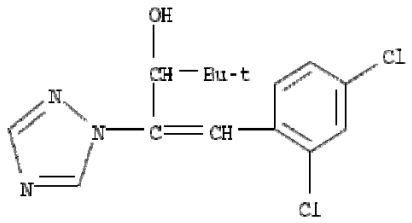
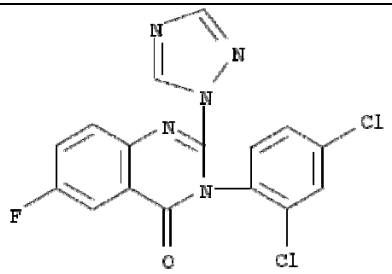
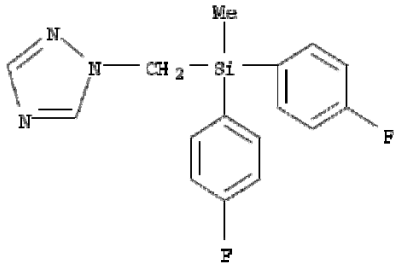
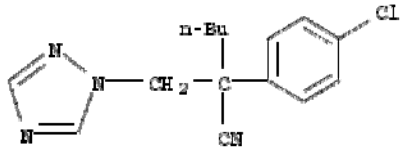
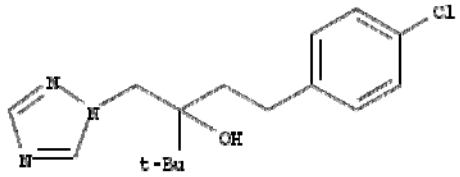
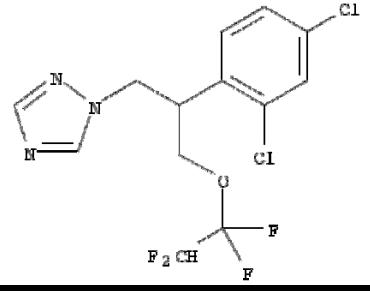
Name [CAS No.]	***Formula / M_w (g mol^{-1})***	***Structure***	***K_{ow} log P***	***Henry constant (Pa m^3 mol^{-1})***	***Solubility in water (mg L^{-1})***
Diniconazole (D) [70217-36-6]	*$C_{15}H_{17}Cl_2N_3O$ / 326.2*		*4.3 (25 °C)*	*$4.0 \cdot 10^{-2}$*	*4 [95 in methanol (g kg^{-1})]*
Fluquinconazole (FQ) [72850-64-7]	*$C_{16}H_8Cl_2FN_5O$ / 376.2*		*3.24 (pH 5.6)*	*$2.1 \cdot 10^{-6}$*	*1 [$3 \cdot 10^{-3}$ in ethanol]*
Flusilazole (FS) [85509-19-9]	*$C_{16}H_{15}F_2N_3Si$ / 315.4*		*3.74 (pH 7, 25 °C)*	*$2.7 \cdot 10^{-4}$*	*45 (pH 7.8) [$> 2 \cdot 10^{-6}$ in many organic solvents]*

Table 1.1 (continuation). *Structures and physico-chemical properties of the studied triazole fungicides.*

Name [CAS No.]	***Formula / M_w (g mol^{-1})***	***Structure***	***K_{ow} log P***	***Henry constant (Pa m^3 mol^{-1})***	***Solubility in water (mg L^{-1})***
Myclobutanil (M) [88671-89-0]	*$C_{15}H_{17}ClN_4$ / 288.8*		*2.94 (pH 7-8, 25 °C)*	*$4.3 \cdot 10^{-4}$*	*142 [$5\text{-}10 \cdot 10^{-4}$ in common organic solvents]*
Tebuconazole (TB) [107534-96-3]	*$C_{16}H_{22}ClN_3O$ / 307.8*		*3.7 (20 °C)*	*$1.0 \cdot 10^{-5}$*	*36 (pH 5-9, 20 °C) [$5\text{-}10 \cdot 10^{-4}$ in toluene]*
Tetraconazole (TT) [112281-77-3]	*$C_{13}H_{11}Cl_2F_4N_3O$ / 372.1*		*3.56 (20 °C)*	*$3.6 \cdot 10^{-4}$*	*156 (pH 7, 20 °C) [Readily soluble in methanol]*

Data from the Pesticide Manual [Tomlin, 2000]

Because of the potential health risks to consumers due to continuous exposure of fruits and vegetables, European Union (EU) has published regulation to establish maximum residue limits (MRLs) for a wide range of pesticides in different food types. First regulations about MRLs were published in 2005 [Regulation EC 396/2005], but the regulations have changed and the MRLs have been updated. The newest regulation and the limits for the studied fungicides in this work are shown in table 1.2. These limits are ranging from 0.01 to 1 mg kg^{-1} depending on the fruit type and the analyte.

Table 1.2. *Updated MRLs allowed for apple and wine grapes according to European Regulations.*

Analyte	Fruit	MRL (mg kg^{-1})	Regulation
Fluquinconazole (FQ)	**Apple**	0.1	[Regulation EC 149/2008]
	Wine Grape	0.5	
Myclobutanil (M)	**Apple**	0.5	[Regulation EC 149/2008]
	Wine Grape	1	
Flusilazole (FS)	**Apple**	0.02	[Regulation EC 459/2010]
	Wine Grape	0.2	
Tetraconazole (TT)	**Apple**	0.3	[Regulation EU 34/2013]
	Wine Grape	0.5	
Diniconazole (D)	**Apple**	0.01	[Regulation EU 1317/2013]
	Wine Grape	0.01	
Tebuconazole (TB)	**Apple**	0.3	[Regulation EU 61/2014]
	Wine Grape	1	

Thus, is necessary to develop methods to determine the pesticide residues remaining in the fruit and therefore establish the security period before harvesting the crops to assure the security in food.

1.2 Extraction techniques

Analytical procedures are developed in order to get information about substances in many different environments or matrices. These procedures involve many steps: sampling, sample preparation, separation, quantification and data analysis. Sample preparation is the bottleneck for the effective and accurate analysis of trace pesticide analysis, not only for preconcentration issues, but also for isolate from various complex matrices as much as possible [Zhang *et al.*, 2012b].

Different techniques for sample preparation have been used. Determinations by chromatographic methods have been mainly selected due to the possibility to separate and quantify many substances at once. But the complexity of some matrices and its impurities can make the analysis very tedious. Apart from that, different techniques have been used for the isolation of target analytes and to preconcentrate them, because the concentration levels found in the samples may not be enough to detect [Ridgway *et al.*, 2007; Gilbert-López *et al.*, 2009; Zhang *et al.*, 2012b].

Among other techniques, during past years, extraction techniques have evolved in order to miniaturize the former methods such as liquid-liquid extraction (LLE) or solid phase extraction (SPE). These techniques, based on the solubility of analytes in organic solvents and their partition coefficient, have been used during a long period due its potential to preconcentrate the analytes before analysis.

Probably LLE might be the oldest and the most common extraction method [Zhang *et al.*, 2012b]. LLE is based on the equilibrium distribution/partition coefficient between two immiscible liquids (acceptor and donor phases). Extraction of an analyte is achieved by the differences in polarity of the phases. Usually is helped with an agitation which increases the contact surface between the two phases in order to facilitate the transfer of molecules from one phase to the other. After the agitation, the two immiscible phases are separated back into two phases one above the other. At this point the two phases are separated with a separating funnel and the phase containing the analytes is analyzed. Sometimes emulsions can be formed, but this can be solved adding salt or centrifugating to separate the two phases [Ridgway *et al.*, 2007]. However, this technique is laborious and time- and solvent-consuming, which is not advisable from the green chemistry point of view.

SPE is based on the selective distribution of analytes between the solid packing material and liquid mobile phase. A solution is passed through the cartridge where the analytes of a high affinity with the stationary phase are caught. The solid phase has been previously conditioned and activated with water or organic solvent. The interferences are removed by pre-washing by organic solvents while the analytes are retained on the absorbent and then, another solution is passed through to elute the analytes trapped to its consequent analysis [Zhang *et al.*, 2012b]. However, limited efficiencies caused by insufficient retention, can be observed for some analytes. Nevertheless, SPE have lately evolved developing many different sorbent types: reversed-phase (C8, C18); normal-phase (silica, alumina), ion exchange phase or functionalized phase [Buszewski and Szultka, 2012].

However, these techniques need important amounts of solvents and resources that make the procedure tedious and difficult to automatize. Apart from the fact that green chemistry supports the idea of reducing the waste, many solvents using in LLE and SPE are difficult to treat after disposal because its toxicity. Moreover, these techniques are performed in many steps and that often results in increasing error sources due to the loss of analytes. Consequently miniaturized techniques started to develop. The so-called microextraction techniques began with solid phase microextraction in the 90's and years later the liquid phase microextraction techniques were showed up into the scientific community. Microextraction techniques are characteristics because its little volume of the extractant phase in microlitres volume scale.

1.2.1 Solid- Phase Microextraction (SPME)

SPME was first introduced by Pawliszyn in 1990 [Arthur and Pawliszyn, 1990] as an alternative to the traditional sample preconcentration techniques. This technique is based on a polymeric fiber which is exposed to a sample to extract the analytes (see figure 1.1). Afterwards analytes are desorbed either thermically or with the help of solvents. The technique preconcentrate and extract analytes in one step allowing saving time and consequently reducing error sources. Desorption is usually placed in chromatographic techniques, where the separation and quantification is performed.

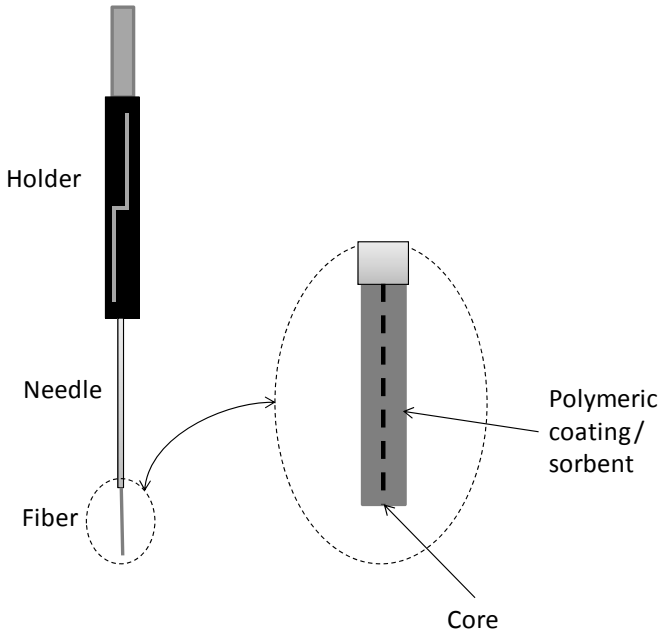


Figure 1.1. *Scheme of a commercial fiber and its holder.*

The fibers can be exposed in three different operation modes [Pawliszyn, 1997]: immersion (DI), headspace (HS) or membrane-protected approach. Depending in the volatility of analytes and the matrix type the appropriate mode will be chosen. In the immersion mode, the fiber is introduced directly to the liquid sample. In the headspace mode, the fiber is placed in the closed space above the liquid. This operational mode is highly recommended for volatile analytes, avoiding direct contact with the matrix that make possible a larger life of the fiber. The third mode is recommended when dirty samples are analyzed. The purpose of the membrane barrier is to protect the fiber against damage caused by interferences of the matrix.

1.2.1.1 SPME principle

Microextraction process is considered finished when the equilibrium is reached between the sample and the fiber. If only two phases are involved (as in the immersion) the mass conservation law can be described with the Equation 1.1 [Pawliszyn, 1997].

$$C_0 \cdot V_s = C_s^{eq} \cdot V_s + C_f^{eq} \cdot V_f \quad (1.1)$$

Where the *C* indicates initial concentration when the subscript is *0*, and sample and fiber concentration of analyte when the subscripts are *s* and *f* respectively. *Eq* superscript reflects the values in the equilibrium. V indicates the volume of the sample and the fiber.

Then the distribution coefficient (K_{fs}) of the analyte can be defined as:

$$K_{fs} = \frac{C_f^{eq}}{C_s^{eq}} \quad (1.2)$$

As a result of rearranging Equation 1.1 and 1.2:

$$C_f^{eq} = C_0 \cdot \frac{K_{fs}V_s}{K_{fs}V_f + V_s} \quad (1.3)$$

Considering the mole amount the equation is transformed:

$$n = C_f^{eq} \cdot V_f = C_0 \cdot \frac{K_{fs}V_s}{K_{fs}V_f + V_s} \cdot V_f \quad (1.4)$$

Taking into account the small size of the fiber, the term $K_{fs}V_f$ can be ignored comparing with the term V_s. Hence, the equation is simplified as:

$$\boldsymbol{n = C_0 \cdot K_{fs} \cdot V_f} \quad (1.5)$$

Therefore, the extracted mole amount is directly proportional to the initial concentration of the sample; because the fiber volume and partition coefficients are constant. Other factors such as the sample volume does not influence in the extraction process.

The evolution of equation in headspace mode include a new term corresponding to the headspace phase, however the final results is comparable to the equation 1.5 and equally the amount of analyte extracted is proportional to the initial concentration as indicated in following equations.

$$C_0 \cdot V_s = C_s^{eq} \cdot V_s + C_f^{eq} \cdot V_f + C_h^{eq} \cdot V_h \quad (1.6)$$

In this case two distribution constants are involved due to the fact that in HS-SPME three phases are included (subscript *h* indicates the headspace phase).

$$K_{hs} = \frac{C_h^{eq}}{C_s^{eq}} \quad (1.7)$$

$$K_{fh} = \frac{C_f^{eq}}{C_h^{eq}} \quad (1.8)$$

$$K_{fs} = K_{fh} K_{hs} \quad (1.9)$$

After the consequent transformations the equation results as:

$$\boldsymbol{n = \frac{K_{fs} V_f V_s}{K_{fs} V_f + K_{hs} V_h + V_s} \cdot C_0} \quad (1.10)$$

Whether the method is, immersion or headspace, the extracted amount is proportional to the initial concentration of the analyte in the sample. Therefore, the extracted amount is independent to the location of the fiber as long as the volume including in the extraction (sample volume, headspace and fiber volume) are kept constant.

1.2.1.2 Factors affecting the SPME process

Many different variables affect the SPME extraction process. Depending on them, extraction could be very successful or not. Although the extraction is directly proportional to the initial concentration, there are several variables to consider [Peñalver *et al.*, 1999]. These factors need to be optimized and fixed to make

reproducible and precise determinations of the samples. Some important variables are described below:

- **Type of coatings**

There are many commercially available fibers [Pawliszyn, 2009] with different coating, thickness, polarities and extraction mechanism. Polyacrylate (PA), polydimethylsiloxane (PDMS), and divinylbenzene (DVB) sorbent materials and its combinations are the most used ones (see figure 1.2).

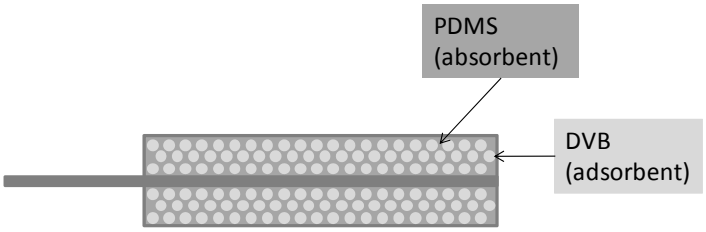


Figure 1.2. *Structure of a PDMS/DVB coating.*

The absorption and/or adsorption occurs depending on the polymeric coating. For example, PDMS fiber is based on absorption, while DVB mainly adsorbs the analyte. The amount extracted in absorption materials depends on the thickness of the fiber. The thicker fibers will extract bigger amounts of analytes, but also need more time. These coatings are "liquid-like" polymers, which have fluid properties. Analytes migrate through the coating, and the retention is based on the thickness of the fiber and its polarity.

On the other hand, the adsorbent material efficiency will depend on the surface area, the amount of porosity and the pores size. The interactions in these cases between the analyte and the adsorbent are intermolecular interactions such as, pi-pi (π-π) bonding, hydrogen bonding or van der Walls interactions.

Fiber coating is choosing depending on the nature of analytes. Polarity and volatility are the crucial factors (see figure 1.3). PDMS is a non-polar phase which in combination with DVB forms a bipolar coating complementing the properties to use in a variety of analytes.

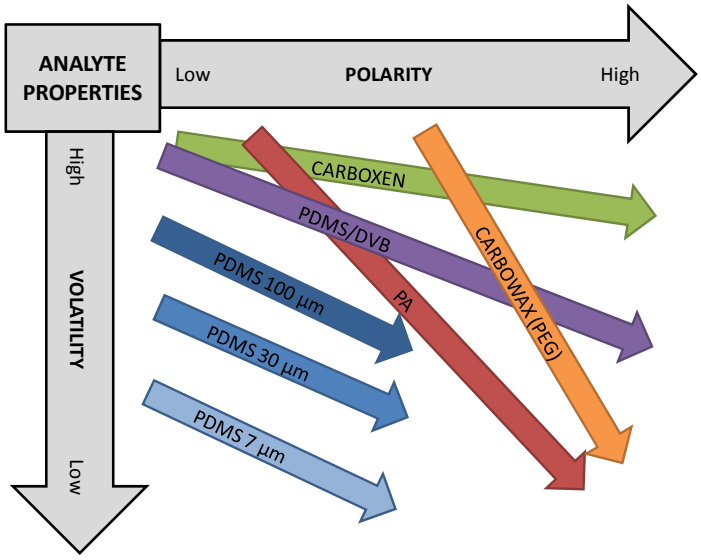


Figure 1.3. *Commercially available fiber coatings guide.*

There are several commercially available coatings. Table 1.3 shows the coatings available by Sigma-Aldrich (Madrid, Spain) and the usage guidelines provided to assure the correct use of the fibers.

PDMS/DVB is widely used in a variety of different analytes and matrices: halogenated fungicides [Millán *et al.*, 2003] and ochratoxin A [Aresta *et al.*, 2006] in wine; various pesticides in strawberries [Wang *et al.*, 2000] and tomatoes [Ravelo-Pérez *et al.*, 2008], pyrethroids in cucumber and watermelon [Parrilla-Vázquez *et al.*, 2008], carbendazim and thiabendazole in apples [Hu *et al.*, 2008], pesticide residues in bovine milk [Fernandez-Alvarez *et al.*, 2008] and strobilurin fungicides in baby foods [Viñas *et al.*, 2009]. Environmental samples also have been analyzed by PDMS/DVB coatings: fenitrothion and its metabolites in environmental waters [Sánchez-Ortega *et al.*, 2005]; organochlorine pesticides in agricultural soils [Vega Moreno *et al.*, 2006]; and different pesticide types in water samples [Beceiro-González *et al.*, 2007; Mmualefe *et al.*, 2009].

Table 1.3. *Fibers usage guidelines provided by the manufacturer.*

Fiber Coating	Film Thickness (µm)	pH range	Maximun Temperature (°C)	Recommended Operating Temperature (°C)
PDMS	100	2-10	280	200-280
	30	2-11	280	200-280
	7	2-11	340	220-320
PDMS/DVB	65	2-11	270	200-270
PA	85	2-11	320	220-300
Carboxen/PDMS	75	2-11	320	250-310
PEG	60	2-9	250	200-250
DVB/CAR/PDMS	50/30	2-11	270	230-270

- **Extraction time**

The extraction time has to be established. The extraction is non exhaustive in SPME, but equilibrium can be easily reached in short times depending on the nature of the analyte and coatings. Time has to be controlled to make the analysis reproducible and quantitatively satisfactory.

- **Extraction temperature**

It is necessary to optimize the temperature because two opposite effects are involved in the process [Abdulra'uf *et al.*, 2012]. Temperature has normally a positive effect on the extraction because temperature enhances the diffusion coefficients of analytes. For HS-SPME analytes is especially helpful because it helps the transfer of analytes from the liquid to the gaseous phase increasing the vapor density [Aulakh *et al.*, 2005]. In the case of direct immersion temperature can also help the mass transfer. On the other hand, the partitioning of the analytes into the coating fiber is exothermic; therefore an excessive increase of the temperature can cause a decrease in the distribution constant leading to lower extractions.

- **Salt addition**

Addition of salt may be advisable because increases the ionic strength of the solution making the organic compounds less soluble in the aqueous phase and increasing the partition coefficient [Aulakh *et al.*, 2005]. Too much salt may have a

negative effect though, the matrix can be saturated and the undissolved salt may occupy the active sites in the coating. Therefore a cleanup step should be considered after each analysis, because the crystallization of the salt can damage the fiber reducing its lifetime. The most used salts are sodium chloride or sodium sulphate.

- **pH effect**

pH may be important for slightly acid or basic compounds. Analytes should be in the undissociated form for extraction and therefore if the analytes are in their dissociated form the extraction efficiency will decrease. In these cases, adjusting pH molecules acquired the undissociated form and consequently fibers coatings extract the analytes.

However, especially in DI-SPME pH should be controlled because fibers can be damaged with very acidic or very basic pH. In HS-SPME, pH range could be larger because there is no direct contact between the sample and the fiber. Manufacturers provide the guidelines for the pH range (table 1.3).

- **Agitation**

Agitation helps the mass transfer and reduces the diffusion layer [Beltran *et al.*, 2000], therefore to achieve faster equilibrium. Nevertheless agitation should be controlled to avoid bubbles and fluttering of the magnetic stirrer. There are various agitation methods in SPME: magnetic stirring, vortex technique, fiber agitation, flow trough and sonication. Magnetic stirring is the most used in SPME, but other methods should be considered depending on the matrix and/or analytes [Alpendurada, 2000].

- **Sample volume and headspace volume**

Mainly when headspace mode used, is important to optimize the sample/headspace volume. Although the sample volume is not a crucial fact looking at the equations written above referring to immersion, these volumes need to be fixed and kept constant during a batch of analyses.

- **Desorption**

Desorption depends on the method following the extraction. Gas chromatography (GC) was firstly used for this purpose, but other techniques such as

high performance liquid chromatography (HPLC) [Lord, 2007] and capillary electrophoresis (CE) [Kumar and Malik, 2009] also have been used during past years.

Desorption in a GC occur in the injector equipped with a SPME suitable liner. Larger volume liners can results in a tailing of peaks. Injector is established in high temperatures, and analytes are thermally desorbed. The coatings maximum allowed temperatures have to be considered in order to avoid fiber damage (table 1.3).

In case of HPLC desorption is made in a desorption chamber that is linked to an injector and fiber is introduced to the chamber. There are two desorption types: the dynamic and the static. In the dynamic mode, the mobile phase of the HPLC is continuously sweeping the analytes while in the static mode, desorption chamber is filled with the mobile phase and kept during the time established without flow. Static desorption is more recommended when the analytes are strongly attached to the coating [Kataoka *et al.*, 2000; Aulakh *et al.*, 2005].

There is some limitation when using solvents for desorption, certain solvents can produce swelling in the fiber damaging the coating when the fiber is retracting in the needle after exposure of solvents. Consequently and as the supplier indicates solvents such as methanol are not recommended to use.

Optimization of these variables is important, because most of the time is not necessary to reach the equilibrium in the process. Quantification is feasible before reaching the equilibrium [Ai, 1997] but is important to fix the variables involved in the process to achieve good precision and accuracy in the method.

Next table 1.4 shows some of the advantages and disadvantages of the SPME technique [Alpendurada, 2000; Kataoka *et al.*, 2000; Pawliszyn and Pedersen-Bjergaard, 2006; Spietelun *et al.*, 2013]. It is remarkable that is a solvent free extraction technique and the availability of different coating types make the method adequate for many different analyte types. On the other hand, the main disadvantage is that the fibers are quite sensitive and can be broken very easily if there are not carefully managed.

Table 1.4. *Advantages and disadvantages of SPME extraction method.*

SPME	
Advantages	***Disadvantages***
• *Solvent free extraction.* • *Fast (comparing with classical LLE, SPE like techniques).* • *Different available fiber coatings.* • *Simplicity of operation.* • *Possibility to automatize.*	• *Fragile fibers.* • *Expensive.* • *Batch to batch differences.* • *Necessary conditioning.* • *High molecular mass compounds irreversibly adsorbed to the fiber.* • *Formation of gas bubbles (sometimes difficult to prevent) affects the mass transfer.* • *Need clean up steps after analysis, because the possible carry over and crystallization of salt content, especially when immersion mode was used.*

1.2.2 Liquid Phase Microextraction (LPME)

To overcome the drawbacks of SPME and other techniques, a method based on a tiny droplet (microlitre scale) of an organic extractant was developed. The method, started developing in 1996 [Jeannot and Cantwell, 1996] that firstly introduced the idea of miniaturizing the classical liquid liquid extraction (LLE) in a water-immiscible organic droplet. In few years the method evolved in many different branches. Basically, the droplet instead of the fiber is put in the contact with the sample (directly in the sample or in headspace) and then the droplet is collected with a variety of techniques.

Liquid phase microextraction (LPME) is also based on equilibrium between the donor and acceptor phase. The extracted amount in the organic droplet will be proportional to the initial concentration as indicates the equation 1.11 [Jeannot and Cantwell, 1996]:

$$\boldsymbol{C_d^{eq} = K_{ds} C_s^{eq} = \frac{K_{ds}\, C_0}{1 + K_{ds}\, V_d / V_s}} \qquad (1.11)$$

Where C_0 and C_s^{eq} are the initial and equilibrium concentration in the aqueous phase or sample, C_d^{eq} is the concentration in equilibrium in the organic droplet; V_d and

V_s are the organic drop and sample phase volume respectively and K_{ds} is the distribution coefficient, defined by:

$$K_{ds} = \frac{C_d^{eq}}{C_s^{eq}} \quad (1.12)$$

For volatile analytes where the headspace can be taken into consideration the equation 1.11 changes into a similar one, but considering headspace terms [Jeannot *et al.*, 2010]:

$$\boldsymbol{C_d^{eq}} = \frac{\boldsymbol{K_{ds}\, C_0}}{\boldsymbol{1+(K_{hs}\, V_h/V_s)+(K_{ds}\, V_d/V_s)}} \quad (1.13)$$

Where the new terms with the *h* subscript are the terms referred to headspace phase.

In any case the concentration of the droplet in equilibrium (C_d^{eq}) is always proportional to the initial concentration of the analyte in the sample (C_0) and will depends on the volumes of sample and organic drop and the partition coefficients that remain constant during all the process.

Enrichment factor can be defined as the ratio of the equilibrium concentration of analyte in the organic phase (C_d^{eq}) to the original concentration (C_0) of the analyte in the sample [Mohamadi and Mostafavi, 2010; Pakade and Tewary, 2010]. High enrichment factors (EF) indicates good extraction efficiency and helps to improve method validation parameters, such as limit of detection.

$$EF = \frac{C_d^{eq}}{C_0} \quad (1.14)$$

There are several classifications of liquid microextraction techniques [Lambropoulou and Albanis, 2007; Pena-Pereira *et al.*, 2010; Pinto *et al.*, 2010; Sarafraz-Yazdi and Amiri, 2010; Mahugo-Santana *et al.*, 2011; Han and Row, 2012; Dehghani Mohammad Abadi *et al.*, 2012; Spietelun *et al.*, 2014; Andrašcíková *et al.*, 2015]. Although different names are given to different technique variations, generally can be summarized as follows:

- **Single drop microextraction (SDME)**

A very little amount of the organic solvent (1-3 μL) is exposed to the sample (DI or HS) at the tip of a microsyringe. After the exposure time, the droplet is collected to further determination. This is basically the method in its starting point, with a slight change using the microsyringe instead of a Teflon tube (fig 1.4.a).

- **Directly-suspended droplet microextraction (DSDME)**

In this case, the droplet is placed in the liquid directly, without any help of external supports. The droplet, depending on the density, will be placed at the bottom of the vial or above the liquid. In any case, after extraction time the droplet has to be collected previous to analysis (fig 1.4.b).

- **Dispersive liquid-liquid microextraction (DLLME)**

As the name says, the organic droplet disperses in the aqueous phase to form a cloudy solution. This dispersion can be made with the help of other solvent (called as dispersive solvent) or with the help of agitation or ultrasonication. Afterwards a centrifugation is needed in order to recollect the organic phase into a one droplet (fig 1.4.c).

- **Hollow-fiber liquid-phase microextraction (HF-LPME)**

A porous hollow fiber is used to keep the organic phase. Therefore, the hollow-fiber is introduced to the sample for the extraction and retracted after the exposure time (fig 1.4.d).

If the extraction is made using “extraction-devices” such as the microsyringe or the hollow-fiber, the collection is also made with the device. But when the droplet during extraction is in contact only with the sample other approaches are needed. Most used is the Solidification of the floating drop (SFO), based on the freezing of the droplet to collect right after with a spatula. Then the organic phase melts before the injection to be analyzed. For this aim, the organic solvent needs melting points low enough to cool with a thermostatic bath but high enough to be liquid in room temperature (between 10-30^{o}C) and the extractant needs to have lower density than water [Ghambarian *et al.*, 2013].

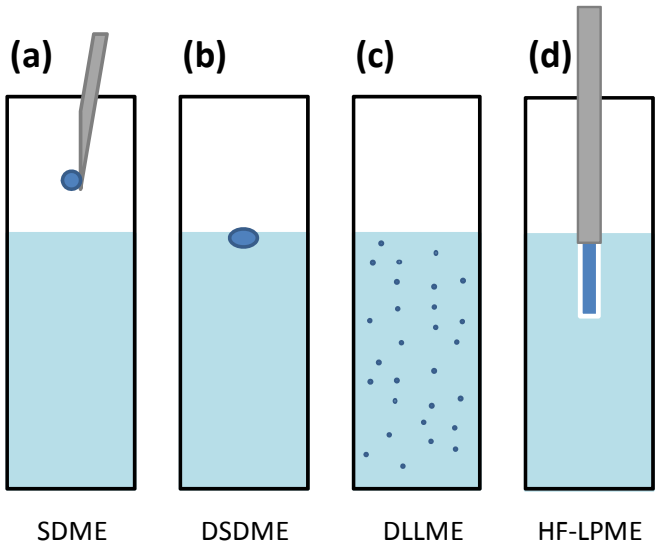


Figure 1.4. *Some liquid microextraction methods scheme corresponding to (a) single drop microextraction; (b) directly-suspended droplet microextraction; (c) dispersive liquid-liquid microextraction and (d) hollow-fiber liquid-phase microextraction.*

As in the SPME case there are several variables that need to be taken into account for a successful extraction using liquid phase microextraction techniques [Regueiro *et al.*, 2008; Ganjali *et al.*, 2010]:

- **Extractant**

There is a big variety of solvents used as extractant, but they must fulfill some conditions [Ganjali *et al.*, 2010; Han and Row, 2012]:

1. To have high affinity with the target analytes.
2. To be immiscible with water.
3. To be stable enough over the extraction time.
4. To have good chromatographic behavior (suitable for GC or HPLC analysis) or other determination techniques.
5. In case of SFO melting points below room temperature but above water melting point (because the drop is usually frozen by a cold water bath).

- **Extractant volume**

Extractant volume can be very important as lower volumes of extractants enhance the Enrichment Factors (EF) but they might be difficult to handle, so is necessary to reach an equilibrium between the EF and the viability of the droplet. Besides, the mass transfer rate increases with the surface area of the droplet [Krylov *et al.*, 2011], but as mentioned before, the dilution of analytes into the droplet is also enhanced.

- **Extraction time**

To increase the repeatability of extraction it is necessary to choose an extraction time during which equilibrium is reached although non-exhaustive extractions also are acceptable for some cases. Depends on the rate of the mass transfer which is affected by the drop volume. Extraction can happen under nonequilibrium conditions, except for DLLME, where the equilibrium is reached in very fast times [Ganjali *et al.*, 2010; Krylov *et al.*, 2011].

- **Extraction temperature**

Temperature usually increases the rate of mass transfer, thus the time required for the preconcentration decreases. But the temperature also increase the solubility of the organic phase into the aqueous phase itself which decrease the partition coefficient of analytes and leads to instabilities in the process [Krylov *et al.*, 2011]. If headspace is involved the situation can be even more complicated due to the extractant can also evaporate and this led to decrease the efficiency of extraction and its reproducibility.

- **Salt addition**

Salt addition may be ambiguous factor for LPME. In some cases the salting out effect can spoil the extraction because it can restrict the transport of the analyte to the drop due to an increase of the sample viscosity. But on the other hand, for some cases the salt promotes the mass transfer into the droplet due to the fact that water molecules form hydration spheres around the salt ions that reduce the concentration of water available to dissolve analyte molecules [Ganjali *et al.*, 2010; Krylov *et al.*, 2011].

- **Agitation**

The efficiency of extraction is increased with the stirring because it helps to the mass transfer, but too fast agitation may be undesirable because the droplet can be unstable [Ganjali *et al.*, 2010; Krylov *et al.*, 2011]. There are several methods for stirring; the most common is the magnetic stirrer though.

- **Disperser solvent**

In DLLME, the dispersive solvent plays a key role helping to form the cloudy solutions that increase the contact surface accelerating the mass transfer process. For its low toxicity and low cost methanol, acetone, ethanol and acetonitrile have been used [Sarafraz-Yazdi and Amiri, 2010]. But its efficiency can be restricted by the use of large volumes that decreases the partition of analytes into the extractant solvent [Saraji and Boroujeni, 2014].

Alternatively, even is not a solvent, ultrasound can be considered as a dispersant agent [Regueiro *et al.*, 2008]. Ultrasound (US) provides the capacity to reach dispersion with the possibility to avoid the dispersive solvent.

The desorption step is not necessary as in SPME, in this case the collected droplet is introduced to the GC injector port or HPLC injector valve. Nevertheless, a dilution may be necessary to make it compatible with the mobile phase used in HPLC.

1.2.2.1 Ultrasound-Assisted Emulsification Microextraction with Solidification of Floating Organic Droplet (USAEME-SFO)

Because its capacity to reduce extraction times DLLME is gaining acceptance. The method is based on the dispersion of the droplet, this dispersion divide the droplet into hundreds of very tiny droplets, forming a cloudy solution. As a consequence, the contact surface is increased to facilitate the transport of the analytes making the extraction times shorter than other cases.

Apart from the conditions mentioned above the extractant should fulfill also other conditions: formation of tiny droplets and the possibility to get back to a one droplet again after centrifugation. To help the formation of the cloudy solution a dispersive solvent is added. This is usually a common organic solvent: methanol, acetone, acetonitrile and ethanol are some of the most used ones [Saraji and Boroujeni, 2014].

Nevertheless, the use of a dispersive solvent is not the only way to form dispersions. Ultrasound (US) also form a cloudy solution (see figure 1.5) with the extractant helping the extraction process [Regueiro *et al.*, 2008].

Figure 1.5. *The cloudy solution formed after ultrasound application.*

Historically ultrasound energy has been used extensively in sample preparation [Luque de Castro and Priego-Capote, 2007]. Ultrasonication provides effectiveness for synthesizing chemicals, emulsifying products, and cleaning materials; even at the hospital is a very used non-invasive technique in surgeries and image development.

The ultrasound waves are transmitted through the substances producing expansion and compression cycles between the molecules. The expansion cycles create bubbles or cavities in liquids and the rapid compression of the gases and vapors in the cavities produces the increase of temperature and pressure. These bubbles are relatively small comparing with the total liquid volume and can be barely appreciable. Therefore the produced heat is rapidly dissipated. Because of that phenomenon the US heat is also known as “cold-boiling”.

The use of US in liquid-liquid microextraction helps in one hand the mass transfer between phases, and in the other hand the formation of a emulsion with tiny droplets of organic phase that increase the contact area of the different phases. One drawback using the US in LLE is the long time needed for the separation of the two phases after the emulsification and extraction, but when microlitre scale drop is used this step can be done in a centrifuge accelerating the separation.

Within ultrasound use for the dispersion there are four varieties of the technique that use ultrasonication. The first one uses only US, without any organic solvent or dispersant to help the process [Ozcan *et al.*, 2010; Wei *et al.*, 2011; Cortada *et al.*, 2011]. Another branch uses some dispersant solvent but in a small scale (0.2-0.5 mL) [Yan *et al.*, 2011; Zhang *et al.*, 2012a]. The third variation uses surfactants that could serve as an emulsifier to enhance the dispersion of the water-immiscible phase into the aqueous phase [Cheng *et al.*, 2011a; Cheng *et al.*, 2011b; Xia *et al.*, 2012; You *et al.*, 2013]. The last ultrasound assisted technique is helped with magnetic nanoparticles that help to retrieve the extractant instead of using centrifugation [Li *et al.*, 2014].

Next table 1.5 shows some of the advantages and disadvantages of the USAME technique [Mohamadi and Mostafavi, 2010; Mahugo-Santana *et al.*, 2011; Han and Row, 2012; Saraji and Boroujeni, 2014; Spietelun *et al.*, 2014]. Although is hard to use with complex matrices is faster, cheaper and simpler than many other techniques.

Table 1.5. *USAEME method advantages and disadvantages.*

USAEME	
Advantages	***Disadvantages***
• *Not carry-over effect.* • *Fast.* • *Low cost.* • *Minimum organic solvent consumption.* • *High EFs achievement.* • *Simplicity to operation.* • *Different branches adaptable for each target.* • *No need for interfaces to use with analysis apparatus.*	• *Complicate to use with complex matrices.*

1.3 Chromatographic techniques

Chromatography was defined by the IUPAC as *"a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction"* [Ettre, 1993]. Although in early sixties there was also a reference about gas chromatography [Stross and Ambrose, 1960]. Therefore, the aim of chromatography is to separate components and afterwards it will be detected by a device that measures the change of composition of the effluent. This measurement gives a value that is proportional to the concentration of the component. Hence chromatography is a very useful tool to quantify analytes in mixtures.

After preconcentration, analytes needs to be separated and determinate by some techniques. In this thesis, the used chromatographic methods are gas chromatography with an electron capture detector (GC-ECD) and high performance liquid chromatography with a diode array detector (HPLC-DAD). Some specifications for each technique are described briefly in this section.

1.3.1 Gas Chromatography with Electron Capture Detection (GC/ECD)

Gas chromatography (GC) is widely used for separation and determination of samples and is based on a capillary column, a gas as the eluent and a temperature gradient.

When SPME was developed the determination was performed in Gas Chromatography. The fiber needle can be inserted to the injector of the GC port without any special equipment or interface. The only feature that needs to be taken into account is the liner. Volume of liner specially affects to volatile compounds; thus, injector should be provided with a narrow liner (see figure 1.6) to avoid tailing in the chromatograms [Kataoka *et al.*, 2000; Lord and Pawliszyn, 2000]. In liners with wider inner diameter (figure 1.6.a), analytes are desorbing and sorbing again many times,

making desorption a slower process, concluding in a broadening of chromatographic peaks.

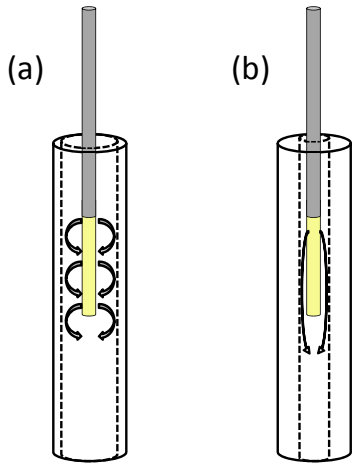


Figure 1.6. *Liner width effect in the desorption step in SPME: (a) liner with wide inner diameter; (b) liner specifically design for SPME with narrower inner diameter.*

Temperature of the port should be higher than least volatile analyte but this temperature never should exceed the maximum temperature allowed for the fiber coating [Abdulra'uf *et al.*, 2012].

The used detector was an Electron Capture Detector (ECD). The detector has an electron emitter, usually ^{63}Ni, the electrons are accelerating to a positively charged anode generating an electrical signal. If an electron acceptor analyte intervene between the electron emitter and the anode the electrical signal changes giving the resultant analyte signal. Halogens are very well detected in this detector because its electronegativity. Triazole compounds have halogen atoms that make the detector a good choice for the determination.

Nevertheless high temperatures of injection port creates problems in fiber life such as degradation of the polymer and thermally unstable compounds could not used in GC due to high temperatures. Hence, other alternative separation techniques have been used such as HPLC.

1.3.2 High-Performance Liquid Chromatography with Diode Array Detector (HPLC/DAD)

High performance liquid chromatography (HPLC) is based on a stationary phase column and a mobile phase formed by different solvents mixtures. Many different solvents are commercially available with different polarities, which can be used for HPLC. The main difficulty when the SPME started to use was the coupling of the fiber with the HPLC injector. In 1990's decade Pawliszyn and his group started to couple fibers with HPLC [Chen and Pawliszyn, 1995]. The first assemblies were homemade, but nowadays, commercially available interfaces are provided by the suppliers. Figure 1.7 shows a SPME-HPLC interface Rheodyne® valve provided by Sigma-Aldrich (Madrid, Spain).

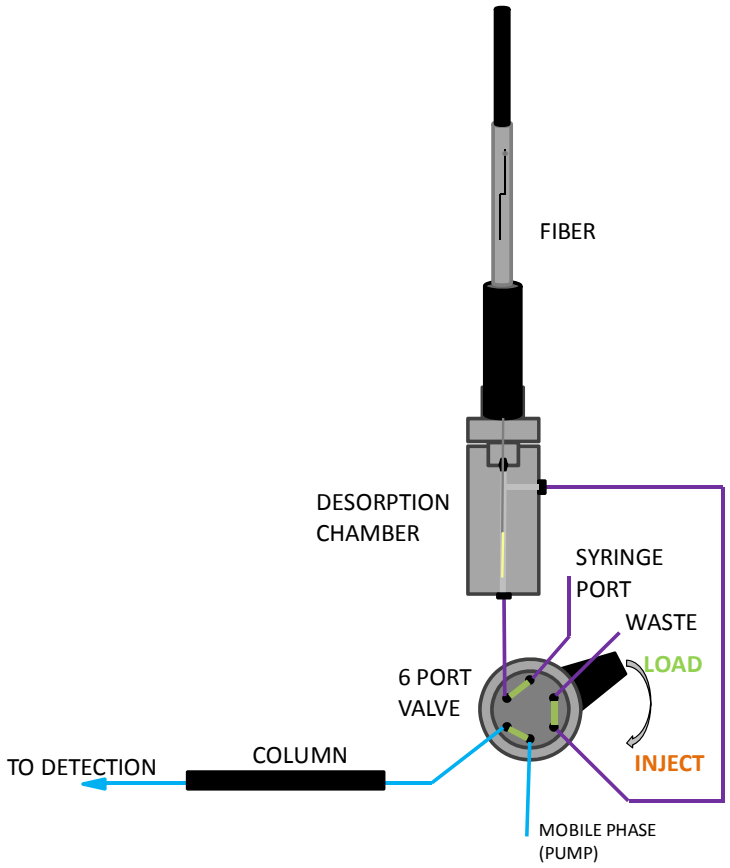


Figure 1.7. *SPME-HPLC interface scheme with Rheodyne® six port valve.*

The assembly replace the original loop used for this valves with a desorption chamber where the fiber is introduced for the subsequent desorption.

The determination was performed by a diode array detector (DAD). The advantage of this detector is that its wide wavelength range allows the detection of many different analytes. Moreover, the second order advantage can be used for multivariate analysis or in case of univariate analysis; the data can be analyzed in more than one wavelength depending on the analyst interests. Apart from that, the ultraviolet (UV) spectra for each elution time can be compared with a library, and if the software contains big enough library the identification of some compounds can be done.

1.4 Chemometrics

Chemometrics: *"a chemical discipline that uses mathematics, statistics, and formal logic (I) to design or select optimal experimental procedures; (II) to provide maximum relevant chemical information by analyzing chemical data; and (III) to obtain knowledge about chemical systems"* [Stalikas *et al.*, 2009].

1.4.1. Experimental design

Microextraction techniques efficiency relies on many variables that need to be fixed for each method. Before starting with calibration and validation processes in order to quantify any substance, is important to optimize the variables involved in the process. Choosing adequate values of the variables can lead to a better response that can improve figures of merit such as limit of detection, repeatability and linear ranges.

When many variables are involved, optimization of each variable or factor independently can be very tedious and time-consuming. The one-variable-at-a-time (OVAT) approach studies each factor at a time. However, each variable would need a number of analyses to decide which values give the maximum response. Apart from that, there may be interactions between factors, which a univariate optimization cannot anticipate [Dejaegher and Vander Heyden, 2009]. The global optimum (for the overall system) might not be found and the found optimal conditions might depend on the starting conditions [Dejaegher and Vander Heyden, 2011]. A multivariate optimization can take under consideration different level interactions between variables. The design of experiments (DoE) is an experimental set up to simultaneously evaluate several factors at given number of levels in a predefined number of experiments.

DoE methodology includes effects of each variable besides the interaction between them. Apart from the fact that allows to reduce the number of experiments the costs involved for the large number of analysis is also reduced leading to more effective laboratory practices.

According to Stalikas [Stalikas *et al.*, 2009] following points are considered essential in the conduct of a straightforward optimization for analytical procedures:

I. Definition of the problem and selection of the appropriate variables and response(s) through screening studies: researcher has to choose variables affecting the considered process. In the case of microextraction: sample volume, extraction time and temperature, agitation, sampling depth, etc. On the other hand the criterion to assess the process or the response wanted to optimize has to be chosen: chromatographic areas, extraction yield, enrichment factor, etc.

II. Choice of design of experiments: among many different designs, depending for each particular case, a design needs to be chosen. Full factorial designs are recommendable if the number of variables is reduced; but with a high number of variables reduced design are much more practical. For instance, Full Factorial, Fractional Factorial, Plackett-Burman, Doehlert, Box-Behnken and Central Composite designs, are able to optimize many variables with enough resolution with a reduced number of experiments.

III. Selection of levels of variables and codification: chose adequate variable ranges to see enough variation of the response to differ the variation from the error and codify as [-1], [+1], [0], [-α] and [+α]. If the range is small the observed variation might be confused with the effect of the random error and as a result wrong interpretation of the variability can be done.

IV. Mathematical model fitting: fit the experimental behavior to a mathematical model (linear, quadratic, with interactions, etc.). When there is not interaction between factors and a simple linear model is proposed, the data will follow next model:

$$y = b_0 + \sum b_i x_i + \varepsilon \qquad (1.15)$$

V. Model adequacy checking: it is necessary to examine the fitted model to ensure that it provides an adequate expectedness to the true system. This can be evaluated using analysis of variance ANOVA, lack-of-fit, least square estimators (R^2) and residual analysis.

VI. Analysis of model and effect estimates: *t*-Student tests is used to verify the significance of the regression coefficient whether differs significantly from the error or not. *p* values and *F* tests are other statistical features that offer information about the significance and finally the graphical outputs gives the information in a very visualized way: Pareto charts, response surfaces, desirability function, etc.

VII. Allocation of the optima: the objective is to find the operating conditions that maximize or minimize the desired response. This can be done graphically (response surfaces, contour plots and using desirability function) or solving mathematically (calculating through the first derivatives of the mathematical function).

VIII. Robustness checking: defined by the ability of the method to provide accurate and precise results. Finding robustness is to discover an experimental region where the response of interest is not influencing by changing significantly the levels of the various operating factors.

Summarizing, the practical aspect of DoE is performed in two steps: screening and optimization. The aim of a screening design is to fix the variables that are significant for the method. That is, to know the most influencing variables that affect to the system. Once the important variables are known, the optimum values need to be found with optimization procedures. At these optimum values the system will give the maximum desirable effect, for example the maximum chromatographic response (area or height) or a high yield in a chemical reaction.

There are several design types which can be used for the mentioned objectives. But, before beginning with these methods, it is convenient to define some terms [Bezerra *et al.*, 2008]:

- *Factors or independent variables:* experimental variables that we need to fix, independent of each other. Typical independent variables are temperature, pH, time of the experiments, etc. Factors can be quantitative (continuous or numerical variables) or qualitative (categorical or discrete variables).
- *Levels of the variables:* the range where the optimization is done. Usually these levels are coded, giving value of 1 to the maximum value, -1 to the

minimum value and 0 to the central value. If the factor is qualitative, 1 is given to an option and -1 is given to the other option.

- *Responses or dependant variables:* variables that are the signal given with an equipment: areas of a chromatogram, absorbance, yield of an experiment, etc. Basically, the measurement we want to optimize to a maximum (or minimum).

The full factorial and the used fractional factorial and central composite designs in this work are briefly explained.

1.4.1.1. Screening. Factorial Designs

Screening designs involve seeing which factors are important for the success of a process. When a new method is developing many possible variables can influence the analysis, but analyst should consider only those factors that are significant for the method. Thus, a significant variable leads to changes in the response when the factor value is changing.

- **Full Factorial Design**

The complete design is the Full Factorial Design. The number of experiment will be $N=l^f$; where l is the number of levels (2 if each factor is evaluated in its two coded levels: the maximum and minimum values [-1,1]) and f is the number of variables. All combinations of the factors are considered, and therefore the number of analyses can be very large if there are many factors (table 1.6) [Brereton, 2003].

Table 1.6. *Number of experiments in a Full Factorial Design regarding the number of independent variables for two levels.*

l=2	**Number of factors or independent variables (f)**									
	2	3	4	5	6	7	8	9	10	11
$N=2^f$	4	8	16	32	64	128	256	512	1024	2048

As it can be seen in the table 1.6, adding more factors to the design results in a high number of experiments, sometimes impossible to perform in laboratory but it can be handled if the number of factors is small.

Usually each factor is measured in two levels different enough to confirm if the variable is important in that system. As mentioned before the levels of the factors are coded: +1 value is given to the highest value and -1 is given to the lowest value in quantitative factors, whereas in qualitative variables the coded values are given randomly. The preference of one of the two choices is that quantitative factor will depend on the sign of the results. If positive, the preferable variable will be the one represented by +1 in the coded values and vice-versa. Table 1.7 shows a full factorial design matrix of a 3 factor system. The full factorial design matrix holds all possible combinations within factors. The number of experiments is given by $N=l^f$. Therefore, if three variables in two factors are considered, the total amount of experiment will be eight ($2^3 = 8$).

Table 1.7. *Experimental for a full factorial design with 3 factors and 2 levels (high [+1] and low [-1] values).*

Exp. No.	Factor A	Factor B	Factor C
1	+1	+1	+1
2	+1	+1	-1
3	+1	-1	+1
4	+1	-1	-1
5	-1	+1	+1
6	-1	+1	-1
7	-1	-1	+1
8	-1	-1	-1

These designs allow estimate all main and interaction effects between considered factors. For instance, for a system with three factors and considering only linear terms (higher level interactions) models are built following next equation:

$$y = b_0 + b_a x_a + b_b x_b + b_c x_c + b_{ab} x_a x_b + b_{ac} x_a x_c + b_{bc} x_b x_c + b_{abc} x_a x_b x_c \quad (1.16)$$

There are 8 unknown coefficients in this equation to solve (b_0, b_a, b_b, b_c, b_{ab}, b_{ac}, b_{bc} and b_{abc}). Once solved, the significance of each coefficient is determined according

with *t*-Student test. Some of the coefficient that results no significant means that the factor defined by this coefficient is not a significant variable for the system. Hence, this variable can be fixed because it has barely effect in the experiment. Nevertheless, the coefficient sign should be consider, although the variable is not significant, it indicates which value give better response.

- **Fractional Factorial Design**

As mentioned before, if there are many independent variables involved in the system, a full factorial design leads to a large amount of experiments; sometimes, unfeasible to perform. There is the need to reduce the number of experiments but without losing important information.

If a full factorial design is built the model is able to calculate all coefficients concerning main effects and all interactions (2-factor, 3-factor, 4-factor… n-factor interactions). Nevertheless many of these high-level interactions become negligible and can properly be disregarded. Therefore, analysts assume some loss of information in exchange of reducing the experiment number. The resolution concept is then defined [Box *et al.*, 1978]:

- Resolution III: does not confound main effects with one another but does confound main effects with other two-factor interactions.
- Resolution IV: No main effect is confounded with any two-factor interaction, but two-factor interactions are confounded with each other.
- Resolution V: does not confound main effects and two-factor interactions with each other, but does confound two-factor interactions with thee-factor interactions, and so on.

There are different design types considering fewer experimental runs: fractional factorial design, Plackett-Burman and Taguchi designs [Brereton, 2003]. Each design reduces the number analyses building different analysis matrices.

In fractional factorial design the number of analysis will be defined by $N=l^{f-k}$ where the new term *k* defines how many times the experiment numbers will be divided by two. Therefore, if k=1 the number of experiment will be half comparing

with a full factorial design; if k=2 the number of experiment will be divided by 4, etc. Consequently, a 5 factor screening if a full factorial design is used, results in 32 experiments. By using k=1 analysis is reduced to a half (16 experiments); and if k=2 results in 8 experiments, etc.

In conclusion, a fractional factorial design reduces the number of experiment to make the design feasible in a reasonable time despite of loosing resolution. In table 1.8 can be seen examples of experimental runs with different fractional factorial designs with 5 and 6 factors.

Table 1.8. *Different screening designs taking into account different resolutions for the different number of variables.*

Number of experiments	5 factors										6 factors					
	2^{5-1} design (Resolution V)					2^{5-2} design (Resolution III)					2^{6-2} design (Resolution IV)					
	Factor					Factor					Factor					
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	F
1	-1	-1	-1	-1	+1	-1	-1	-1	+1	+1	-1	-1	-1	-1	-1	-1
2	+1	-1	-1	-1	-1	+1	-1	-1	-1	-1	+1	-1	-1	-1	+1	-1
3	-1	+1	-1	-1	-1	-1	+1	-1	-1	+1	-1	+1	-1	-1	+1	+1
4	+1	+1	-1	-1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	-1	+1
5	-1	-1	+1	-1	-1	-1	-1	+1	+1	-1	-1	-1	+1	-1	+1	+1
6	+1	-1	+1	-1	+1	+1	-1	+1	-1	+1	+1	-1	+1	-1	-1	+1
7	-1	+1	+1	-1	+1	-1	+1	+1	-1	-1	-1	+1	+1	-1	-1	-1
8	+1	+1	+1	-1	-1	+1	+1	+1	+1	+1	+1	+1	+1	-1	+1	-1
9	-1	-1	-1	+1	-1						-1	-1	-1	+1	-1	+1
10	+1	-1	-1	+1	+1						+1	-1	-1	+1	+1	+1
11	-1	+1	-1	+1	+1						-1	+1	-1	+1	+1	-1
12	+1	+1	-1	+1	-1						+1	+1	-1	+1	-1	-1
13	-1	-1	+1	+1	+1						-1	-1	+1	+1	+1	-1
14	+1	-1	+1	+1	-1						+1	-1	+1	+1	-1	-1
15	-1	+1	+1	+1	-1						-1	+1	+1	+1	-1	+1
16	+1	+1	+1	+1	+1						+1	+1	+1	+1	+1	+1

The results usually are interpreted statistically with the help of analysis of variance (ANOVA). The effect of each factor is compared with the error and the *F* test is carried out. The $F_{critical}$ value is chosen according to the degree of freedom of the system and if this value is higher than the calculated *F* value, means the variable is not significant. On the other hand the *p* value shows the probability of a variability to occur

for pure chance. Therefore if the value given by ANOVA is higher than the threshold established (usually, *p*=0.05), means that the variation occurred by chance.

Apart from ANOVA tables, Pareto charts visualize the significant effects. Hence, the bars above the threshold limit indicate the influencing variables. The sign of each bar indicates the preferred coded value. That is, if the sign is positive, the preferred coded value is [+1] and the negative sign represents the [-1] coded value. After the analyzing the screening results, the significant variables are taken into optimization procedures, and those with results no-significant are fixed.

1.4.1.2 Optimization. Central Composite Design

Optimization step involved to find an optimum value where the best responses are obtained. Usually only two or three factors are optimized, because with more variables the optimization design matrix requires a high number of experiments. The number of variables is reduced by a previous screening design and qualitative variables cannot be included in optimization. The reason is that the responses considered are modeled as a function of the factors [Dejaegher and Vander Heyden, 2011].

Then, a second degree polynomial mathematical model is built to describe the data (equation 1.17).

$$y = b_0 + \sum_{i=1}^{f} b_i x_i + \sum_{1 \le i < j}^{f} b_{ij} x_i x_j + \sum_{i=1}^{f} b_{ii} x_i^2 \qquad (1.17)$$

Equation 1.17 represents the general mathematical model for f variables, including main effects coefficients (b_i), linear interactions (b_{ij}) and quadratic effects (b_{ii}).

For a central composite design, the factorial design is increased with additional points: star points and replicates at center point. The replicates in central point have two main objectives; firstly to provide a measure of pure error and secondly to stabilize the variance of the predicted response [Ferreira *et al.*, 2007]. The number of total experiments is calculated combining the number of factorial points, axial (or star) point and central points:

$$N = N_f + N_s + N_c \quad (1.18)$$

Where N_f is defined by $N_f = 2^f$; N_s is calculated as $N_s = 2f$ and N_c is the number of central points. As an example, if a three variable system is considered (f=3) the total amount of experiment if five central points are included will be nineteen in total [N= 2^3 + (2 x 3) + 5 = 8 + 6 + 5 = 19].

Star points are fixed from the central point adding a value of α fulfilling one of the next conditions [Brereton, 2003]:

a) Rotatability: implies that the confidence in the predictions depends only on the distance from the centre of design. Therefore the farther is the value from the central point lower is the confidence in that value. Star points are located at the value α calculated with the equation 1.19.

$$\alpha = \sqrt[4]{N_f} \quad (1.19)$$

b) Orthogonality: implies that all the terms in equation 1.17 (linear, quadratic and interactions) are orthogonal to each other, so, there is no correlation between any two terms (correlation coefficient will be equal to zero). A condition more complicated than rotatability because is not easy to find correlation 0 between squared terms. Star points using orthogonality conditions are calculated using α calculated with equation 1.20.

$$\alpha = \sqrt{\frac{\sqrt{N \cdot N_f} - N_f}{2}} \quad (1.20)$$

Figure 1.8 shows the experiments points in a 3 factor system. The experiments are planned to be as a full factorial design (figure 1.8, blue dots) increasing the design by the so-called start points or axial points (figure 1.8, orange stars) plus central point (figure 1.8, green dot).

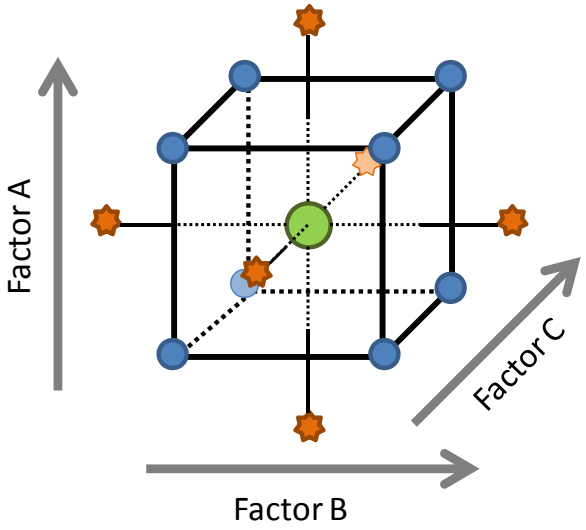


Figure 1.8. *Central composite design for a three factor system.*

Once the second order polynomial model is built, results are given graphically or statistically. Generally, models are visualized using 2D contour plots or 3D surface plots, where the optimal values are derived. But when many variables are involved, interpretation of these plots should be done together.

Desirability approaches are used to find an overall effect when optimizing variables simultaneously. Desirability function-based approach consist on converting estimated response models ($\hat{y}$) into desirability functions (d), which will be maximized (or minimized) [Costa *et al.*, 2011]. Derringer and Suich [Derringer and Suich, 1980] define the desirability function as follows: the individual desirability function transforms the response variable into a range of values between 0 and 1, where 1 is the most favorable.

$$d = \begin{Bmatrix} 0 & , & \hat{y} \leq U \\ \left(\frac{\hat{y}-U}{T-U}\right)^s & , & U < \hat{y} < T \\ 1 & , & T \leq \hat{y} \end{Bmatrix} \tag{1.21}$$

Where T is the target value (1), U is the minimum acceptable value (0) and s is user specified parameters (s >0). Consequently, d=1 for $\hat{y}$=T. Once the responses are

changed to desirability values, a global desirability surface is plotted, where the optimum values can be easily interpreted.

The optimum values are chosen from the plots or from the mathematical function and the values which obtain the desirable response are fixed to continue with the validation of the method.

1.4.2. Multivariate analysis

Chemometrics provides powerful tools to extract information and understand our data. Multivariate analysis involves a wide range of methods which extract information reducing the large amount of variables into others in a simple and visual way. Basically most of the methods are based on creating new variables (latent variables) or components that describe the interesting chemical information. Those methods are solved mathematically with different algorithms that are briefly explained in the following part.

1.4.2.1. Nomenclature

In order to clarify the terms used when describing models, nomenclature will be fixed. Regarding letters, scalars are indicated with lower case (e.g. b), vectors are expressed in bold lower case (e.g. **a**) and matrices are indicated with bold upper cases if 2-way (e.g. **X**) and underlined if 3-way (e.g. $\underline{\mathbf{X}}$). The term mode expressed the order of the matrix. Even larger orders matrices exist (multi-way) are not considered in this thesis. Letters I, J and K are reserved for indicating the dimension of the modes; therefore, when (I x J x K) is expressed means that the matrix has three modes: first mode has I number of variables, second mode J variables and the third mode has K number of variables. The superscript T means the transpose of a matrix (e.g. $\mathbf{X}^T$). Other symbols, apart from the explained in this section, will be explained in the text.

1.4.2.2. Principal Component Analysis (PCA)

Principal Component Analysis (PCA) is a decomposition method that reduces the dimensionality of the data consisting of a large number of interrelated variables, while retaining as much as possible of the variation present in the data [Kumar *et al.*, 2014]. It transforms the original variables into new variables called principal components (PC) in order to seek the maximum variability (see figure 1.9). First PC, will describe the maximum variability, second PC will be orthogonal to the first PC and find the second maximum variability. Third PC will describe the third maximum variability and so on. Each PC explains a certain variance of the data, which is expressed as a cumulative explained variance. The explained variance will give the clue about how many PCs are necessary to describe the data, although the analyst will make the final decision taking into account other factors.

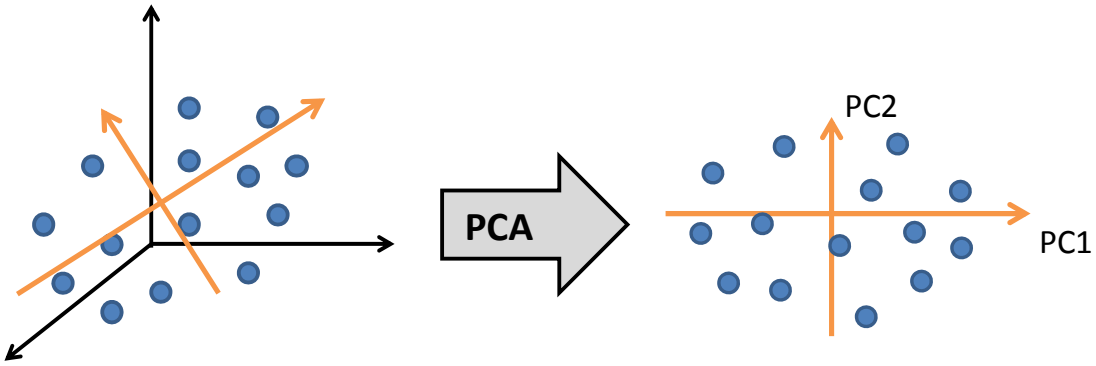


Figure 1.9. *PCA transformation of a set of samples into two principal components.*

Basically PCA is a transformation of the original variables to a new reduced number of variables. In the simple example in figure 1.9 can be seen how a PCA can describe the data variability from a 3 dimension system to a 2 PCs. The new latent variables or PCs are orthogonal among them and will be the new "axes" to describe the variability of the data. Nevertheless, PCA interpretation can be difficult, because it has not represented true chemical profiles as in other cases.

PCA is a bilinear model, that is, follows the equation 1.22, where **X** is the data matrix formed by I samples and J variables and its elements x_{ij} can be described as:

$$x_{ij} = \sum_{r=1}^{R} t_{ir} p_{jr} + e_{ij} \quad (1.22)$$

The aim of the PCA is to decompose the original matrix into a score matrix (**T** (IxR)) and a loading (**P** (JxR)) matrix. The loadings are the relationship of the PCs with the old axes and the scores are the projected value of the samples on these new variables (loadings) [Bro and Smilde, 2014]. **E** (IxJ) is the residual matrix, with equal dimensions of the initial matrix **X** (IxJ), that contains the variances that the model cannot fit, in other words, the representative part of the error.

$$\boldsymbol{X} = \boldsymbol{T}\boldsymbol{P}^{T} + \boldsymbol{E} \quad (1.23)$$

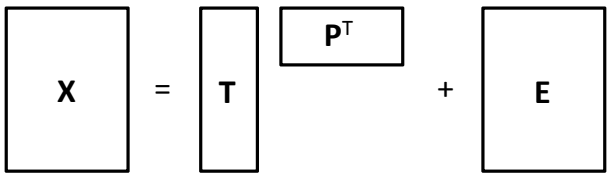


Figure 1.10. *Representation of the PCA mathematical model with R components. Lines represent vectors, while squares are representative for 2-way matrices.*

PCA is expressed as in equation 1.23, and this equation can be represented by the figure 1.10 in the matricial form. PCA can be very useful for exploratory analysis but is not useful for calibration. An extended literature can be found with extended information about PCA mathematical and practical aspects [Munck *et al.*, 1998; Bro, 2003; Smilde *et al.*, 2004; Bro and Smilde, 2014]. Many of the multivariate methods are based on PCA principles, such as, PCR, PLS, PARAFAC and MCR.

1.4.2.3. Parallel Factor Analysis (PARAFAC)

Parallel Factor Analyisis (PARAFAC) is a generalization of PCA to higher order arrays [Bro, 1997], that provides more interpretable models. The aim is the same, to reduce number of the variables to a reduced number of components. While the interpretation of a PCA can be abstract, the PARAFAC can be easily interpreted as each component show the chemical variation source. As it can be seen in the example in figure 1.11, a range with overlapped chromatographic peaks can be decomposed with PARAFAC into three components contributions: a tail at the beginning of the selected range (red), the main peak (green) and another peak (purple).

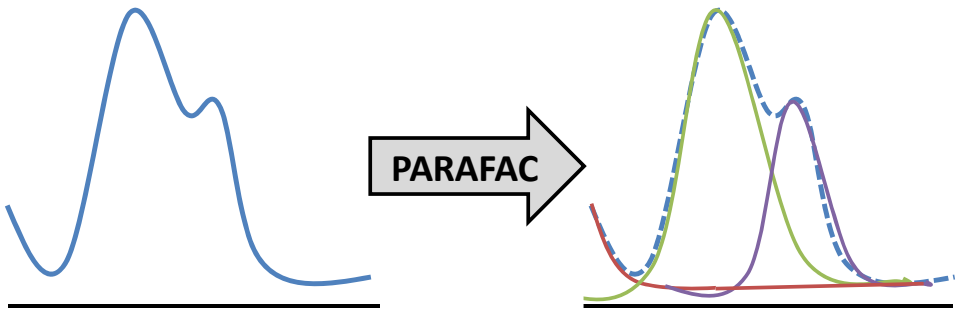


Figure 1.11. *PARAFAC decomposition with three components.*

In the chromatography case, each component obtained with PARAFAC can be representative of each analyte elution and spectral profile [Amigo *et al.*, 2010].

Even graphically are not interpreted the same way, PCA and PARAFAC have strong similarities. As PCA is considered a bilinear model, PARAFAC is extended to three dimensions following a trilinear model [Smilde *et al.*, 2004] (see fig. 1.12):

$$x_{ijk} = \sum_{r=1}^{R} a_{ir} b_{jr} c_{jr} + e_{ijk} \qquad (1.24)$$

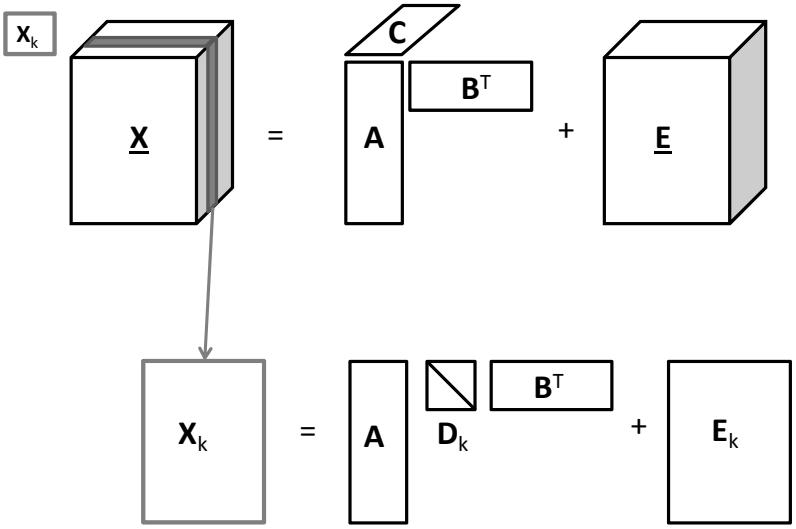


Figure 1.12. *Representation of the PARAFAC mathematical model with R components. Lines represent vectors, squares and cubes are representative for 2-way and 3-way matrices respectively.*

x_{ijr} elements are part of the **X** matrix described as follows in a matricial form, where $\mathbf{X}_k$ will be the k^{th} slice of **X** (IxJxK) [Smilde *et al.*, 2004].

$$\boldsymbol{X}_k = \boldsymbol{A}\boldsymbol{D}_K\boldsymbol{B}^T + \boldsymbol{E}_k \quad (1.25)$$

Each component $\mathbf{X}_k$ is modeled by the same **A** and **B** loadings (elution and spectral loadings) but different weights or relative concentrations ($\mathbf{D}_k$). This $\mathbf{D}_k$ is the diagonal matrix with the k^{th} row of **C** (KxR) on its diagonal (completed by the elements c_{k1}, c_{k2}…, c_{kR}) where **C** is the loading representing the components concentrations (see figure 1.12).

PARAFAC can be used as exploratory analysis, but also for calibration due to the fact that it extracts information about relative concentrations of the obtained pure spectra. The main advantage of PARAFAC is its uniqueness if there is not a rank deficiency problem [Sidiropoulos and Bro, 2000; Omidikia *et al.*, 2013].

1.4.2.4. Parallel Factor Analysis 2 (PARAFAC2)

Most multi-way methods assume that the profiles between different runs are the same. But in some cases there are little differences between batches. For example in chromatography is very usual to see retention time shifts [Bro *et al.*, 1999]. Little experimental conditions changes, such as pressure, flow, or even changes in the chromatographic columns, led to shifts difficult to avoid that many multi-way methods cannot deal. PARAFAC2 can handle those little variations in one profile [Kiers *et al.*, 1999].

Ideally PARAFAC2 separate the data into three contributions as PARAFAC: relative concentrations (weights), elution profiles and spectra. But the main difference is that PARAFAC2 extract an elution profile for each sample, while PARAFAC extract just one profile for all the samples. Moreover, PARAFAC2 does not impose strong restrictions that assuming the same shape and length for every sample [Amigo *et al.*, 2008].

Mathematically PARAFAC2 is very similar to PARAFAC. The difference is that there is not one **B** matrix describing just one elution profile. In this case, **B** matrices are as much as K variables (samples) (see figure 1.13).

$$\boldsymbol{X}_k = \boldsymbol{A}\boldsymbol{D}_k\boldsymbol{B}_k^T + \boldsymbol{E}_k \tag{1.26}$$

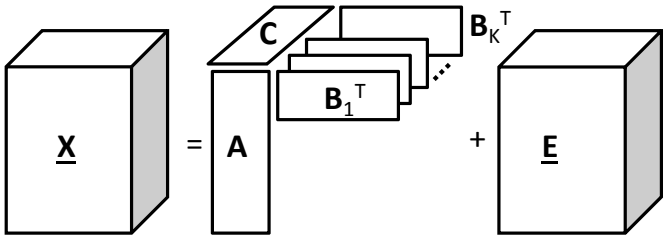


Figure 1.13. *Representation of the PARAFAC2 mathematical model with R components. Squares and cubes are representative for 2-way and 3-way matrices respectively.*

Therefore, the result of a PARAFAC2 model will result in one concentration or relative weight profile (**A**), one spectral profile (**C**) and as many as variables elution profiles (**B**).

The main advantage of PARAFAC2 in front of PARAFAC is that is possible to handle elution time shifts without any previous time-consuming treatment, such as alignment.

1.4.2.5. Multivariate Curve Resolution (MCR)

Multivariate Curve Resolution (MCR) is a bilinear model, but can also handle trilinear data, although an unfolding is necessary. For this aim, the (**X** (IxJxK)) matrix is unfolded column-wise (**D** (IKxJ)) as it can be seen in figure 1.14. The main goal of MCR is the determination of true concentration (**C** (IKxR) and spectra (**S** (JxR)) matrices, with R number of components, beginning from an initial estimation that is optimized using alternating least squares (ALS) as the iterative method [Tauler, 1995]. MCR can be used for trilinear data, as well as non-trilinear data, in contrast to PARAFAC based methods which are designed to be used only for trilinear second order data structures.

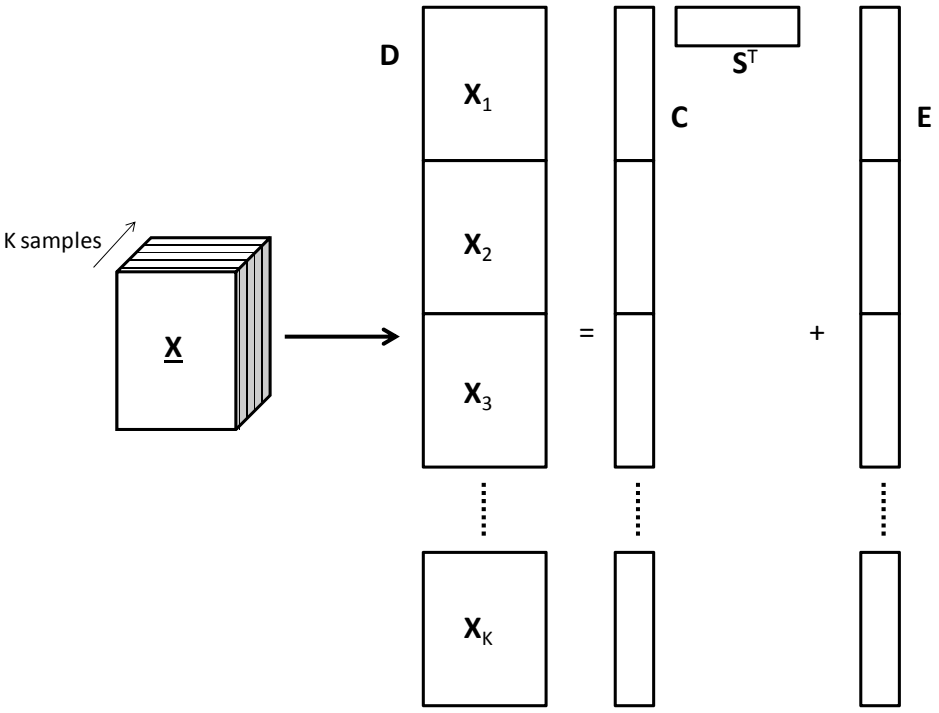


Figure 1.14. *Representation of the MCR mathematical model. First unfolding step is represented and then the decomposition into the concentration (**C**) and spectra (**S**) and residual (**E**) matrices.*

The mathematical model appearance of MCR is very similar to PCA although the graphical meaning is very different. MCR components are not orthogonal as in PCA and has more physical meaning and easier interpretability [Jaumot and Tauler, 2010].

$$\boldsymbol{D} = \boldsymbol{C}\boldsymbol{S}^T + \boldsymbol{E} \quad (1.27)$$

MCR model does not provide unique solutions if there is rotational ambiguity. To get true unique solutions this rotational ambiguity needs to be restricted [Abdollahi and Tauler, 2011]. For this aim, constraints are applied in the model, this constraints are translated into mathematical language and force the iterative optimization process to model the profiles respecting the conditions desired [de Juan and Tauler, 2003]. These constraints include non-negativity, unimodality or closure (constraints with chemical properties) or local rank and selective windows, trilinear structure, etc. (constraints with mathematical meaning) [de Juan and Tauler, 2006].

1.4.2.6. Parallel Factor Analysis with Linear Dependencies (PARALIND)

Parallel Factor Analysis with Linear Dependencies (PARALIND) is a PARAFAC based method for rank deficient data matrices. Sources of variations (such as a chromatographic signal) can have independent effect in two modes but can be linearly dependent in the third mode. This rank deficient problem lead to non-unique solutions and confusing results if a PARAFAC model is used [Bro *et al.*, 2009].

PARALIND include a dependency matrix (**H**) to incorporate the rank deficiency into the model. Then, the model will be expressed as in PARAFAC case [Bahram and Bro, 2007]:

$$\boldsymbol{X}_k = \overline{\boldsymbol{A}}\boldsymbol{D}_K\boldsymbol{B}^T + \boldsymbol{E}_k \quad (1.28)$$

Where,

$$\overline{\boldsymbol{A}} = \boldsymbol{A}\boldsymbol{H} \quad (1.29)$$

Where **A** is a (IxR) matrix and **H** is a (RxS) matrix. Therefore $\overline{\boldsymbol{A}}$ is a (IxS) matrix, where R represents the number of components in the rank deficient mode and S the

number of components in the other two modes. For instance, for a N + M → P reaction, where the initial concentrations of N and M are the same and its consumption for the reaction is the same, the dependency matrix is formed by 3 components in two modes and only 2 in the rank deficient mode (because the concentrations profiles of N and M are the same) [Bro *et al.*, 2009]:

$$\boldsymbol{H} = \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 1 \end{bmatrix} \tag{1.30}$$

With this dependency matrix the model specifies that two of the profiles are identical. Because considering $\boldsymbol{A} = [\bar{\boldsymbol{a}}_1 \bar{\boldsymbol{a}}_2]$ due to its rank deficiency, then $\bar{\boldsymbol{A}} = \boldsymbol{AH} = [\bar{\boldsymbol{a}}_1 \bar{\boldsymbol{a}}_2 \bar{\boldsymbol{a}}_2]$. This can be chemically interpreted as two phenomena in the first mode, have mathematical "interactions" with three in the other two modes. In other words, $\bar{\boldsymbol{A}}$ represents three phenomena as the other two modes, but this matrix, $\bar{\boldsymbol{A}}$, is composed by **A** and its dependency matrix **H** (see figure 1.15).

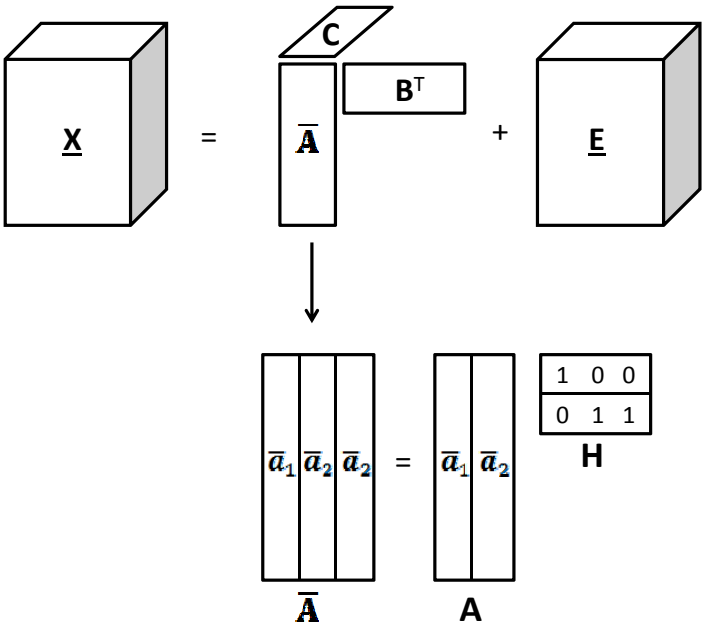


Figure 1.15. *Representation of the PARALIND mathematical model where the* $\bar{\boldsymbol{A}}$ *matrix is composed with the rank-deficient* ***A*** *matrix and the dependency matrix* ***H****.*

The PARALIND algorithm will provide the **A** profile (concentration profile in this example) with the description of the two phenomena, as well as **B**, **C** (elution and spectral profiles) with three phenomena and the residual matrix **E**.

1.5 References

Abdollahi, H. and Tauler, R.; *Uniqueness and rotation ambiguities in Multivariate Curve Resolution methods.* **2011.** Chemometrics and Intelligent Laboratory Systems, 108(2), 100-111.

Abdulra'uf, L.B., Chai, M.K. and Tan, G.H.; *Applications of Solid-Phase Microextraction for the Analysis of Pesticide Residues in Fruits and Vegetables: A Review.* **2012.** Journal of AOAC International, 95(5), 1272-1290.

Ai, J., *Headspace Solid Phase Microextraction. Dynamics and Quantitative Analysis before Reaching a Partition Equilibrium.* **1997.** Analytical Chemistry, 16, 3260-3266.

Allen, J.W., Wolf, D.C., George, M.H., Hester, S.D., Sun, G., Thai, S., Delker, D.A., Moore, T., Jones, C., Nelson, G., Roop, B.C., Leavitt, S., Winkfield, E., Ward, W.O. and Nesnow, S.; *Toxicity Profiles in Mice Treated with Hepatotumorigenic and Non-Hepatotumorigenic Triazole Conazole Fungicides: Propiconazole, Triadimefon, and Myclobutanil.* **2006.** Toxicologic Pathology, 34(7), 853-862.

Alpendurada, M.F., *Solid-phase microextraction: a promising technique for sample preparation in environmental analysis.* **2000.** Journal of Chromatography A, 889(1–2), 3-14.

Amigo, J.M., Skov, T. and Bro, R.; *ChroMATHography: Solving Chromatographic Issues with Mathematical Models and Intuitive Graphics.* **2010.** Chemical Reviews, (110), 4582-4605.

Amigo, J.M., Skov, T., Bro, R., Coello, J. and Maspoch, S.; *Solving GC-MS problems with PARAFAC2.* **2008.** Trends in Analytical Chemistry, 27(8), 714-725.

Andrašcíková, M., Matisová, E. and Hrouzková, S.; *Liquid Phase Microextraction Techniques as a Sample Preparation Step for Analysis of Pesticide Residues in Food.* **2015.** Separation & Purification Reviews, 44, 1-18.

Aresta, A., Vatinno, R., Palmisano, F. and Zambonin, C.G.; *Determination of Ochratoxin A in wine at sub ng/mL levels by solid-phase microextraction coupled to liquid chromatography with fluorescence detection.* **2006.** Journal of Chromatography A, 1115(1-2), 196-201.

Arthur, C.L. and Pawliszyn, J.; *Solid Phase Microextraction with Thermal Desorption Using Fused Silica Optical Fibers.* **1990.** Analytical Chemistry, 62, 2145-2148.

Aulakh, J.S., Malik, A.K., Kaur, V. and Schmitt-Kopplin, P.; *A Review on Solid Phase Micro Extraction—High Performance Liquid Chromatography (SPME-HPLC) Analysis of Pesticides.* **2005.** Critical Reviews in Analytical Chemistry, 35(1), 71-85.

Bahram, M. and Bro, R.; *A novel strategy for solving matrix effect in three-way data using parallel profiles with linear dependencies.* **2007.** Analytica Chimica Acta, 584(2), 397-402.

Beceiro-González, E., Concha-Graña, E., Guimaraes, A., Gonçalves, C., Muniategui-Lorenzo, S. and Alpendurada, M.F.; *Optimisation and validation of a solid-phase microextraction method for simultaneous determination of different types of pesticides in water by gas chromatography–mass spectrometry.* **2007.** Journal of Chromatography A, 1141(2), 165-173.

Beltran, J., López, F.J. and Hernández, F.; *Solid-phase microextraction in pesticide residue analysis.* **2000.** Journal of Chromatography A, 885(1-2), 389-404.

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S. and Escaleira, L.A.; *Response surface methodology (RSM) as a tool for optimization in analytical chemistry.* **2008.** Talanta, 76(5), 965-977.

Box, G.E.P., Hunter, W.G. and Hunter, J.S.; *Statistics for experimenters. An introduction to design, data analysis, and model building.* **1978.** John Wiley & Sons. New York (USA).

Brereton, R.G.; *Chemometrics. Data Analyisi for the Laboratory and Chemical Plant.* **2003.** 1 edn. John Wiley & Sons Ltd. Chichester (UK).

Bro, R., *PARAFAC. Tutorial and applications.* **1997.** Chemometrics and Intelligent Laboratory Systems, 38(2), 149-171.

Bro, R., *Multivariate calibration: What is in chemometrics for the analytical chemist?* **2003.** Analytica Chimica Acta, 500(1–2), 185-194.

Bro, R., Andersson, C. and Kiers, H.A.L.; *PARAFAC2: Part II. Modeling chromatographic data with retention time shifts.* **1999.** Journal of Chemometrics, 13(3-4), 295-309.

Bro, R., Harshman, R.A., Sidiropoulos, N.D. and Lundy, M.E.; *Modeling multi-way data with linearly dependent loadings.* **2009.** Journal of Chemometrics, 23(7-8), 324-340.

Bro, R. and Smilde, A.K.; *Principal component analysis.* **2014.** Analytical Methods, 6, 2812-2831.

Buszewski, B. and Szultka, M.; *Past, present, and future of solid phase extraction: A review.* **2012.** Critical Reviews in Analytical Chemistry, 42, 198-213.

Chen, J. and Pawliszyn, J.; *Solid Phase Microextraction Coupled to High- Performance Liquid Chromatography.* **1995.** Analytical Chemistry, 67(15), 2530-2533.

Cheng, J., Matsadiq, G., Liu, L., Zhou, Y. and Chen, G.; *Development of a novel ultrasound-assisted surfactant-enhanced emulsification microextraction method and its application to the analysis of eleven polycyclic aromatic hydrocarbons at trace levels in water.* **2011a.** Journal of Chromatography A, 1218(18), 2476-2482.

Cheng, J., Xia, Y., Zhou, Y., Guo, F. and Chen, G.; *Application of an ultrasound-assisted surfactant-enhanced emulsification microextraction method for the analysis of diethofencarb and pyrimethanil fungicides in water and fruit juice samples.* **2011b.** Analytica Chimica Acta, 701(1), 86-91.

Cortada, C., Vidal, L. and Canals, A.; *Determination of geosmin and 2-methylisoborneol in water and wine samples by ultrasound-assisted dispersive liquid–liquid microextraction coupled to gas chromatography–mass spectrometry.* **2011.** Journal of Chromatography A, 1218(1), 17-22.

Costa, N.R., Lourenço, J. and Pereira, Z.L.; *Desirability function approach: A review and performance evaluation in adverse conditions.* **2011.** Chemometrics and Intelligent Laboratory Systems, 107(2), 234-244.

de Juan, A. and Tauler, R.; *Multivariate curve resolution (MCR) from 2000: progress in concepts and applications.* **2006.** Critical Reviews in Analytical Chemistry, 36(3-4), 163-176.

de Juan, A. and Tauler, R.; *Chemometrics applied to unravel multicomponent processes and mixtures: Revisiting latest trends in multivariate resolution.* **2003.** Analytica Chimica Acta, 500(1–2), 195-210.

Dehghani Mohammad Abadi, M., Ashraf, N., Chamsaz, M. and Shemirani, F.; *An overview of liquid phase microextraction approaches combined with UV–Vis spectrophotometry.* **2012.** Talanta, 99, 1-12.

Dejaegher, B. and Vander Heyden, Y.; *The use of experimental design in separation science.* **2009.** Acta Chromatographica, 21(2), 161-201.

Dejaegher, B. and Vander Heyden, Y.; *Experimental designs and their recent advances in set-up, data interpretation, and analytical applications.* **2011.** Journal of pharmaceutical and biomedical analysis, 56(2), 141-158.

Derringer, G. and Suich, R.; *Simultaneous optimization of several response variables.* **1980.** Journal of Quality Technology, 12(4), 214-219.

Echeverría-Sáenz, S., Mena, F., Pinnock, M., Ruepert, C., Solano, K., de la Cruz, E., Campos, B., Sánchez-Avila, J., Lacorte, S. and Barata, C.; *Environmental hazards of pesticides from pineapple crop production in the Río Jiménez watershed*

(Caribbean Coast, Costa Rica). **2012.** Science of The Total Environment, 440, 106-114.

Ekman, D.R., Keun, H.C., Eads, C.D., Furnish, C.M., Murrell, R.N., Rockett, J.C. and Dix, D.J.; *Metabolomic evaluation of rat liver and testis to characterize the toxicity of triazole fungicides.* **2006.** Metabolomics, 2(2), 63-73.

Ettre, L.S., *Nomenclature for chromatography (IUPAC recommendations 1993).* **1993.** Pure and Applied Chemistry, 65(4), 819-872.

Fernandez-Alvarez, M., Llompart, M., Lamas, J.P., Lores, M., García-Jares, C., Cela, R. and Dagnac, T.; *Development of a solid-phase microextraction gas chromatography with microelectron-capture detection method for a multiresidue analysis of pesticides in bovine milk.* **2008.** Analytica Chimica Acta, 617(1-2), 37-50.

Ferreira, S.L.C., Bruns, R.E., da Silva, E.G.P., dos Santos, W.N.L., Quintella, C.M., David, J.M., de Andrade, J.B., Breitkreitz, M.C., Jardim, I.C.S.F. and Neto, B.B.; *Statistical designs and response surface techniques for the optimization of chromatographic systems.* **2007.** Journal of Chromatography A, 1158(1-2), 2-14.

Ganjali, M.R., Sobhi, H.R., Farahani, H., Norouzi, P., Dinarvand, R. and Kashtiaray, A.; *Solid drop based liquid-phase microextraction.* **2010.** Journal of Chromatography A, 1217(16), 2337-2341.

Ghambarian, M., Yamini, Y. and Esrafili, A.; *Liquid-phase microextraction based on solidified drops of organic solvents.* **2013.** Microchimica Acta, 180, 519-535.

Gilbert-López, B., García-Reyes, J.F. and Molina-Díaz, A.; *Sample treatment and determination of pesticide residues in fatty vegetable matrices: A review.* **2009.** Talanta, 79(2), 109-128.

Goetz, A.K., Ren, H., Schmid, J.E., Blystone, C.R., Thillainadarajah, I., Best, D.S., Nichols, H.P., Strader, L.F., Wolf, D.C., Narotsky, M.G., Rockett, J.C. and Dix, D.J.; *Disruption of Testosterone Homeostasis as a Mode of Action for the Reproductive Toxicity of Triazole Fungicides in the Male Rat.* **2007.** Toxicological Sciences, 95(1), 227-239.

Han, D. and Row, K.; *Trends in liquid-phase microextraction, and its application to environmental and biological samples.* **2012.** Microchimica Acta, 176(1), 1-22.

Hu, Y., Yang, X., Wang, C. and Zhao J.; *A sensitive determination method for carbendazim and thiabendazole in apples by solid-phase microextraction−high performance liquid chromatography with fluorescence detection.* **2008.** Food Additives & Contaminants: Part A, 25(3), 314-319.

Jaumot, J. and Tauler, R.; *MCR-BANDS: A user friendly MATLAB program for the evaluation of rotation ambiguities in Multivariate Curve Resolution.* **2010.** Chemometrics and Intelligent Laboratory Systems, 103(2), 96-107.

Jeannot, M.A. and Cantwell, F.F.; *Solvent Microextraction into a Single Drop.* **1996.** Analytical Chemistry, 68(13), 2236-2240.

Jeannot, M.A., Przyjazny, A. and Kokosa, J.M.; *Single drop microextraction—Development, applications and future trends.* **2010.** Journal of Chromatography A, 1217(16), 2326-2336.

Kataoka, H., Lord, H.L. and Pawliszyn, J.; *Applications of solid-phase microextraction in food analysis.* **2000.** Journal of Chromatography A, 880(1-2), 35-62.

Kiers, H.A.L., ten Berge, J.M.F. and Bro, R.; *PARAFAC2: Part I. A direct fitting algorithm for the PARAFAC2 model.* **1999.** Journal of Chemometrics, 13(3-4), 275-294.

Krylov, V., Krylov, A., Mosyagin, P. and Matkivskaya, Y.; *Liquid-phase microextraction preconcentration of impurities.* **2011.** Journal of Analytical Chemistry, 66(4), 331-350.

Kumar, A. and Malik, A.K.; *A Review on the Hyphenation of Solid Phase Microextraction with Capillary Electrophoresis and Mass Spectrometry.* **2009.** Critical Reviews in Analytical Chemistry, 39, 81-88.

Kumar, N., Bansal, A., Sarma, G.S. and Rawal, R.K.; *Chemometrics tools used in analytical chemistry: An overview.* **2014.** Talanta, 123, 186-199.

Lambropoulou, D.A. and Albanis, T.A.; *Liquid-phase micro-extraction techniques in pesticide residue analysis.* **2007.** Journal of Biochemical and Biophysical Methods, 70(2), 195-228.

Li, Y., Yang, X., Zhang, J., Li, M., Zhao, X., Yuan, K., Li, X., Lu, R., Zhou, W. and Gao, H.; *Ultrasound-assisted emulsification magnetic microextraction: a fast and green method for the determination of triazole fungicides in fruit juice.* **2014.** Analytical Methods, 6, 8328-8336.

Lord, H.L., *Strategies for interfacing solid-phase microextraction with liquid chromatography.* **2007.** Journal of Chromatography A, 1152(1-2), 2-13.

Lord, H. and Pawliszyn, J.; *Evolution of solid-phase microextraction technology.* **2000.** Journal of Chromatography A, 885(1–2), 153-193.

Luque de Castro, M.D. and Priego-Capote, F.; *Ultrasound-assisted preparation of liquid samples.* **2007.** Talanta, 72(2), 321-334.

Mahugo-Santana, C., Sosa-Ferrera, Z., Torres Padrón, M. and Santana Rodríguez, J.J.; *Application of new approaches to liquid-phase microextraction for the*

determination of emerging pollutants. **2011.** Trends in Analytical Chemistry, 30(5), 731-748.

Millán, S., Sampedro, M.C., Unceta, N., Goicolea, M.A., Rodríguez, E. and Barrio, R.J.; *Coupling solid-phase microextraction and high-performance liquid chromatography for direct and sensitive determination of halogenated fungicides in wine.* **2003.** Journal of Chromatography A, 995(1-2), 135-142.

Mmualefe, L.C., Torto, N., Huntsman-Mapila, P. and Mbongwe, B.; *Headspace solid phase microextraction in the determination of pesticides in water samples from the Okavango Delta with gas chromatography-electron capture detection and time-of-flight mass spectrometry.* **2009.** Microchemical Journal, 91(2), 239-244.

Mohamadi, M. and Mostafavi, A.; *A novel solidified floating organic drop microextraction based on ultrasound-dispersion for separation and preconcentration of palladium in aqueous samples.* **2010.** Talanta, 81(1–2), 309-313.

Munck, L., Nørgaard, L., Engelsen, S.B., Bro, R. and Andersson, C.A.; *Chemometrics in food science—a demonstration of the feasibility of a highly exploratory, inductive evaluation strategy of fundamental scientific significance.* **1998.** Chemometrics and Intelligent Laboratory Systems, 44(1–2), 31-60.

Navarro, S., Pérez, G., Navarro, G., Mena, L. and Vela, N.; Influence of Fungicide Residues on the Primary Fermentation of Young Lager Beer. **2007.** *Journal of Agricultural and Food Chemistry,* 55(4), 1295-1300.

Navarro, S., Vela, N., Pérez, G. and Navarro, G.; *Effect of sterol biosynthesis-inhibiting (SBI) fungicides on the fermentation rate and quality of young ale beer.* **2011.** Food Chemistry, 126(2), 623-629.

Noguerol-Pato, R., González-Rodríguez, R.M., González-Barreiro, C., Cancho-Grande, B. and Simal Gándara, J.; *Influence of tebuconazole residues on the aroma composition of Mencía red wines.* **2011.** Food Chemistry, 124(4), 1525-1532.

Omidikia, N., Abdollahi, H. and Kompany-Zareh, M.; *Visualization and establishment of partial uniqueness and uniqueness rules in parallel factor analysis.* **2013.** Journal of Chemometrics, 27(10), 330-340.

Ozcan, S., Tor, A. and Aydin, M.E.; *Determination of polycyclic aromatic hydrocarbons in waters by ultrasound-assisted emulsification-microextraction and gas chromatography–mass spectrometry.* **2010.** Analytica Chimica Acta, 665(2), 193-199.

Pakade, Y.B. and Tewary, D.K.; *Development and applications of single-drop microextraction for pesticide residue analysis: A review.* **2010.** Journal of Separation Science, 33(23-24), 3683-3691.

Parrilla-Vázquez, P., Mughari, A.R. and Martínez-Galera, M.; *Solid-phase microextraction (SPME) for the determination of pyrethroids in cucumber and watermelon using liquid chromatography combined with post-column photochemically induced fluorimetry derivatization and fluorescence detection.* **2008.** Analytica Chimica Acta, 607(1), 74-82.

Pawliszyn, J.; *Handbook of Solid Phase Microextraction.* **2009.** 1 edn. Chemical Industry Press. Beijing (China).

Pawliszyn, J.; *Solid Phase Microextraction. Theory and Practice.* **1997.** 1 edn. Wiley-VCH. New York (USA).

Pawliszyn, J. and Pedersen-Bjergaard, S.; *Analytical Microextraction: Current Status and Future Trends.* **2006.** Journal of Chromatographic Science, 44(6), 291-307.

Pena-Pereira, F., Lavilla, I. and Bendicho, C.; *Liquid-phase microextraction techniques within the framework of green chemistry.* **2010.** Trends in Analytical Chemistry, 29(7), 617-628.

Peñalver, A., Pocurull, E., Borrull, F. and Marcé, R.M.; *Trends in solid-phase microextraction for determining organic pollutants in environmental samples.* **1999.** Trends in Analytical Chemistry, 18(8), 557-568.

Pinto, M.I., Sontag, G., Bernardino, R.J. and Noronha, J.P.; *Pesticides in water and the performance of the liquid-phase microextraction based techniques. A review.* **2010.** Microchemical Journal, 96(2), 225-237.

Ravelo-Pérez, L.M., Hernández-Borges, J., Borges-Miquel, T.M. and Rodríguez-Delgado, M.A.; *Pesticide analysis in tomatoes by solid-phase microextraction and micellar electrokinetic chromatography.* **2008.** Journal of Chromatography A, 1185(1), 151-154.

Regueiro, J., Llompart, M., Garcia-Jares, C., Garcia-Monteagudo, J.C. and Cela, R.; *Ultrasound-assisted emulsification–microextraction of emergent contaminants and pesticides in environmental waters.* **2008.** Journal of Chromatography A, 1190(1–2), 27-38.

Regulation EC 396/2005 of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. Official Journal of the European Union. **(16.3.2005)** L 70/1-16. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EC 149/2008 of 29 January 2008 amending Regulation (EC) No 396/2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto. Official Journal of the European Union. **(1.3.2008)** L 58/1-398. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EC 459/2010 of 27 May 2010 amending Annexes II, III and IV to Regulation (EC) No 396/2005 of hte European Parliament and of the council as regards maximum residue levels for certain pesticides in or on certain products. Official Journal of the European Union. **(28.5.2010)** L 129/3-49. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 34/2013 of 16 January 2013 amending Annexes II, III and IV to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residuel levels for 2-phenylphenol, ametoctradin, *Aureobasidium pullulans* strains DSM 14940 and DSM 14941, cyproconazole, difenoconazole, dithiocarbamates, folpet, propamocarb, spinosad, spirodiclofen, tebufenpyrad and tetraconazole in or on certain products. Official Journal of the European Union. **(26.1.2013)** L 25/1-48. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 1317/2013 of 16 December 2013 amending Annexes II, III and V to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for 2,4-D, beflubutamid, cyclanilide, diniconazole, florasulam, metolachlor and S-metolachlor, and milbemectin in or on certain products. Official Journal of the European Union. **(17.12.2013)** L 339/1-43. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 61/2014 of 24 January 2014 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for cyromazine, fenopropidin, formetanate, oxamyl and tebuconazole in or on certain products. Official Journal of the European Union. **(24.1.2014)** L 22/1-32. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Ridgway, K., Lalljie, S.P.D. and Smith, R.M.; *Sample preparation techniques for the determination of trace residues and contaminants in foods.* **2007.** Journal of Chromatography A, 1153(1–2), 36-53.

Sánchez-Ortega, A., Sampedro, M.C., Unceta, N., Goicolea, M.A. and Barrio, R.J.; *Solid-phase microextraction coupled with high performance liquid chromatography using on-line diode-array and electrochemical detection for the determination of fenitrothion and its main metabolites in environmental water samples.* **2005.** Journal of Chromatography A, 1094(1-2), 70-76.

Sarafraz-Yazdi, A. and Amiri, A.; *Liquid-phase microextraction.* **2010.** Trends in Analytical Chemistry, 29(1), 1-14.

Saraji, M. and Boroujeni, M.K.; *Recent developments in dispersive liquid–liquid microextraction.* **2014.** Analytical and Bioanalytical Chemistry, 406(8), 2027-2066.

Scherm, H., Christiano, R.S.C., Esker, P.D., Del Ponte, E.M. and Godoy, C.V.; *Quantitative review of fungicide efficacy trials for managing soybean rust in Brazil.* **2009.** Crop Protection, 28(9), 774-782.

Sidiropoulos, N.D. and Bro, R.; *On the uniqueness of multilinear decomposition of N-way arrays.* **2000.** Journal of Chemometrics, 14, 229-239.

Smilde, A., Bro, R. and Geladi, P.; *Multi-way analysis. Applications in the chemical sciences.* **2004.** John Wiley & Sons Ltd. West Sussex (England).

Spietelun, A., Marcinkowski, Ł, de la Guardia, M. and Namieśnik, J.; *Recent developments and future trends in solid phase microextraction techniques towards green analytical chemistry.* **2013.** Journal of Chromatography A, 1321, 1-13.

Spietelun, A., Marcinkowski, Ł, de la Guardia, M. and Namieśnik, J.; *Green aspects, developments and perspectives of liquid phase microextraction techniques.* **2014.** Talanta, 119, 34-45.

Stalikas, C., Fiamegos, Y., Sakkas, V. and Albanis, T.; *Developments on chemometric approaches to optimize and evaluate microextraction.* **2009.** Journal of Chromatography A, 1216(2), 175-189.

Stross, F.H. and Ambrose, D.; *Preliminary reccomendations on nomenclature and presentation of data in gas chromatography.* **1960.** Pure and Applied Chemistry, 1(1), 177-186.

Tauler, R., *Multivariate curve resolution applied to second order data.* **1995.** Chemometrics and Intelligent Laboratory Systems, 30(1), 133-146.

Taxvig, C., Hass, U., Axelstad, M., Dalgaard, M., Boberg, J., Andeasen, H.R. and Vinggaard, A.M.; *Endocrine-Disrupting Activities In Vivo of the Fungicides Tebuconazole and Epoxiconazole.* **2007.** Toxicological Sciences, 100(2), 464-473.

Tomlin, C.D.S.; *The Pesticide Manual.* **2000.** 12 edn. The British Crop Protection Council. Farnham (UK).

Vega Moreno, D., Sosa Ferrera, Z. and Santana Rodríguez, J.J.; *Sample Extraction Method Combining Micellar Extraction−SPME and HPLC for the Determination of Organochlorine Pesticides in Agricultural Soils.* **2006.** Journal of Agricultural and Food Chemistry, 54(20), 7747-7752.

Viñas, P., Campillo, N., Martínez-Castillo, N. and Hernández-Córdoba, M.; *Method development and validation for strobilurin fungicides in baby foods by solid-phase microextraction gas chromatography–mass spectrometry.* **2009.** Journal of Chromatography A, 1216(1), 140-146.

Wang, Z., Hennion, B., Urruty, L. and Montury, M.; *Solid-phase microextraction coupled with high performance liquid chromatography: a complementary technique to solid-phase microextraction-gas chromatography for the analysis of pesticide residues in strawberries.* **2000.** Food Additives and Contaminants, 17(11), 915-923.

Wei, S., Leong, M., Li, Y. and Huang, S.; *Development of liquid phase microextraction based on manual shaking and ultrasound-assisted emulsification method for analysis of organochlorine pesticides in aqueous samples.* **2011.** Journal of Chromatography A, 1218(51), 9142-9148.

Wolf, D.C., Allen, J.W., George, M.H., Hester, S.D., Sun, G., Moore, T., Thai, S., Delker, D.A., Winkfield, E., Leavitt, S., Nelson, G., Roop, B.C., Jones, C., Thibodeaux, J. and Nesnow, S.; *Toxicity profiles in rats treated with tumorigenic and nontumorigenic triazole conazole fungicides: propiconazole, tridimefon and myclobutanil.* **2006.** Toxicologic Pathology, 34(7), 895-902.

Xia, Y., Zhi, X., Wang, X., Chen, M. and Chen, J.; *Ultrasound-enhanced surfactant-assisted dispersice liquid-liquid microextraction and high-performance liquid chromatography for determination of ketoconazole and econazole nitrate in human blood.* **2012.** Analytical and Bioanalytical Chemistry, 402, 1241-1247.

Yan, H., Wang, H., Qin, X., Liu, B. and Du, J.; *Ultrasound-assisted dispersive liquid–liquid microextraction for determination of fluoroquinolones in pharmaceutical wastewater.* **2011.** Journal of Pharmaceutical and Biomedical Analysis, 54(1), 53-57.

You, X., Wang, S., Liu, F. and Shi, K.; *Ultrasound-assisted surfactant-enhanced emulsification microextraction based on the solidification of a floating organic droplet used for the simultaneous determination of six fungicide residues in juices and red wine.* **2013.** Journal of Chromatography A, 1300, 64-69.

Zhang, J., Gao, H., Peng, B., Li, Y., Li, S. and Zhou, Z.; *Simultaneous determination of four synthesized metabolites of mequindox in urine samples using ultrasound-assisted dispersive liquid–liquid microextraction combined with high-performance liquid chromatography.* **2012a.** Talanta, 88, 330-337.

Zhang, L., Liu, S., Cui, X., Pan, C., Zhang, A. and Chen, F.; *A review of sample preparation methods for pesticide residue analysis.* **2012b.** Central European Journal of Chemistry, 10(3), 900-925.

Zhang, Y., Zhang, X., Chen, C., Zhou, M. and Wang, H.; *Effects of fungicides JS399-19, azoxystrobin, tebuconazloe, and carbendazim on the physiological and biochemical indices and grain yield of winter wheat.* **2010.** Pesticide Biochemistry and Physiology, 98(2), 151-157.

2. Objectives

2. Objectives

The main objective of the thesis was to get procedures that allow easily and confidently the determination of the triazole fungicides in different samples. Diniconazole, fluquinconazole, flusilazole, myclobutanil, tebuconazole and tetraconazole were the studied triazoles in this work. The main objective was accomplished with the following specific objectives:

1. To develop procedures based on microextraction techniques. Microextraction techniques were selected due to its inherent green characteristics. Thus, techniques with absence of organic solvents such as Solid Phase Microextraction (SPME) or with a very limited use of organic solvent as Ultrasound Assisted Emulsification Microextraction (USAEME) were chosen.

2. To extend the use of GC/ECD and HPLC/DAD chromatographic techniques. The equipments were considered since they can be easily coupled to the microextraction procedures and its affordable cost allows the acquisition by routine laboratories.

3. To improve the procedures and the results using chemometric tools. Firstly, with the use of experimental design to select and optimize the variables involved in the microextraction processes. Secondly, with the use of multivariate analysis in the post-processing of the obtained data.

4. To apply the optimized procedures to fruit and liquid samples. Due the occurrence of these pesticides in apple and grape orchards in the Basque Country region, these samples were selected to check the adequacy of the methods.

3. Development of a method for triazole fungicides determination in juice samples using solid-phase microextraction and GC/ECD

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3. Development of a method for triazole fungicides determination in juice samples using solid-phase microextraction and GC/ECD

3.1 Introduction

Triazole fungicides are one of the most common groups of pesticides applied to vineyards and apple orchards. Besides its antifungal activity, those compounds are also discussed as a group that disturbs endocrine activity in humans. Azole fungicides used in agriculture are moderately lipophilic and fairly persistent with typical half lives of weeks to months. They may reach the aquatic environment mainly by surface runoff and spray drift. Those chemicals can reach plant tissues leaving residues that can be detected in fruits and processed products. The determination of residue levels is necessary for food safety monitoring and regulatory purposes. In order to protect the health of consumers maximum residue levels (MRLs) in raw products have been established. European Union has fixed MRLs ranging from 0.01 to 1 mg kg^{-1} in apples and grapes for diniconazole, myclobutanil, tebuconazole, and tetraconazole [Regulation EC 149/2008; Regulation EU 34/2013; Regulation EU 1317/2013; Regulation EU 61/2014]. However, there is no legislation for processed products such as fruit juices.

After some preliminary treatment steps, the determination of pesticides in food matrices mostly use gas chromatography (GC) or liquid chromatography (LC) with mass spectrometry (MS) detectors. In recent years, considerable efforts have been made trying to develop new sample preparation techniques that save time, labor, and solvent consumption. In this respect, several procedures based on solid-phase extraction (SPE), quick, easy, cheap, effective, rugged and safe (QuEChERS) methodology, stir bar sorptive extraction and solid-phase microextraction (SPME) have been used in multiple pesticide residue determination [Lambropoulou and Albanis, 2007; Picó *et al.*, 2007; Cunha *et al.*, 2009; Jiang *et al.*, 2009; Jin *et al.*, 2012]. Referring to azole fungicides methods based on solid-phase microdispersion followed by LC with ultraviolet diode array detection in fruit pulps, LC/MS/MS and dispersive liquid–liquid micro-extraction, and GC/MS or GC/ECD in wine samples have been developed [Bicchi

et al., 2001; Trösken *et al.*, 2005; Montes *et al.*, 2009]. SPME is a useful technique that does not require solvents and can be carried out directly from the liquid phase (direct immersion (DI)) or from the headspace (HS) over the samples [Pawliszyn, 1997]. SPME has been used as a sample preparation method followed by GC/MS for the analysis of different pesticides in water [Beceiro-González *et al.*, 2007], triazole residues in wine and strawberries [Zambonin *et al.*, 2002], pesticide residues in fruit juice [Cortés-Aguado *et al.*, 2008] and multi class pesticides in mango fruit [Menezes Filho *et al.*, 2010]. Also, SPME coupled to LC/MS has been used for fungicide determination in fruits [Blasco *et al.*, 2003] and for determination of carbamate and phenyl urea pesticide residues in fruit juices [Sagratini *et al.*, 2007].

There are several variables affecting the SPME procedure, among them fiber type, extraction temperature, extraction time, stirring rate, and desorption conditions. The design of experiment (DoE), that takes into account simultaneously several variables and its interaction effects, seems to be an appropriate way to find the convenient experimental conditions with a reduced number of experiments. Response surface designs are used during method optimization to determine optimal conditions for the factors that have the most influence on the response of interest [Van den hauwe *et al.*, 2002]. Also, factorial designs have considered to assess the influence of several factors and to obtain the best conditions. This approach has been presented in the developing a SPME followed by the GC/ECD for multiresidue analysis of pesticides in milk [Fernandez-Alvarez *et al.*, 2008]. Although choice of an experimental design ultimately depends on the objectives of the study and the number of factors to be investigated, a thorough approach takes into account two steps, the screening and the optimization. Screening techniques, such as factorial designs, allow to select which factors are significant and at what levels. During the optimization response surface designs, including central composite design (CCD), are often applied to determine the optimal experimental conditions [Stalikas *et al.*, 2009]. For instance, the latter approach in two steps has been used for organochlorine pesticides and polychlorinated biphenyls determination in human serum using SPME and GC/ECD [López *et al.*, 2007]. More recently, the DoE has been applied to organotin compounds determination in water samples [Coscollà *et al.*, 2014] and hydrazine determination in drinking water [Gionfriddo *et al.*, 2014]. In the first work, factors affecting SPME in HS mode and GC/MS/MS were considered in DoE. In the second one, SPME process and tandem mass spectrometry parameters were taken into account in the multivariate optimization.

The goal of the work was to develop an appropriate green method for the determination of triazoles in juices samples using SPME followed by GC/ECD. In the method the advantageous characteristics of GC and its inherently green character compared with LC, and the SPME technique that avoid the use of clean-up steps and the hazardous solvents were considered. The experimental design was planned in two steps, the first one using a screening design to find which experimental conditions were significant. Secondly, with an optimization design trying to obtain the best values of significant variables for the determination of triazoles. The application of the optimized procedure was checked by analysis of different apple and grape juice samples.

3.2 Experimental

3.2.1 Reagents and equipment

Diniconazole (99.8%, PESTANAL®) and tebuconazole (99.6%, PESTANAL®) were supplied by Sigma- Aldrich (Madrid, Spain), myclobutanil (99.4%) was acquired at LGC Standards (Barcelona, Spain) and tetraconazole (97.5%) from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The methanol used for the stock solutions was purchased from Teknokroma (Barcelona, Spain).

In the sample treatment, a Beckman Coulter centrifuge (Fullerton, USA) with a 25.5 rotor (with maximum and minimum radium of 108 mm and 38.5 mm respectively) and 0.45 µm sterilized filters (Albet- Hahnemuehle, Barcelona, Spain) were used.

SPME holders and fibers [85µm polyacrilate (PA) and 65 µm polydimethylsiloxane/divinylbencene (PDMS/DVB)], sample vials (40 mL amber glass) and PTFE silicone septa were obtained from Supelco (Bellefonte, PA, USA). For the extraction a vessel with thermostatic jacket joined to a cooling thermostat (Lauda RE 104) was used. Inside this vessel a 40 mL amber vial with a 20 mm PTFE coated stir bar was placed.

The separation and quantification were performed in a HP 6890N (Agilent, Wilmington, DW, USA) gas chromatograph with an electron capture detector. The liner

inner diameter was 0.75 mm and was acquired in Supelco. The column used was an HP-5 [(5%-Phenyl)-methylpolysiloxane] (30 m x 0.250 mm x 0.25 µm film thickness) capillary column (Agilent). The analytes were desorbed in the injector at 260°C, where the valve was opened 2 min after injection. The carrier gas was Helium with a 2.0 mL min^{-1} flow. The oven was programmed to separate the analytes as following: initial temperature starts at 60°C for 2 min, then a ramp of 20°C min^{-1} until 280°C held for 4 min. The overall chromatogram time was 15 minutes. The electron capture detector temperature was fixed at 280°C.

Using the above mentioned gradient temperature the studied analytes eluted as follows: tetraconazole 11.5 min, myclobutanil 12.4 min, diniconazole 12.7 min and the last analyte, tebuconazole 13.3 min.

The software used for the experimental design and the results analysis was STATISTICA (StatSoft, Tulsa, USA).

Individual analyte stock solutions were prepared in 1000 mg L^{-1} concentration for tetraconazole, myclobutanil and diniconazole. Tebuconazole was added later due its sensitivity with the method was not as high as the other analytes and need to be in much higher concentrations. Five different mix solutions were prepared from these stock solutions adding also tebuconazole in this step. These were the solution spiked to the vial adding 25 µL to 25 mL of water. The final concentrations obtained for analysis are shown in table 3.1.

Table 3.1. *Final analyte concentrations for SPME analysis.*

	Standard Concentrations (µg L^{-1})				
	Standard 1	**Standard 2**	**Standard 3**	**Standard 4**	**Standard 5**
Tetraconazole (TT)	0.78	3.90	5.20	7.80	10.40
Myclobutanil (M)	8.7	43.5	58.0	87.0	106.0
Diniconazole (D)	0.38	1.91	2.55	3.83	5.10
Tebuconazole (TB)	190	950	1280	1900	2560

3.2.2 Samples

Apple and grape samples were collected in different places over Basque Country, Navarra and La Rioja (Northern Spain) in September 2009. These samples were the fruits used for the production of typical alcoholic beverages in the area: Rioja wine, txakolí (white wine) and cider. The varieties of harvested grapes were different; two were "Tempranillo" and the other three, "Hondarribi Zuri". The first variety was used to make red wine and the last one, white wine. The apple samples collected in different farmhouses were used to making cider. Cidermakers did not specify the varieties of the apples.

Five grape samples were collected in Alesanco (1), Hormilleja (2), Gamiz-Fika (3) and Zarautz (4-5). Eight apple samples were collected in Renteria (7-14), two in Ataun (18-19) and three in Santesteban (15-17). Besides, two control samples were collected in Orio, one for grape (6) and other for apple (20). The control samples, located in the countryside, did not have any pesticide treatment. Figure 3.1 shows the locations in the map.

Figure 3.1. *Northern Spain map with the sampling location points.*

After collecting the samples were blended and centrifuged at 21000 rpm (53300 x g) during 8 minutes. Afterward the juices were filtered with a 0.45 µm pore size filter and were kept at the freezer in amber vials at -20°C until the analysis.

3.2.3 SPME procedure

For SPME extraction procedure, firstly, samples were defrosted and homogenized. Then 1 mL was diluted with 24 mL of water in a 40 mL amber vial capped with a PTFE-coated silicone septum. The vial was placed in a water jacket connected to a thermostatic bath controlling the temperature at 60°C. The magnetic stirrer was put at 500 rpm to keep homogenized the liquid phase in the vial. Figure 3.2 shows the assembly of the heating jacket vessel with the extraction vial and the SPME holder.

The 65 µm PDMS/DVB fiber needle pierced the cap of the vial and the coating was exposed to the sample in direct immersion (DI) mode during the extraction time (45 min). After the extraction time the fiber was withdrawn into the holder and placed in the GC injector port. The analytes were desorbed thermally at 260°C during 3 minutes. A cleanup step was done to avoid the possible carry over effect. After desorption in the GC inlet port, the fiber was introduced in water with a gentle agitation for fifteen minutes previous to subsequent analysis.

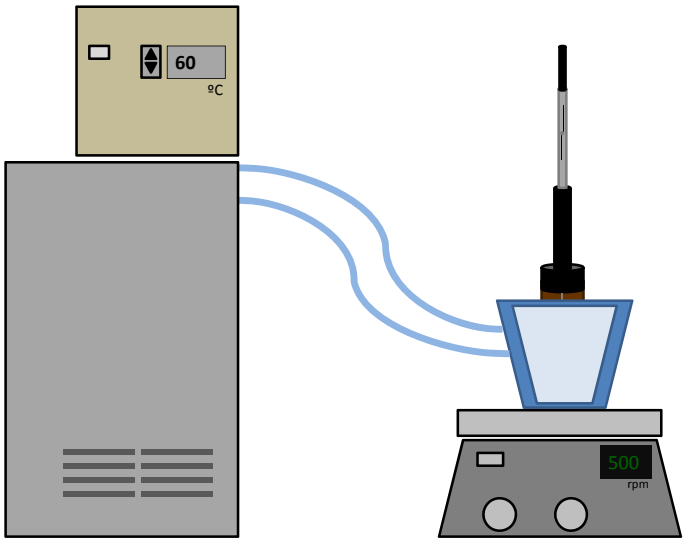

Figure 3.2. *Schematic diagram of the assembly for extraction procedure.*

The quantification of triazoles was performed using standard addition method using mixed standards (see table 3.1). The final concentrations in vials varied between 0.78 and 10.40 μg L^{-1} for tetraconazole, 8.7 and 106.0 μg L^{-1} for myclobutanil, 0.38 and 5.10 μg L^{-1} for diniconazole and 190-2560 μg L^{-1} for tebuconazole. Blanks were run periodically to assess the absence of contaminants.

3.3 Results and discussion

The aim of the work was to develop a suitable method using SPME for the determination of triazole fungicides in fruit juice samples. There are many variables in the extraction process that can lead to a different response. Firstly, following an experimental design, an election of these variables and its best values were fixed. Once the optimum values were known the work was continued with the calibration and validation. Finally, the method was applied to real samples.

3.3.1 Experimental design

Firstly, it is necessary to define the experimental conditions. Based on the literature and previous experience in laboratory the variables and its ranges were chosen [Beltran *et al.*, 2000; Zambonin *et al.*, 2002; Lasa *et al.*, 2006; López *et al.*, 2007; Cortés-Aguado *et al.*, 2008; Zuazagoitia *et al.*, 2009]. An experimental design with two steps (screening and optimization) was used for searching the best experimental conditions. The screening was performed with a fractional factorial design and for the optimization a central composite design was chosen.

The analyte concentrations in the vials used for the whole screening design, and also the following optimization step were: 1 μg L^{-1} for diniconazole and tetraconazole, 100 μg L^{-1} for myclobutanil and 1 mg L^{-1} for tebuconazole.

3.3.1.1 Screening. Fractional Factorial Design

A screening design was performed in order to evaluate the significance of the variables. For this study six variables were chosen: fiber type (PA, PDMS/DVB), extraction temperature and time, desorption temperature and time and agitation speed. Among variables there were qualitative or quantitative ones. The qualitative or categorical variables are not numerical variables, such as fiber type. The quantitative variables are considered the continuous variables; that is, the numerical variables, for instance, temperature, time or agitation speed.

The salt addition variable that has been mentioned in the introduction chapter was not considered. Preliminary experiments showed troubles with NaCl addition due to the crystallization. Although a cleaning step was included in the procedure, the crystallization when the fiber was exposed to high temperatures was almost unavoidable. This implies some maintenance problems due to the frequent breakage of the fiber. Therefore this variable was not included in the DoE.

The applied design was a 2^{6-2} design. With this fractional factorial design a resolution IV was obtained. This resolution is able to distinguish the principal effects and also second order interaction of the main effects. Although this resolution is not a complete resolution and leads to confuse minor interactions between effects, the experiment number was reduced to a quarter. Considering a complete design (2^6), the 64 total number of experiments was reduced to 16. In this study with the inclusion of two more central the total number of experiments was 18.

Table 3.2 shows the variables and levels taken into account for this study. Fiber type was the only qualitative variable, the chosen coatings were PA (85 µm) and PDMS/DVB (65 µm). Extraction temperature was considered between 20 and 50°C; time was established 15 to 30 minutes and desorption temperature and time ranges were 250-270°C and 3-9 min respectively. Agitation with the magnetic stirrer was between 300 to 500 rpm. From now on the actual data will be shown in its coded values. The coded values were [-1] for the lowest value and [+1] for the highest value. In case of the categorical variable the coded value for PA was [-1] and for PDMS/DVB was [+1].

Table 3.2. *Considered variables and its levels in the 2^{6-2} fractional factorial design.*

Variables	Type	Levels	
		Low [-1]	High [+1]
Fiber type [Fiber]	qualitative	PA	PDMS/DVB
Extraction temperature [Ext T] (°C)	quantitative	20	50
Extraction time [Ext t] (min)	quantitative	15	30
Desorption temperatura [Des T] (°C)	quantitative	250	270
Desorption time [Des t] (min)	quantitative	3	9
Agitation (rpm)	quantitative	300	500

Table 3.3 shows the experimental matrix and the runs that were randomly carried out. The levels of the variables are indicated in the coded values. The code 0 indicates the central point (only for quantitative variables). That was the mean value between the low and the high level. For instance, 35 °C for extraction temperature, 260 °C for desorption temperature, or 400 rpm for agitation. The right part of the table shows the areas obtained for each analyte in a specific experimental run.

Table 3.3. *Coded matrix of the 2^{6-2} fractional factorial design with the random order of experiments and the analytes responses in peak areas.*

Experiment	Variables						Areas			
	Fiber	Ext T	Ext t	Des T	Des t	Agitation	TT	M	D	TB
10	-1	-1	-1	1	1	1	11377	15164	3214	3867
17 C	1	0	0	0	0	0	44538	105932	8491	18957
4	-1	1	-1	-1	-1	1	20093	31703	5532	10013
9	1	-1	-1	1	-1	1	34630	67005	6358	11892
6	-1	-1	1	-1	-1	1	17776	23507	4467	6804
8	-1	1	1	-1	1	-1	24935	40915	6634	12538
1	1	-1	-1	-1	-1	-1	21960	52025	3987	9209
18 C	-1	0	0	0	0	0	36202	54243	9122	14379
14	-1	-1	1	1	-1	-1	24184	36605	6945	8678
3	1	1	-1	-1	1	1	27890	61164	7836	12075
12	-1	1	-1	1	-1	-1	21924	38062	8123	11199
13	1	-1	1	1	1	-1	42976	92470	8319	15857
5	1	-1	1	-1	1	1	44827	89165	9030	15311
11	1	1	-1	1	1	-1	19785	54645	4647	10762
16	-1	1	1	1	1	1	26337	38707	7247	12995
15	1	1	1	1	-1	1	53145	96196	10862	22795
2	-1	-1	-1	-1	1	-1	6275	11320	1598	3074
7	1	1	1	-1	-1	-1	46046	110346	9290	23232

C: Central point
TT: Tetraconazole
M: Myclobutanil
D: Diniconazole
TB: Tebuconazole

The data obtained were analyzed using analysis of variance (ANOVA) (table 3.4) and visualized using Pareto charts (Figures 3.3 and 3.4). With these charts the main effects of the fractional factorial design were observed. Interactions were not considered as significant, therefore the table and the figures show only the principal effects in the process.

F-test is used for comparing two variables. Fischer coefficient (*F-test*) was calculated for each case dividing the variable mean square effect by the error mean square effect and this coefficient is compared with *F*-distribution table, taking into account the degrees of freedom. If the calculated *F* is higher than the table-*F* means the variable is significant. The *p*-value, shows the probability that a certain phenomena occurred by pure chance, therefore if this value is little means that the variation in the system was not pure chance. The border is established in p=0.05. Values lower than 0.05 are significant variables.

Table 3.4 shows the ANOVA results for each analyte. Numbers in red are the significant variables. As it can be seen in the table, fiber and extraction time were important variables for tetraconazole and myclobutanil. For diniconazole extraction time was also a significant variable but the fiber coating type was not significant. Nevertheless extraction temperature was important for this analyte. At last, for tebuconazole fiber type, extraction temperature and time were the significant variables.

Table 3.4 *ANOVA for the studied analytes showing the significance of the variables.*

		SS[a]	df[b]	MS[c]	*F*[f]	*p*[g]
TETRACONAZOLE						
Variables	**Fiber**	1.195491E+09	1	1.195491E+09	21.15666	0.000765
	Ext T	8.167912E+07	1	8.167912E+07	1.44548	0.254497
	Ext t	8.452393E+08	1	8.452393E+08	14.95824	0.002619
	Des T	3.768609E+07	1	3.768609E+07	0.66693	0.431454
	Des t	7.813057E+07	1	7.813057E+07	1.38268	0.264467
	Agitation	4.896711E+07	1	4.896711E+07	0.86657	0.371886
ERROR		6.215726E+08	11	5.650660E+07		
Total SS		2.908766E+09	17			
MYCLOBUTANIL						
		SS[a]	df[b]	MS[c]	*F*[f]	*p*[g]
Variables	**Fiber**	1.069315E+10	1	1.069315E+10	48.48304	0.000024
	Ext T	4.460206E+08	1	4.460206E+08	2.02227	0.182737
	Ext t	2.421240E+09	1	2.421240E+09	10.97797	0.006913
	Des T	2.187807E+07	1	2.187807E+07	0.09920	0.758691
	Des t	1.683364E+08	1	1.683364E+08	0.76324	0.400986
	Agitation	1.186389E+07	1	1.186389E+07	0.05379	0.820851
ERROR		2.426098E+09	11	2.205544E+08		
Total SS		1.618858E+10	17			
DINICONAZOLE						
		SS[a]	df[b]	MS[c]	*F*[f]	*p*[g]
Variables	**Fiber**	14112391	1	14112391	4.640053	0.054261
	Ext T	16512032	1	16512032	5.429038	0.039867
	Ext t	28887400	1	28887400	9.497970	0.010436
	Des T	3365941	1	3365941	1.106697	0.315362
	Des t	3099184	1	3099184	1.018990	0.334448
	Agitation	1565627	1	1565627	0.514767	0.488039
ERROR		33455716	11	3041429		
Total SS		100998291	17			
TEBUCONAZOLE						
		SS[a]	df[b]	MS[c]	*F*[f]	*p*[g]
Variables	**Fiber**	177613500	1	177613500	23.76105	0.000491
	Ext T	104641647	1	104641647	13.99891	0.003258
	Ext t	132938594	1	132938594	17.78446	0.001443
	Des T	2095691	1	2095691	0.28036	0.606986
	Des t	18798728	1	18798728	2.51488	0.141083
	Agitation	90541	1	90541	0.01211	0.914347
ERROR		82224853	11	7474987		
Total SS		518403554	17			

[a]SS: Sum of Squares

[b]df: degrees of freedom

[c]MS: Mean Square effect

[f]*F*: Fischer coefficient

[g]*p*: Probability

Pareto charts show the estimation of standardized effects of the variables. The line of the *p*-value drew in the charts separate the significant values at the right. The positive and negative sign in Pareto charts indicate that the response is enhanced or reduced, respectively, when passing a given factor from the lowest to the highest level.

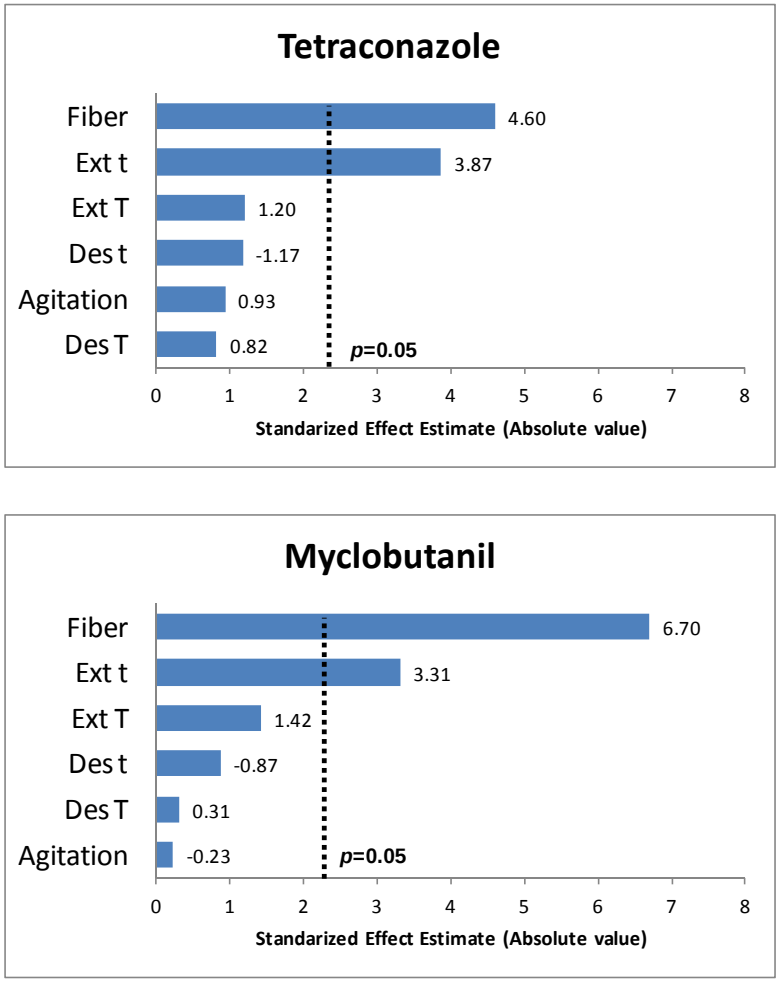

Figure 3.3. *Pareto charts for tetraconazole and myclobutanil. Values after the bars indicate the absolute values of the main effects and the dotted line indicates 95% confidence level (p=0.05).*

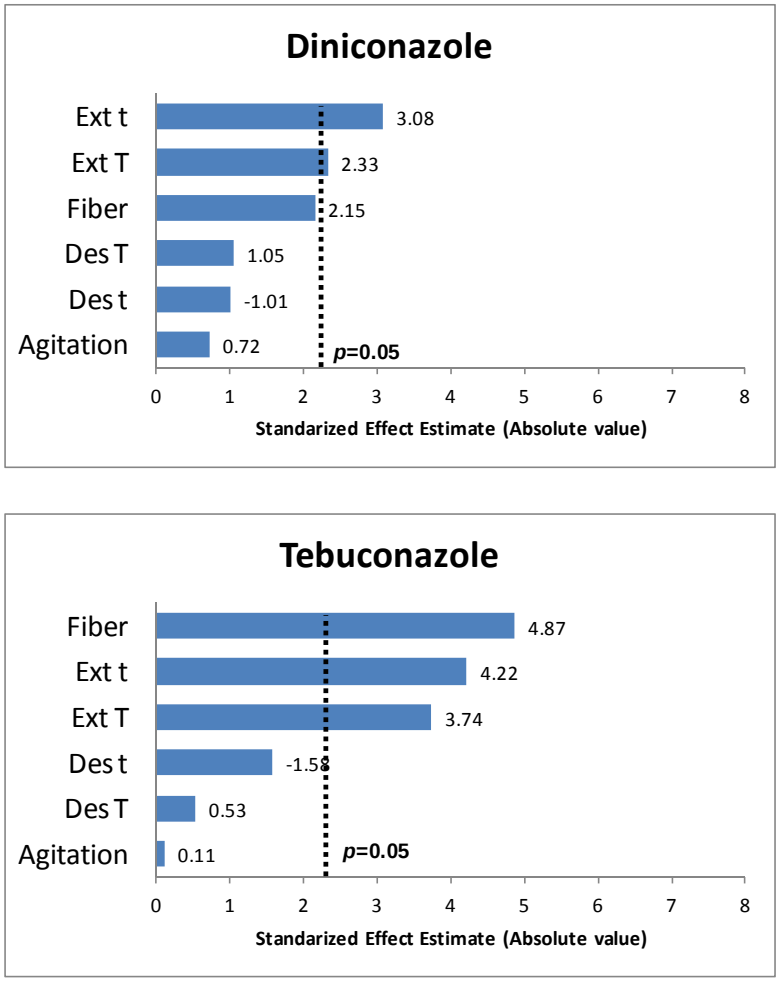

Figure 3.4. *Pareto charts for diniconazole and tebuconazole. Values after the bars indicate the absolute values of the main effects and the dotted line indicates 95% confidence level (p=0.05).*

Taking into account ANOVA results and Pareto charts, the fiber type was significant. The positive sign was assigned to the PDMS/DVB (see table 3.2); therefore, PDMS/DVB was the chosen coating. Furthermore, the main effect indicated by the large of the bar suggests that in tetraconazole, myclobutanil and tebuconazole the fiber was the most important variable. For diniconazole extraction with PDMS/DVB fiber was also preferred, but was not significant even though it was very close to the *p*=0.05 line.

The extraction time was considered significant for all the analytes. The positive sign indicates that the best values for the experimental system were the highest values. This fact was taking into consideration for following optimization studies.

The extraction temperature was significant for diniconazole and tebuconazole and both indicated better results with higher values. For tetraconazole and myclobutanil this factor was not significant, but higher extraction temperatures gave also better results.

The other three factors, agitation in the extraction and desorption time and temperature, were not considered significant. Therefore, these variables were fixed at the convenient values. Table 3.5 summarizes the results. The underlined values indicate significant variables and positive and negative values indicate the preferred coded value. Remark that even the preferred desorption temperature was 270°C the chosen temperature was 260°C trying to avoid excessive damage of the fiber coating, due to the maximum temperature recommended by the supplier of the fiber was 270°C.

Once the qualitative and not-significant variables are fixed the method followed with the optimization step (shaded variables in table 3.5) considering the two significant variables: extraction time and temperature.

Table 3.5. R*esults of the screening design.*

Variable	TT	M	D	TB	Action
Fiber type	+1	+1	+1	+1	Fixed: **PDMS/DVB**
Ext T	+1	+1	+1	+1	**To optimization**
Ext t	+1	+1	+1	+1	**To optimization**
Des T	+1	+1	+1	+1	Fixed: **260º C**
Des t	-1	-1	-1	-1	Fixed: **3 min**
Agitation	+1	-1	+1	+1	Fixed: **500 rpm**

3.3.1.2 Optimization. Central Composite Design

Once the screening step was done, the number of the experiments for optimization was reduced focusing in the two significant variables. A central composite design (CCD) was used for optimization. Since both variables in the Pareto charts indicated the highest values as the best values, the range for optimization was closer to the higher values.

A 2^2 factorial design was increased by [(2 x 2) + 1] star or axial points. Star points were located at [–α] and [+α] from the central point using the rotatability condition. Using $\alpha = \sqrt[4]{N_f} = 1.4142$ ($N_f = 2^2$) was established the axial distances: 46 and 74°C for the temperature and 16 and 44 minutes for the time. The different experimental condition points are shown in figure 3.5. Six extra central points were added to the design to estimate the experimental error. In total, the number of assays was fifteen (2^2 + [(2 x 2) + 1] + 6).

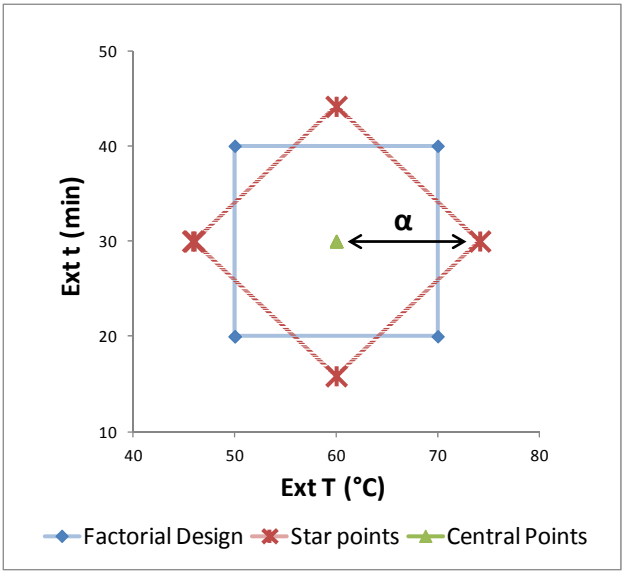

Figure 3.5. *Graphical output of the CCD with the extraction time and temperature variables.*

The variables taking into account for optimization and its values are shown in table 3.6. Extraction time ranged from 20 to 40 min adding star points at 16 and 44

min. On the other hand, the extraction temperature range was established between 50 and 70°C plus the star points at 46 and 74°C.

Table 3.6. *The considered variables and its levels in the central composite design.*

Variables	Levels				
	Low [-1]	Central [0]	High [+1]	[-α]	[+α]
Extraction temperature [Ext T] (°C)	50	60	70	46	74
Extraction time [Ext t] (min)	20	30	40	16	44

Once the experimental matrix was designed, the experiments were randomly carried out as indicated in table 3.7. The statistical analysis was performed using chromatographic areas as the response.

Table 3.7. *The randomly carried out experimental matrix with the coded variables and the chromatographic responses for the analytes.*

Experiment	Variables		Areas			
	Ext T	Ext t	TT	M	D	TB
2	-1	1	10111	108549	17784	26102
11 C	0	0	7792	86180	14438	22414
3	1	-1	5612	63572	10631	17495
13 C	0	0	12192	94700	19482	31734
5	-α	0	10110	82943	14315	24125
10 C	0	0	14493	89770	16059	27743
15 C	0	0	16780	84968	15977	27214
7	0	-α	6386	25928	7813	9301
4	1	1	14080	72142	17803	24537
12 C	0	0	10644	96053	16694	28070
1	-1	-1	6718	64856	10125	17616
6	α	0	9302	99772	17377	30942
14 C	0	0	9814	107411	19141	31338
8	0	α	13152	134843	25815	39141
9 C	0	0	8646	99807	17683	28269

C: Central point

The obtained data were analyzed using STATISTICA software. These data is used to build models in order to know the best experimental conditions. A second-degree polynomial model was used including the main effects for the two factors, their interaction and their quadratic components. The R^2 obtained for each triazole was considered as indication of adequacy of the model. The regression coefficients obtained are used to compute predicted values of the dependent variable for the different combinations of the levels of the independent variables.

The most common way to summarize the results of a CCD experiment is in the form of a response surface. This can be done in 3D plots selecting two factor (i.e. temperature and time). In this study, four 3D plots belonging to each of the triazole compounds can be obtained. Instead of these independent response surfaces, the global desirability function was chosen.

Among different desirability function approaches [Costa *et al.*, 2011] Derringer and Suich proposed one of the most used method for simultaneous optimization of several variables [Derringer and Suich, 1980]. The method is based on desirability transformations. The desirability function for each dependant variable was fixed by assigning desirability values of 0.0 (for undesirable, lowest chromatographic response in this work), 0.5 (medium) and 1.0 (for very desirable, highest chromatographic response in this work).

Figure 3.6 shows individual plots of predicted values and desirability for each analyte, plus the last plot line an overall desirability trend of analytes. Plots in the first column express extraction temperature trend and second column shows the extraction time trend. For each analyte the evolution among variables differed a bit, but the results shows the same conditions as the optimum variables as it can be seen the last line plots.

Instead of individual desirability function, an overall desirability surface plot was also obtained (Figure 3.7). Extraction time and temperature were drawn in x and y-axis and desirability was expressed in the z-axis. Color scale indicated the desirability, that is, red intense correspond to the best response conditions and the green color indicate the worse response.

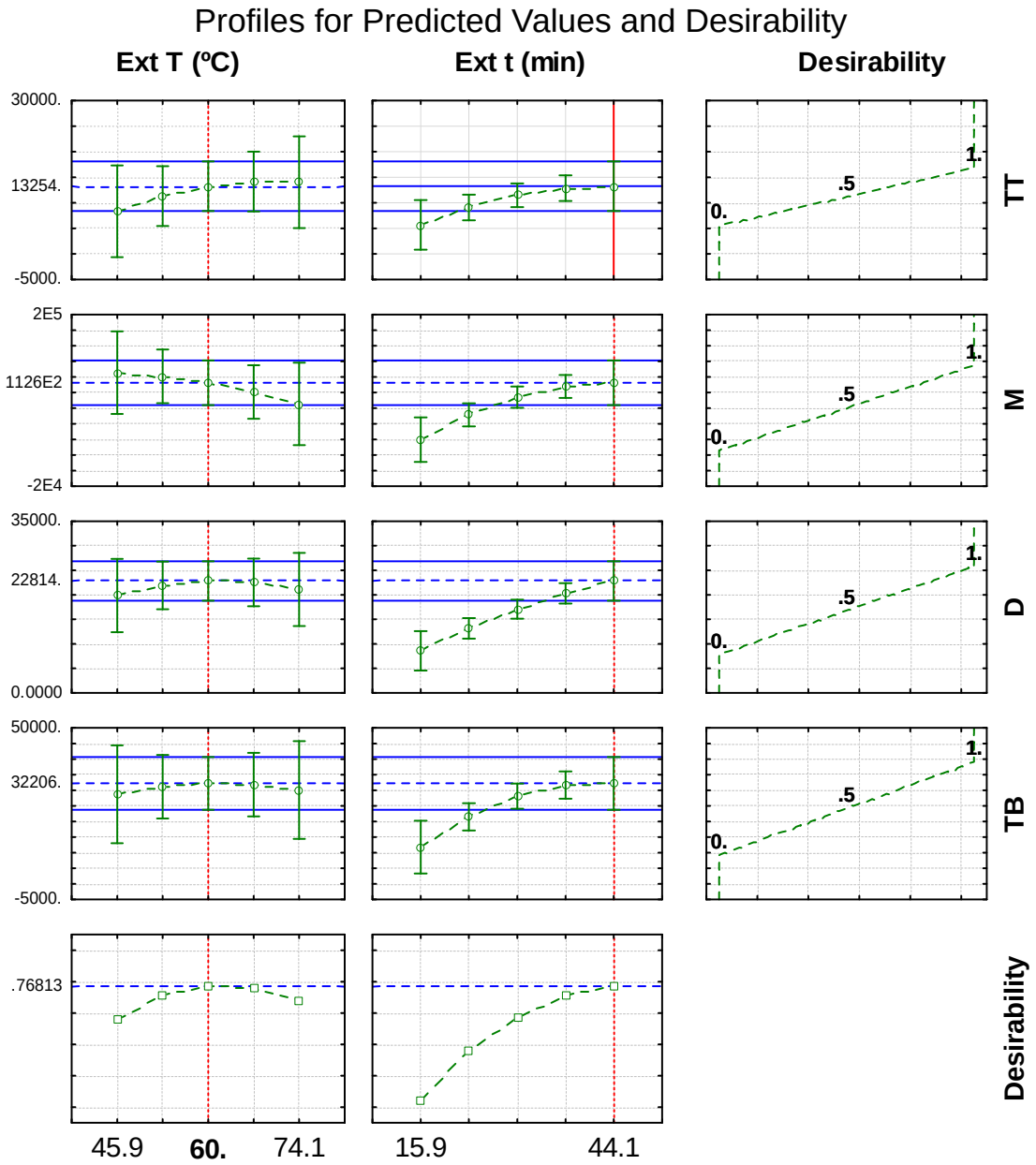

Figure 3.6. *Profiles for predicted values and desirability in each of the analytes.*

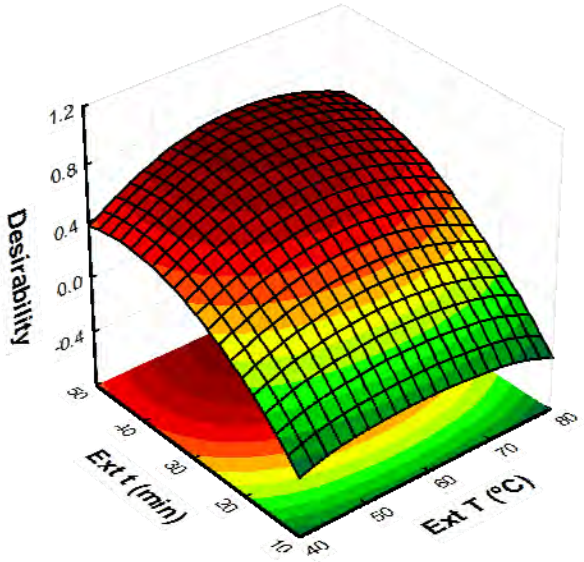

Figure 3.7. *Global desirability surface response from CCD design considering time and temperature extraction for the triazole determination.*

As it can be seen, according to figures 3.6 and 3.7, optimum values were obtained with 60°C for extraction temperature and 45 minutes for extraction time.

Taking into account the results from the screening and optimization studies, the working extraction conditions to obtain the best response for determination of the triazoles were:

- **Fiber coating: PDMS/DVB**
- **Extraction temperature: 60°C**
- **Extraction time: 45 min**
- **Desorption temperature: 260°C**
- **Desorption time: 3 min**
- **Agitation: 500 rpm**

Following studies were carried out with these fixed conditions.

3.3.2. Analytical characteristics

Once the variables are fixed the method needs to be assessed for quality parameters. Method validation is needed to "confirm the fitness for purpose of a particular analytical method" [Taverniers *et al.*, 2004]. Parameters used for the validation are described below [Rambla-Alegre *et al.*, 2012]:

- Accuracy: an agreement between the measured and the real value. Often represented as recovery values.
- Precision: an agreement between series of replicate measurements.
- Linear range: a concentration interval where the method is precise, accurate and linear.
- Limit of Detection (LOD): the lowest amount of analyte to be detected.
- Sensitivity: the capacity of giving a measurable signal with the change of concentration.

The method was evaluated with those parameters. For this purpose the control samples were used.

The calibration ranges were assessed depending on the intensity in the chromatogram. The fibers and the detector sensitivity with each analyte may make difference in the intensity of the chromatogram, therefore different ranges were established. Linearity was also different in each matrix because the saturation of the fiber may be different. Figure 3.8 shows the obtained chromatogram for each standard extraction and determination. Table 3.8 shows the calibration range and the calibration curves for each analyte and matrix type. Regression coefficients (R^2) obtained in a first order calibration curve equation within the calibration ranges were higher than 0.99 for the analytes except myclobutanil where the values were lower (0.9780 and 0.9416 for grape and apple matrices).

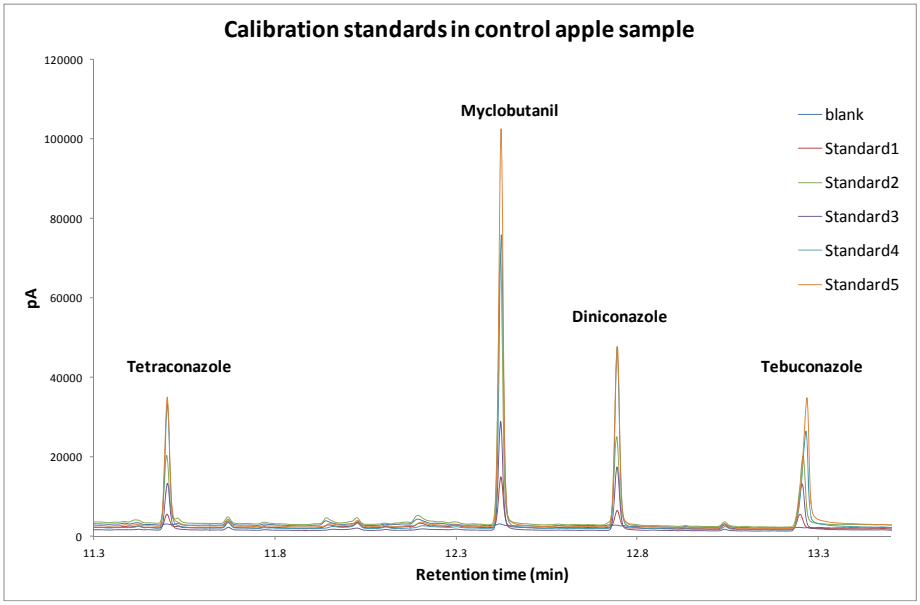

Figure 3.8. *Chromatograms obtained in the analyte range for the addition standard calibration made for the control apple sample.*

The calculation of LOD has been a discussed point because a large number of definitions are proposed for calculation [Compañó-Beltran and Ríos-Castro, 2002; Konieczka and Namieśnik, 2009]. The LOD is based on the calculation standard deviation that could be calculated from different sources: the standard deviation of the blank signal (s_B) or the standard deviation obtained from the uncertainty of the slope of the calibration curve ($s_{y/x}$).

The IUPAC recommended [Currie, 1995] the method based on measuring the blank signal several times to calculate the noise level. The standard deviation of these measurements is calculated (s_B) and the limit of detection is calculated three times this standard deviation. But in the case of chromatography this methodology might be tedious because it could be very time consuming (if there are long chromatogram runs). Moreover to take into account a value of a parameter such as an area of a peak interval, the retention time of the peaks could be very unstable, with retention time shifts.

Foley and Dorsey proposed a parameter called peak-to-peak noise (N_{p-p}) based on the baseline fluctuation within a region in the chromatogram [Foley and Dorsey, 1984] that is measured manually. Blank signal is considered the peak-to-peak noise divided with a parameter (r) dependent of the type of the signal. If the signal was

considered random and normally distributed, then r=5. With these parameters the standard deviation of a blank chromatogram is calculated (s_B):

$$s_B = N_{p-p}/r = N_{p-p}/5 \quad (3.1)$$

The peak-to-peak noise level value was led to the equation 3.1 and then the limit of detection was calculated as IUPAC recommended: as three times the blank signal.

Limits of Detection (LOD) were calculated using blank samples (control samples) in the analyte range of the chromatogram. The noise was measured manually within the analytes retention time range and the peak-to peak noise (N_{p-p}) was calculated. Limits of detection were very different depending on the analyte, due to the different sensitivity of the analytes with the method. Lower limits of detections were obtained in apple juices comparing with grape juices. Values were 0.73 and 0.48 μg L^{-1} for tetraconazole, 7.5 and 4.3 μg L^{-1} for myclobutanil, 0.29 and 0.23 μg L^{-1} for diniconazole and 162 and 106 μg L^{-1} for tebuconazole for grape and apple juices respectively (see Table 3.8).

Table 3.8. *Calibration characteristics for the four analytes in apple and grape control samples.*

Analyte	Sample	Calibration range (μg L^{-1})	R^2	Calibration curve	LOD (μg L^{-1})
TT	Grape	0.78-10.40	0.9955	y=5596.4x - 228.42	0.73
	Apple	0.78-7.80	0.9919	y= 3810.3x - 256.57	0.48
M	Grape	8.7-106.0	0.9780	y=1220.1x - 5294.4	7.5
	Apple	8.7-87.0	0.9416	y=1260.1x - 3824.4	4.3
D	Grape	0.38-5.10	0.9947	y=16966x - 1062.6	0.29
	Apple	0.38-3.80	0.9917	y=10733x - 883.5	0.23
TB	Grape	190-2560	0.9917	y=29.88x -962.62	162
	Apple	190-1900	0.9919	y=22.919x -871.43	106

AOAC defines precision as *the closeness of agreement between independent test results obtained under stipulated condition expressed as a standard deviation of the test results* [AOAC International, 2012].

Inter day precision was calculated with three different measurements in three different days within a week in two different concentrations corresponding to standard mixtures number one and four. The values expressed into the relative standard deviation (RSD) were ranged from 6.8 to 18.0% in the lowest concentration and from 1.2 to 10.2% in the highest concentration considering both juice matrices (see table 3.9).

Table 3.9. *Recovery and inter-day precision (in two spiked levels) for the four analytes in apple and grape control samples.*

Analyte	Sample	Recovery		Precision (Inter-day)	
		Spiked level ($\mu g\ L^{-1}$)	Mean (%) (n=3)	Spiked level ($\mu g\ L^{-1}$)	RSD (%) (n=3)
TT	Grape	7.8	103.2	0.78	11.7
				7.8	6.8
	Apple	3.9	101.7	0.78	6.8
				7.8	3.3
M	Grape	87.0	105.0	8.7	17.6
				87	10.2
	Apple	43.5	112.1	8.7	11.6
				87	4.1
D	Grape	2.5	93.6	0.38	18.0
				3.8	6.7
	Apple	2.5	96.4	0.38	7.7
				3.8	1.2
TB	Grape	450	94.8	190	10.8
				1900	7.8
	Apple	950	97.7	190	10.1
				1900	5.6

Recovery values were calculated to find the accuracy of the method. Recovery was defined by the AOAC as *the fraction or percentage of the analyte that is recovered when the test sample is analyzed using the entire method* [AOAC International, 2012].

For this aim, a known concentration was spiked into the matrix and it was quantified with the method. Each result is the mean value of three measurements. The values were ranging from 93.6 to 105.0% for grapes and from 96.4 to 112.1% for apples. Table 3.9 shows the results for the recovery concentration and its results.

AOAC establishes some precision and recovery values to consider the method acceptable (see in table 3.10) [AOAC International, 2012].

According to precision values, the results obtained for the analytes in the developed method were considered satisfactory. The concentration range of the study was focused in the marked part of the table 3.10 and the obtained highest result was 18.0 % for a concentration comparable to the last line in the table.

Regarding recoveries from table 3.9 and AOAC recommendations these values are also within the expected range. The worst recovery (112.1%) was achieved with myclobutanil in apple juice matrix, for 43.5 μg L^{-1}. In table 3.10 this concentration is between the 10 and 100 ppb, where the acceptable recovery values are 80-110 and 60-115% respectively. Therefore this analyte in this specific matrix was near to the acceptable results limit. Other recovery values were considered satisfactory.

Table 3.10. *AOAC expected precision and recovery values for a method.*

Analyte fraction	Unit	RSD (%)	Mean Recovery (%)
1	100 %	1.3	98-102
10^{-1}	10 %	1.9	98-102
10^{-2}	1 %	2.7	97-103
10^{-3}	0.1 %	3.7	95-105
10^{-4}	100 ppm	5.3	90-107
10^{-5}	10 ppm	7.3	80-110
10^{-6}	1 ppm	11	80-110
10^{-7}	100 ppb	15	80-110
10^{-8}	10 ppb	21	60-115
10^{-9}	1 ppb	30	40-120

Other works that determined triazoles in wine [Trösken *et al.*, 2005; Jiang *et al.*, 2009] found similar recovery and precision results than obtained in this study. However, LODs presented more variation; we obtained better results for diniconazole

and higher values for myclobutanil and tebuconazole than obtained LODs using QuEChERS-GC/MS and LC/MS/MS methodologies.

The comparison with the data obtained for triazoles in wine and fruit juices using SPME-GC/MS [Cortés-Aguado *et al.*, 2008] showed similar precision and recovery results. The LODs obtained in this work were higher for tebuconazole, slightly higher for myclobutanil, and similar for tetraconazole.

A DI-SPME-GC/ToF/MS method was applied to grape and strawberry samples [Souza-Silva *et al.*, 2013], where recovery and precision studies in various triazoles in three different concentrations were applied. For myclobutanil the recoveries were ranging from 91 to 104% with a 2.1-14.6% RSD. In diniconazole case, the values for recovery were between 96-111% and RSD from 7.9 to 13.8%. Finally for tebuconazole obtained recoveries were 98 to 107% and the precision 1.7-10.6%. These results were comparable with the results obtained with the presented method.

3.3.3. Application to samples

The developed procedure was applied to collected apple and grape samples. Always the standard addition method was used trying to avoid possible matrix interferences [Ouyang and Pawliszyn, 2008]. For each sample different standard concentrations were added. For grape samples calibration curves correlations higher than 0.9897 were obtained showing good linearity. For apple samples the correlations were higher than 0.9906.

Grape and apple samples were collected and blended to make juices, were kept until analysis frozen in amber vials. These juices were defrosted at the time of analysis and there were analyzed using the developed method.

Diniconazole residue was found in the sample collected in Hormilleja (sampling point 2). In other grape juices some residues were also detected, but less than the limit of detection. This means that these residues are not reliable quantified in the found amount. The obtained results are shown in table 3.11.

Myclobutanil residues were found above the limit of detection in three apple samples. Other fungicide residues were also found below the limit of detection. It was known that these crops had been treated with some pesticide products to fight against fungus that can damage the apple trees. These studies indicate that some residues were in the apples at the moment the samples were collected.

***Table 3.11**. Found residue amounts in grape and apple samples.*

		Concentration (μg L^{-1})			
	Sample No.	**TT**	**M**	**D**	**TB**
Grape	**1**	< LOD	< LOD	-	< LOD
	2	-	-	9.4	-
	3	< LOD	-	-	-
	4	< LOD	-	-	-
	5	-	-	-	-
Apple	**7**	< LOD	119	-	< LOD
	8	-	-	-	-
	9	-	< LOD	-	< LOD
	10	-	< LOD	-	< LOD
	11	-	-	-	-
	12	-	122	-	< LOD
	13	-	109	-	< LOD
	14	-	-	-	-
	15	-	-	-	-
	16	-	-	-	-
	17	-	-	-	-
	18	-	-	-	-
	19	-	-	-	-

As mentioned before the used calibration method was based in addition of standards. The figure 3.9 and 3.10 show the standard addition calibration in the sample number two for diniconazole in grape sample and sample number twelve for myclobutanil. The blank sample and three of the standards were spiked in order to get the calibration: the used standards were the standards number one, three and five expressed in table 3.1. Each solution was analyzed three times and the point showed in figure 3.9 and 3.10 are the mean values of the three analysis.

The regression equations and its correlation coefficients are also shown in the figures. The correlation coefficients values show good linearity in both cases: 0.9979 for sample number two and 0.9945 for sample number twelve.

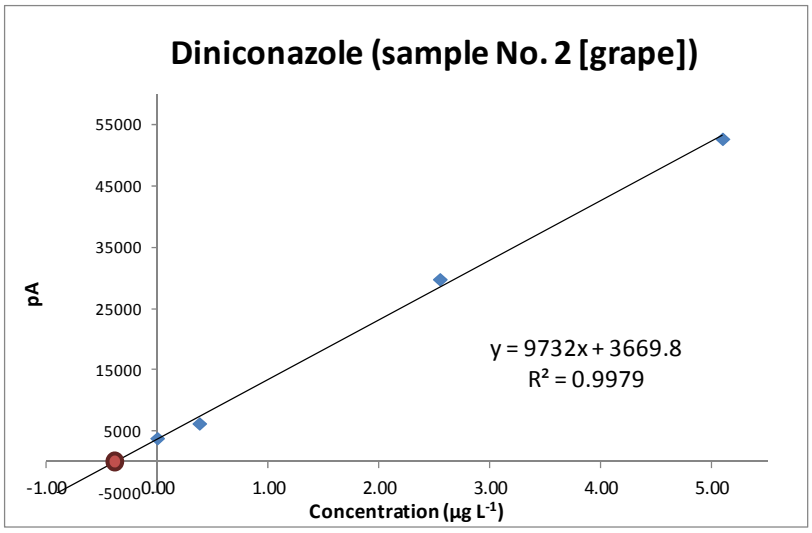

Figure 3.9. *Standard addition calibration for diniconazole in grape sample number two.*

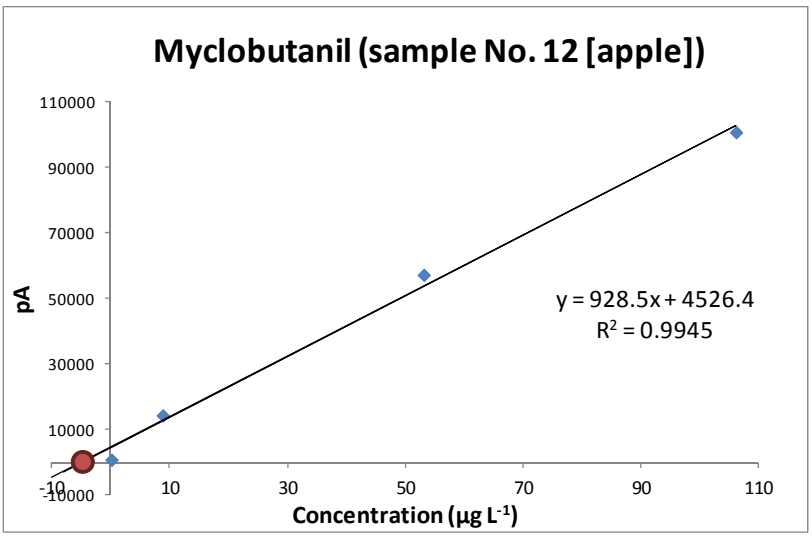

Figure 3.10. *Standard addition calibration for myclobutanil in apple sample number twelve.*

Figures 3.11 and 3.12 show sample chromatograms for samples number two and twelve. Total chromatogram time was fifteen minutes (upper/right position, little chromatogram). The analyte chromatographic range is amplified in the main chromatogram of each figure. Analytes elutes as follows: tetraconazole 11.5 min,

myclobutanil 12.4 min, diniconazole 12.7 min and the last analyte, tebuconazole 13.3 min.

It can be seen that the blank samples are clean at the retention times where the analytes elute, except in the case where there is presence of the analyte: time 12.7 min (for diniconazole in sample number two) and time 12.4 min (for myclobutanil in sample number twelve). The rest of the chromatogram shows similar pattern comparing the blank and the spiked samples. However the chromatograms are quite dirty at the beginning stages of the chromatograms, this means that the fiber extracts other components from the matrix.

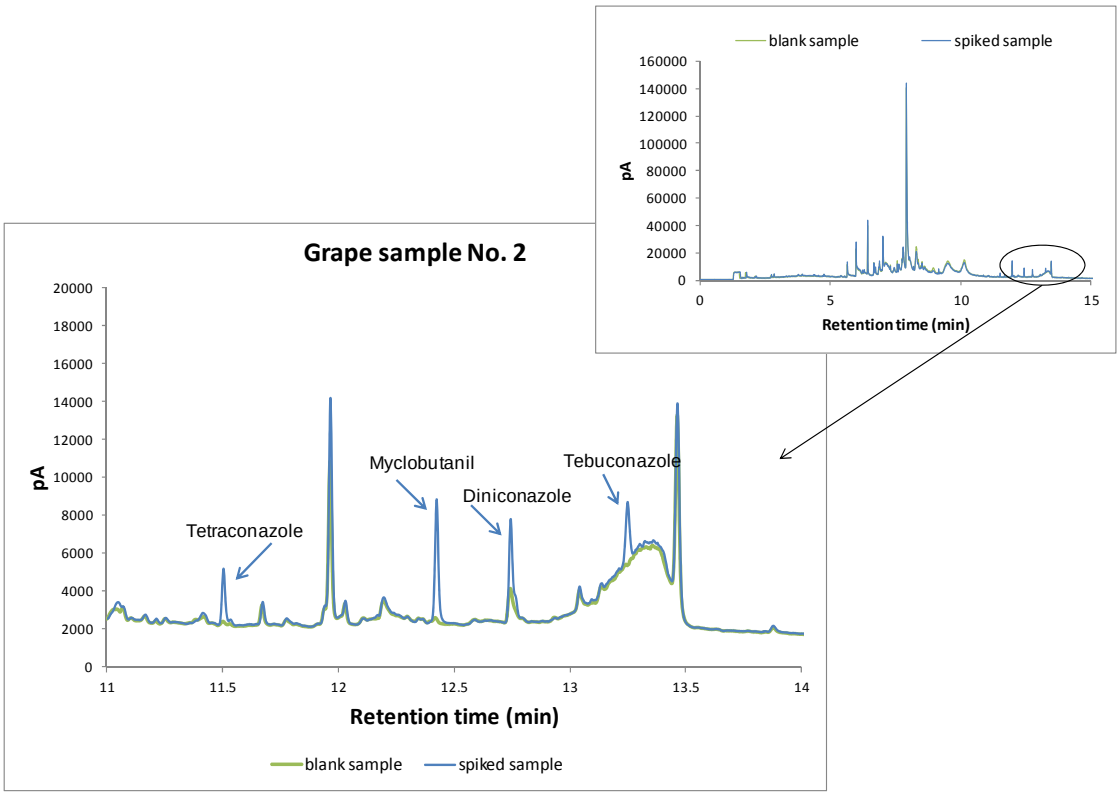

Figure 3.11. *Whole chromatogram (upper/right part) and amplified chromatogram of analyte range for the extraction of the triazole fungicides in a grape juice (sample number two).*

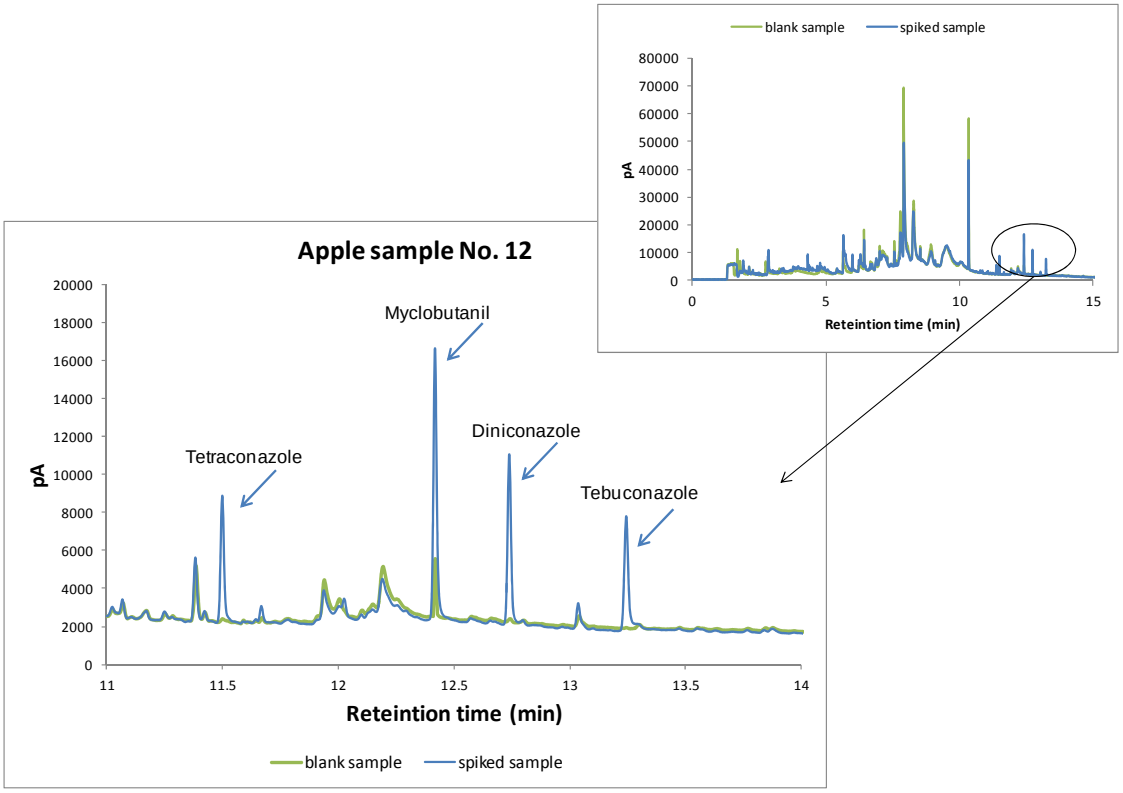

Figure 3.12. *Whole chromatogram (upper/right part) and amplified chromatogram of analyte range for the extraction of the triazole fungicides in an apple juice (sample number twelve).*

To sum it up, the study of the samples was concluded with one positive sample for grape samples in diniconazole and other three positive samples for apple samples in myclobutanil.

Other works with other techniques for triazole determination in grape and juice samples found residues. In the analysis of various juice and water samples with a SEV-DLLME-GC/FID one positive sample in grape at 5 µg L^{-1} level of tebuconazole was obtained [Farajzadeh *et al.*, 2011]. In other study applies DI-SPME-GC/ToF/MS to grapes and strawberries. Three positive in grape samples with myclobutanil (9.2 to 18.2 µg kg^{1}) and one positive grape sample in tebuconazole (36.5 µg kg^{-1}) were obtained [Souza-Silva *et al.*, 2013].

The European Regulations establish MRLs in fruits. The regulation expressed the residue limits in units of mg kg^{-1} (see table 3.12) and the content corresponds to the whole fruit pieces. In these studies juices were analyzed, and the concentrations were in units of weight/volume (mg L^{-1} or µg L^{-1}).

Table 3.12. *MRLs allowed for apple and wine grapes according to European Regulations.*

Analyte	Fruit	MRL (mg kg^{-1})	Regulation	Regulation Date
M	**Apple**	0.5	Reg. (EC) No 149/2008	29 January 2008
	Wine Grape	1		
TT	**Apple**	0.3	Reg. (EU) No 34/2013	16 January 2013
	Wine Grape	0.5		
D	**Apple**	0.01	Reg. (EU) No 1317/2013	16 December 2013
	Wine Grape	0.01		
TB	**Apple**	0.3	Reg. (EU) No 61/2014	24 January 2014
	Wine Grape	1		

Taking into account that just blended juices density range was between 1.03-1.05 mg mL^{-1}, the founded amount of triazoles in the samples, was below these limits. But it should be remarked that those MRLs were expected to be in the whole fruit and not only in juices as it was approached for this case.

3.4 Conclusions

A simple and reliable method has been developed for the determination of four triazole fungicides: tetraconazole, myclobutanil, diniconazole and tebuconazole. For this purpose solid-phase microextraction technique was used to preconcentrate the analytes and afterwards the determination was carried out using a gas chromatograph with an electron capture detector.

The procedure was developed using screening and optimization designs in order to select the optimum variables to carry out the best possible performance. The method was evaluated using figures of merit such as linearity, limits of detection, precision and recovery. Taking into account the presented results, the method is considered satisfactory for the aim of this work.

The method was applied successfully to real samples with the assumption that crops had been treated with some pesticide products to fight against harmful agents. One positive sample was found in grape samples and other three apple samples results in positive residue content.

The method provide various advantages such as the process barely need sample treatment making the method faster comparing with methods like classical solid-phase or liquid-liquid phase extraction. Besides the SPME method avoids the environmentally dangerous organic solvents.

On the other hand, there are some drawbacks. The low volatility of the analytes forces the extraction to be in immersion mode as well as long extraction times (45 min). This extraction mode could result in early fiber damage and consequently this affect in the figures of merit as well as the fiber life.

3.5 References

AOAC INTERNATIONAL, **2012.** AOAC Official Methods of Analysis, Guidelines for standard method performance requirements. Appendix F, .

Beceiro-González, E., Concha-Graña, E., Guimaraes, A., Gonçalves, C., Muniategui-Lorenzo, S. and Alpendurada, M.F.; *Optimisation and validation of a solid-phase microextraction method for simultaneous determination of different types of pesticides in water by gas chromatography–mass spectrometry.* **2007.** Journal of Chromatography A, 1141(2), 165-173.

Beltran, J., López, F.J. and Hernández, F.; *Solid-phase microextraction in pesticide residue analysis.* **2000.** Journal of Chromatography A, 885(1-2), 389-404.

Bicchi, C., Cordero, C., Rubiolo, P. and Occeli, A.; *Simultaneous determination of six triazolic pesticide residues in apple and pear pulps by liquid chromatography with ultraviolet diode array detection.* **2001.** Journal of AOAC International, 84, 1543-1550.

Blasco, C., Font, G., Mañes, J. and Picó, Y.; *Solid-Phase Microextraction Liquid Chromatography/Tandem Mass Spectrometry To Determine Postharvest Fungicides in Fruits.* **2003.** Analytical Chemistry, 75(14), 3606-3615.

Compañó-Beltran, R. and Ríos-Castro, Á; *Garantía de la calidad en los laboratorios analíticos.* **2002.** 1 edn. Síntesis S.A. Madrid (Spain).

Cortés-Aguado, S., Sánchez-Morito, N., Arrebola, F.J., Garrido-Frenich, A. and Martínez-Vidal, J.L.; *Fast screening of pesticide residues in fruit juice by solid-phase microextraction and gas chromatography–mass spectrometry.* **2008.** Food Chemistry, 107(3), 1314-1325.

Coscollà, C., Navarro-Olivares, S., Martí, P. and Yusà, V.; *Application of the experimental design of experiments (DoE) for the determination of organotin compounds in water samples using HS-SPME and GC–MS/MS.* **2014.** Talanta, 119, 544-552.

Costa, N.R., Lourenço, J. and Pereira, Z.L.; *Desirability function approach: A review and performance evaluation in adverse conditions.* **2011.** Chemometrics and Intelligent Laboratory Systems, 107(2), 234-244.

Cunha, S.C., Fernandes, J.O., Alves, A. and Oliveira, M.B.P.P.; *Fast low-pressure gas chromatography–mass spectrometry method for the determination of multiple pesticides in grapes, musts and wines.* **2009.** Journal of Chromatography A, 1216(1), 119-126.

Currie, L.A., *Nomenclature in evaluation of analytical methods including detection and quantification capabilities (IUPAC Recommendations 1995).* **1995.** Pure and Applied Chemistry, 67, 1699-1723.

Derringer, G. and Suich, R.; *Simultaneous optimization of several response variables.* **1980.** Journal of Quality Technology, 12(4), 214-219.

Farajzadeh, M.A., Bahram, M., Jafary, F. and Bamorowat, M.; *Combination of Extraction by Silylated Vessel-Dispersive Liquid–Liquid Microextraction as a High-Enrichment Factor Technique: Optimization and Application in Preconcentration of Some Triazole Pesticides from Aqueous Samples Followed by GC-FID Determination.* **2011.** Chromatographia, 73(3), 393-401.

Fernandez-Alvarez, M., Llompart, M., Lamas, J.P., Lores, M., García-Jares, C., Cela, R. and Dagnac, T.; *Development of a solid-phase microextraction gas chromatography with microelectron-capture detection method for a multiresidue analysis of pesticides in bovine milk.* **2008.** Analytica Chimica Acta, 617(1-2), 37-50.

Foley, J.P. and Dorsey, J.G.; *Clarification of the limit of detection in chromatography.* **1984.** Chromatographia, 18(9), 503-511.

Gionfriddo, E., Naccarato, A., Sindona, G. and Tagarelli, A.; *Determination of hydrazine in drinking water: Development and multivariate optimization of a rapid and simple solid phase microextraction-gas chromatography-triple quadrupole mass spectrometry protocol.* **2014.** Analytica Chimica Acta, 835, 37-45.

Jiang, Y., Li, X., Xu, J., Pan, C., Zhang, J. and Niu, W.; *Multiresidue method for the determination of 77 pesticides in wine using QuEChERS sample preparation and gas chromatography with mass spectrometry.* **2009.** Food Additives & Contaminants: Part A, 26(6), 859-866.

Jin, B., Xie, L., Guo, Y. and Pang, G.; *Multi-residue detection of pesticides in juice and fruit wine: A review of extraction and detection methods.* **2012.** Food Research International, 46(1), 399-409.

Konieczka, P. and Namieśnik, J.; *Quality Assurance and Quality Control in the Analytical Chemical Laboratory.* **2009.** 1 edn. CRC Press, Taylor & Francis Group. Boca Raton (USA).

Lambropoulou, D.A. and Albanis, T.A.; *Methods of sample preparation for determination of pesticide residues in food matrices by chromatography–mass spectrometry-based techniques: a review.* **2007.** Analytical and Bioanalytical Chemistry, 389(6), 1663-1683.

Lasa, M., Garcia, R. and Millán, E.; *A Convenient Method for Epichlorohydrin Determination in Water using Headspace–Solid-Phase Microextraction and Gas Chromatography.* **2006.** Journal of Chromatographic Science, 44(7), 438-443.

López, R., Goñi, F., Etxeandia, A. and Millán, E.; *Determination of organochlorine pesticides and polychlorinated biphenyls in human serum using headspace solid-phase microextraction and gas chromatography-electron capture detection.* **2007.** Journal of Chromatography B, 846(1-2), 298-305.

Menezes Filho, A., dos Santos, F.N. and de Paula Pereira, P.A.; *Development, validation and application of a methodology based on solid-phase micro extraction followed by gas chromatography coupled to mass spectrometry (SPME/GC–MS) for the determination of pesticide residues in mangoes.* **2010.** Talanta, 81(1–2), 346-354.

Montes, R., Rodríguez, I., Ramil, M., Rubí, E. and Cela, R.; *Solid-phase extraction followed by dispersive liquid–liquid microextraction for the sensitive determination of selected fungicides in wine.* **2009.** Journal of Chromatography A, 1216(29), 5459-5466.

Ouyang, G. and Pawliszyn, J.; *A critical review in calibration methods for solid-phase microextraction.* **2008.** Analytica Chimica Acta, 627(2), 184-197.

Pawliszyn, J.; *Solid Phase Microextraction. Theory and Practice.* **1997.** 1 edn. Wiley-VCH. New York (USA).

Picó, Y., Fernández, M., Ruiz, M.J. and Font, G.; *Current trends in solid-phase-based extraction techniques for the determination of pesticides in food and environment.* **2007.** Journal of Biochemical and Biophysical Methods, 70(2), 117-131.

Rambla-Alegre, M., Esteve-Romero, J. and Carda-Broch, S.; *Is it really necessary to validate an analytical method or not? That is the question.* **2012.** Journal of Chromatography A, 1232, 101-109.

Regulation EC 149/2008 of 29 January 2008 amending Regulation (EC) No 396/2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto. Official Journal of the European Union. **(1.3.2008)** L 58/1-398. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 34/2013 of 16 January 2013 amending Annexes II, III and IV to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residuel levels for 2-phenylphenol, ametoctradin, *Aureobasidium pullulans* strains DSM 14940 and DSM 14941, cyproconazole, difenoconazole, dithiocarbamates, folpet, propamocarb, spinosad, spirodiclofen, tebufenpyrad and tetraconazole in or on certain products. Official Journal of the European Union. **(26.1.2013)** L 25/1-48. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 1317/2013 of 16 December 2013 amending Annexes II, III and V to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for 2,4-D, beflubutamid, cyclanilide, diniconazole, florasulam, metolachlor and S-metolachlor, and milbemectin in or on certain

products. Official Journal of the European Union. **(17.12.2013)** L 339/1-43. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 61/2014 of 24 January 2014 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for cyromazine, fenopropidin, formetanate, oxamyl and tebuconazole in or on certain products. Official Journal of the European Union. **(24.1.2014)** L 22/1-32. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Sagratini, G., Mañes, J., Giardiná, D., Damiani, P. and Picó, Y.; *Analysis of carbamate and phenylurea pesticide residues in fruit juices by solid-phase microextraction and liquid chromatography–mass spectrometry.* **2007.** Journal of Chromatography A, 1147(2), 135-143.

Souza-Silva, E.A., Lopez-Avila, V. and Pawliszyn, J.; *Fast and robust direct immersion solid phase microextraction coupled with gas chromatography–time-of-flight mass spectrometry method employing a matrix compatible fiber for determination of triazole fungicides in fruits.* **2013.** Journal of Chromatography A, 1313, 139-146.

Stalikas, C., Fiamegos, Y., Sakkas, V. and Albanis, T.; *Developments on chemometric approaches to optimize and evaluate microextraction.* **2009.** Journal of Chromatography A, 1216(2), 175-189.

Taverniers, I., De Loose, M. and Van Bockstaele, E.; *Trends in quality in the analytical laboratory. I. Traceability and measurement uncertainty of analytical results.* **2004.** Trends in Analytical Chemistry, 23(7), 480-490.

Trösken, E.R., Bittner, N. and Völkel, W.; *Quantitation of 13 azole fungicides in wine samples by liquid chromatography–tandem mass spectrometry.* **2005.** Journal of Chromatography A, 1083(1-2), 113-119.

Van den hauwe, O., Dumoulin, F., Antignac, J.P., Bouche, M.P., Elliott, C. and Van Peteghem, C.; *Liquid chromatographic–mass spectrometric analysis of 11 glucocorticoid residues and an optimization of enzymatic hydrolysis conditions in bovine liver.* **2002.** Analytica Chimica Acta, 473(1–2), 127-134.

Zambonin, C.G., Cilenti, A. and Palmisano, F.; *Solid-phase microextraction and gas chromatography–mass spectrometry for the rapid screening of triazole residues in wine and strawberries.* **2002.** Journal of Chromatography A, 967(2), 255-260.

Zuazagoitia, D., Millán, E. and García-Arrona, R.; *A Screening Method for Polycyclic Aromatic Hydrocarbons Determination in Sediments by Headspace SPME with GC–FID.* **2009.** Chromatographia, 69(1), 175-178.

4. Development of a method for triazole fungicides determination in fruit samples using solid-phase microextraction and HPLC/DAD

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4. Development of a method for triazole fungicides determination in fruit samples using solid-phase microextraction and HPLC/DAD

4.1 Introduction

Triazole pesticides are widely used as antifungal agents to fight against *Oidium*, a common fungus that can affect fruit trees. In general, the action mode of azole fungicides is the inhibition of ergosterol, an essential component in fungal cell membranes, by influencing the cytocrome P450 enzyme activity [Ekman *et al.*, 2006]. There are observed side effects with the exposure of some triazole fungicides in some *in vivo* assays. Also, some studies have shown that those effects in rats lead to disturbances in reproductive systems [Taxvig *et al.*, 2007] or even induce some tumors in mice liver [Allen *et al.*, 2006].

Because of the potential health risks for consumers resulting from acute and chronic dietary exposure, European Union (EU) has published new regulations establishing the Maximum Residual Limits (MRL) to a wide range of pesticides in different vegetable and fruits [Regulation EC 149/2008; Regulation EC 459/2010; Regulation EU 750/2010; Regulation EU 34/2013; Regulation EU 1317/2013; Regulation EU 61/2014]. Those MRLs for triazole fungicides were ranging from 0.01 to 1 mg kg^{-1} based on the analyte and the type of fruit, including apples and grapes for wine production. Moreover, commercial products establish a pre-harvest interval for safety since in the crops may remain fungicide residues. These fungicide residues may disappear by degradation of the product or by sweeping because of the precipitations. In the Basque Country (North of Spain) the following triazole fungicides diniconazole (D), fluquinconazole (FQ), flusilazole (FS), myclobutanil (M), tebuconazole (TB) and tetraconazole (TT) are widely used in apple orchards and vineyards.

The amount of those analytes in environmental samples is usually in a very low concentration. Thus, very precise and sensitive techniques are needed. Gas or liquid chromatography (GC, LC) techniques coupled to mass spectrometry (MS) are

frequently used for pesticide analysis in fruits and vegetables [Araoud *et al.*, 2007; Walorczyk, 2008; Kmellár *et al.*, 2010]. Sample preparation steps previously to chromatographic analysis are required in most of the samples. Historically, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have been the most used preparative methods, but those techniques are time consuming and need large amounts of solvents. Nowadays, the trends in sample preparation are the solvent-minimized and the solventless techniques [Tankiewicz *et al.*, 2011]. The solid-phase microextraction (SPME) is an useful technique that does not require solvents and can be carried out directly from the liquid phase (direct immersion, DI) or from the headspace (HS) over the samples [Pawliszyn, 1999]. It has been used in many different applications including pesticide residues determination [Aulakh *et al.*, 2005; Picó *et al.*, 2007; Sagratini *et al.*, 2007]. Although the pesticide studies often include one triazole fungicide, mainly tebuconazole, few works considering simultaneously several triazoles in fruits and liquid extracts using SPME have been done. SPME coupled to GC/MS for triazole residues in wine and strawberries have been used [Zambonin *et al.*, 2002]. Also, SPME and GC with electron capture detector (ECD) have been showed adequate for determining diniconazole, myclobutanil and tetraconazole in juice samples [Bordagaray *et al.*, 2011]. However, in the latter work the results obtained with tebuconazole were not good as the others studied triazoles. Due to the analytical characteristics of the triazole compounds a solvent desorption using LC from the SPME fiber could result in a better performance that allows a wider and versatile triazole determination.

There are several experimental variables affecting the SPME procedure such us type of fiber, temperature, extraction time, salt addition, and desorption conditions. An experimental design that could take into account simultaneously several variables seems to be a convenient approach to get the best experimental conditions. A screening design, such as fractional factorial, is recommended to perform in a previous step to reduce the number of variables. In the next step, the chosen variables, usually two or three, are optimized using response surface methodology. Among the designs, Central Composite CCD or Box-Behnken are frequently used [González-Barreiro *et al.*, 2000; Zou *et al.*, 2012].

The aim of the work was to develop a convenient method that allows simultaneous determination of various triazole fungicides in liquid, fruit and related samples using simple equipment (SPME coupled to HPLC/DAD). In order to select the

experimental conditions, a planned experimental design with screening and optimization steps was run out. The optimized procedure was applied to check and to evaluate the triazole residues into peel, pulp and juice of apple and grapes during a pre-harvest interval of two weeks.

4.2 Experimental

4.2.1 Reagents and equipment

Diniconazole (99.8%, Pestanal®) and tebuconazole (99.6%, Pestanal®) were acquired in Sigma-Aldrich (Madrid, Spain), flusilazole (99.3%) and myclobutanil (99.4%) were supplied by LGC Standards (Barcelona, Spain) and tetraconazole (97.5%) and fluquinconazole (98.5%) from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were prepared in methanol (Superpure Solvent, SpS) supplied by Teknocroma (Barcelona, Spain) in a concentration of 1000 mg L^{-1}, and calibration standard were prepared in a range from 5 to 30 mg L^{-1}. Just previously to each use the dilution of the standards was made adding 20 µL of these standards to 20 mL of salted water (see table 4.1).

The 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers were acquired in Supelco (Bellefonte, PA, USA). Sample vials (40 mL amber glass), PTFE-faced silicone septa and holders for extractions were also supplied by Supelco.

HPLC analysis was performed in a LC- 20AD liquid chromatographer coupled with a SPD-M20A diode array detector (Shimadzu Corporation, Duisburg, Germany). Data were collected and processed by LC Solution software (1.2. version). An interface with a Rheodyne® Valve (Supelco) with 60 ± 10 µL chamber volume was used for desorption. All separations were carried out with a XDB- C18 column (4.6 mm x 250 mm, 5 µm) (Agilent, Wilmington, DW, USA).

Mobile phase for HPLC was formed by acetonitrile (SpS) acquired in Teknocroma and a pH 4 buffer. The buffer was made by 0.01 mol L^{-1} acetic acid and

adjusted with sodium acetate solution 0.01 mol $^{L-1}$ to pH 4. The buffer was stored at 4^{o}C and the necessary amount for each day was taken out every morning before analysis in order to get the room temperature. Mobil phase was eluted in isocratic mode; therefore, there were not solvent ratio changes during the whole chromatogram: 78% acetonitrile and 22% acetic/acetate buffer. Flow was established at 0.5 mL min^{-1}. The election of the organic solvent used for SPME desorption, and therefore the mobile phase for HPLC has to be done carefully. Some solvents can cause swelling in the fiber that can break the coating when it is retracted. With those conditions the sequence and elution time was tetraconazole (7.2 min), myclobutanil (7.5 min), flusilazole (7.8 min), fluquinconazole (8.1 min), tebuconazole (8.4 min) and diniconazole (9.9 min).

The chromatographic UV-Vis absorption spectra were recorded from 190 to 800 nm using 221 nm as a detection wavelength. The spectra recorded for preliminary studies were obtained in 8453 UV-Vis Diode Array System (Agilent Technologies, Spain) with the software "UV-Visible Chemstation".

The extractions were made in a vessel with a thermostatic jacket joined to a Lauda RE 104 thermo bath (Lauda, GmbH & Co. KG, Lauda-Königshofen, Germany). The agitation was performed by Heidolph MR 3003 magnetic stirrer (Heidolph, GmbH & Co. KG, Schwabach, Germany). For sample treatment, an ultrasonic bath and an Eppendorf Centrifuge with a maximum speed of 14000 rpm were used.

Experimental designs were performed and the results were evaluated using Statistica software (StatSoft, Tulsa, USA).

Table 4.1. *Calibration concentrations (µg L^{-1}) used for SPME-HPLC method.*

	TT	M	FS	FQ	TB	D
Standard 1	5.1	20.4	10.0	31.2	15.6	26.5
Standard 2	10.2	25.5	15.0	5.2	20.8	31.8
Standard 3	15.3	30.6	20.0	10.4	26.0	5.3
Standard 4	20.4	5.1	25.0	15.6	31.2	10.6
Standard 5	25.5	10.2	30.0	20.8	5.2	15.9
Standard 6	30.6	15.3	5.0	26.0	10.4	21.2
Standard 7	15.3	15.3	15.0	15.6	15.6	15.9
Standard 8	25.5	25.5	25.0	26.0	26.0	26.5

TT: Tetraconazole
M: Myclobutanil
FS: Flusilazole
FQ: Fluquinconazole
TB: Tebuconazole
D: Diniconazole

4.2.2 Samples

In order to check and evaluate the occurrence of triazole residues in the apple and grape during a two weeks pre-harvest interval, a mixture of two commercial products (named *Genius WG* and Poltix, see figure 4.1) containing triazole pesticides were applied according to manufacturer instructions using a spray applicator. The manufacturer indicated that the security period of these products were fifteen days; thus, the product can remain in the fruit during this period and it cannot be collected. The established sampling days were according to this period of two weeks and were fixed as can be seen in table 4.2: the day after application, the fourth day, a week after application and two weeks after application. Trying to see the rain carry over effect, also the collection of rain samples was planned. However, during the selected period rain was not frequent and one simulated rain sample was collected. The simulation

was done spraying tap water to the trees and was collected in the recipient located under the plants.

Figure 4.1. *The applied commercial products with myclobutanil (Poltix) and tebuconazole (Genius WG).*

Table 4.2. *Pesticide application and sampling days during harvest period of autumn 2011.*

September (2011)						
			1	2	3	4
5	6	7	8	9	10	11
12	13	14	15	16	17	18
19	20	21	22	23	24	25
26	27	28	29	30		

October (2011)						
					1	2
3	4	5	6	7	8	9
10	11	12	13	14	15	16
17	18	19	20	21	22	23
24	25	26	27	28	29	30
31						

RAIN		pesticide application
SIMULATED	02/10/2011	sampling days
REAL	07/10/2011	

Five apples from separate locations in the tree and 2-3 grapes from different bunches until complete at least 200 g were taken. The samples were divided in 3

groups: peel, pulp and juice and they were separately analyzed. The peel was removed, and the fruit was blended separating the pulp and the juice. The samples were kept in the freezer until the analysis.

The peel or pulp samples (*ca.* 2 g) were covered by acetonitrile (5 mL) and put into an ultrasonic bath during 20 min. Then, the supernatant was transferred to a 2 mL eppendorf vial and was centrifuged at 14000 rpm for 10 min. After centrifugation, 1.0 mL was transferred to 40 mL glass vials and diluted to 20 mL with salted water (NaCl, 180 g L^{-1}) before extraction with the SPME fibers. The juice was also analyzed, adding directly salted water to 1 mL sample until complete 20 mL of volume.

With the rain samples, filtering to remove the solids, the analysis was done taking from 2 to 20 mL in 40 mL glass vials, completing with double distilled water to 20 mL and adding the amount of needed salt into the vial to obtain 180 g L^{-1} concentration.

4.2.3 SPME procedure

Extraction procedure needs to follow some steps. Firstly the fibers were previously conditioned during 30 min in the desorption chamber of the HPLC. The thermostatic bath was heated to 60ºC and the magnetic stirrer was fixed in 500 rpm. A 40 mL amber glass vial with the 20 mL of liquid extracts previously prepared and with a magnetic stirrer was placed in the water jacket connected to the thermostatic bath. In the case of standards adequate volumes of analyte solutions were added to the vial containing 20 mL of salted water. Then, the fiber was immersed into the aqueous solution and after 90 min for exposition time was withdrawn and put into the SPME-HPLC interface to desorb the analytes. The scheme of the procedure is shown in figure 4.2.

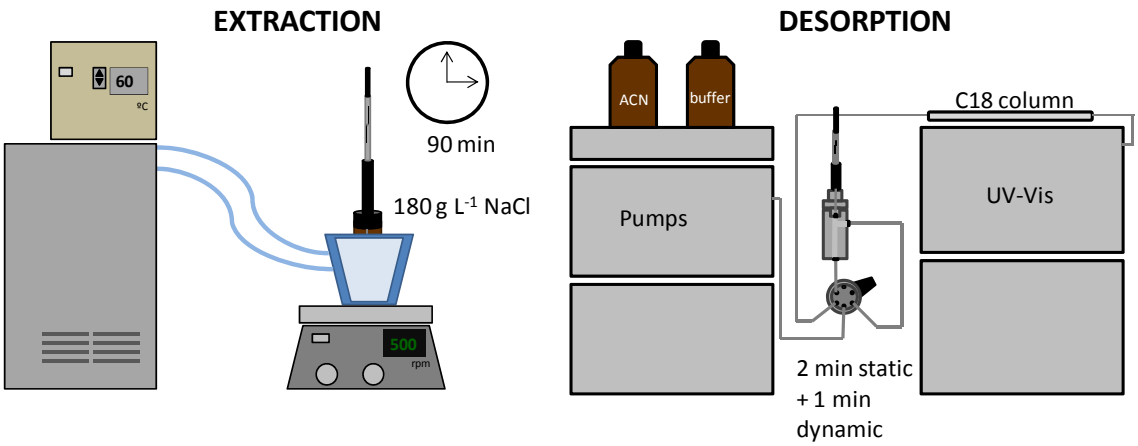

Figure 4.2. *Representation of the extraction and desorption arranged conditions for the SPME-HPLC procedure.*

The chosen desorption mode was the static mode (2 min), following by another dynamic mode step of 1 min to swept all the mobile phase with the analytes of the desorption chamber. Some dynamic mode was necessary because it was not possible to swap all the mobile phase with analytes in the desorption chamber without the fiber inside the interface. To maintain the pressure in the interface is compulsory to keep the circuit closed, and this can only happen with the fiber assemblage inside the interface. Figure 4.3 shows both desorption modes. As it can be seen in the figure, in the static mode the injector is on *load* position where the previously filled chamber is closed and the mobile phase on the chamber is not on movement. On the other hand, in dynamic mode, injector is on *inject* position and the mobile phase flows through the desorption chamber. Take note that the chamber was homogenized and filled with the mobile phase before each insertion of the fiber. The valve was led to *inject* position and the mobile phase was sucked with a syringe.

After each analysis the fiber was maintained in stirred water for 15 min as cleaning step and trying to avoid the crystallization of the salt and the possible carry over effect that can happen because analytes are not totally desorbed. With these precautions the fiber was reused around sixty times.

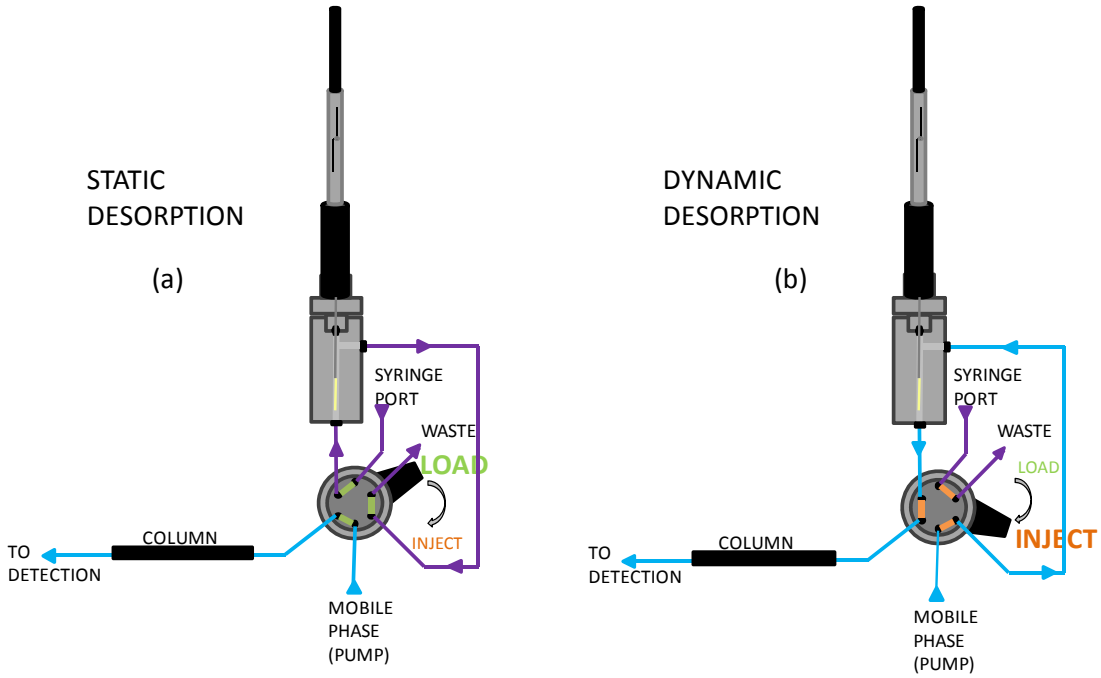

Figure 4.3. *SPME desorption modes in SPME-HPLC interface. (a) static desorption and (b) dynamic desorption.*

4.3 Results and discussion

A reliable method for determination of triazole fungicides using SPME and HPLC/DAD was the goal of the work. For this aim, firstly the attainment of the best conditions was performed through two steps: screening and optimization designs. Afterwards, the method was validated and finally was applied to peel, pulp, and juice from apple and grape and rain samples.

4.3.1 UV spectra. Wavelength election

Data used in this procedure were provided by a diode array detector (DAD). The chromatogram obtained with this detector is a three-dimensional chromatogram where the wavelength range is in x axis and the retention time is in the y axis. For

univariate quantification a specific wavelength needs to be chosen. The election of a correct wavelength could be crucial in terms of reproducibility and other analytical characteristics. Usually in low wavelengths the used mobile phase can affect, and it is recommended to choose a wavelength were analytes show a maximum, to increase the sensitivity with the signal. A 3D chromatogram is shown in figure 4.4. The analytes elution time ranged from 6.8 to 10.5 minutes and the wavelengths were from 190 nm to 250 nm.

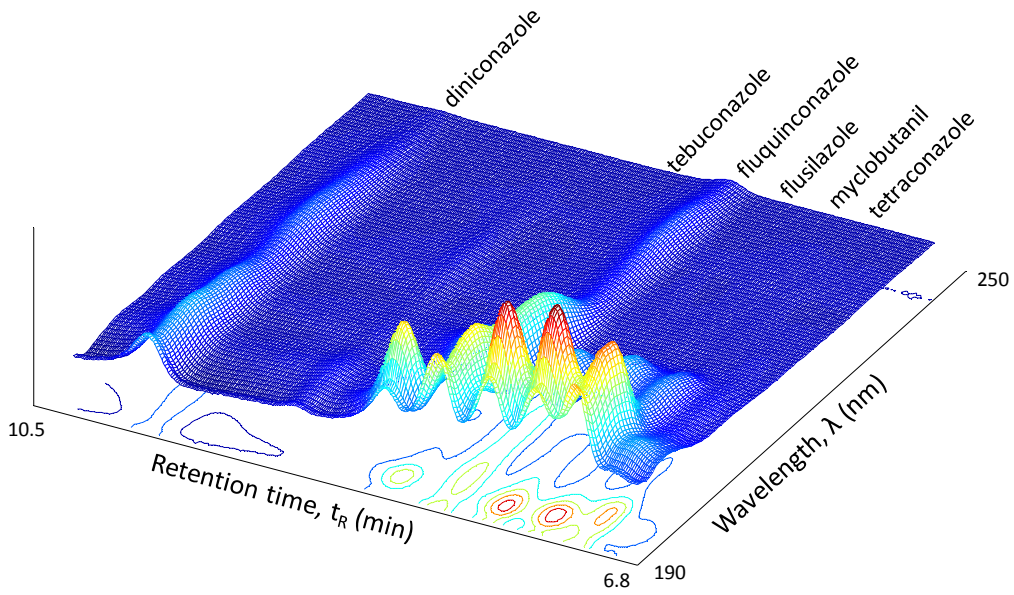

Figure 4.4. *3D chromatogram for the mixture of the standard number 7 (see table 4.1).*

In multivariate analysis the whole chromatogram in 3D can be considered for studies. The fact that the analyst has a whole UV spectrum (or a vector instead of a scalar) for each retention time is called the second-order advantage. This advantage will be useful for further studies explained in the multivariable treatment chapter.

The determination for univariate analysis was done in a specific wavelength. To select which of the wavelength could be a good choice, a spectrum was recorded separately for each analyte in a UV spectrometer. Figure 4.5 shows the individual UV spectra in the wavelength range from 190 to 300 nm. In every spectrum the maximum

absorbance is reached in wavelengths below 200 nm; but as mentioned before, in HPLC the organic mobile phase can affect to the determination. If the solvent absorb in some wavelengths the measurement performed can be affected. Solvents cut-off wavelengths are established to avoid these problems. Acetonitrile effective cut-off wavelength was established at 213 nm [Burgess and Frost T., 1999]. Therefore, the wavelengths above that limit were chosen. The spectra below this cut-off wavelength are shaded in red (see figure 4.5).

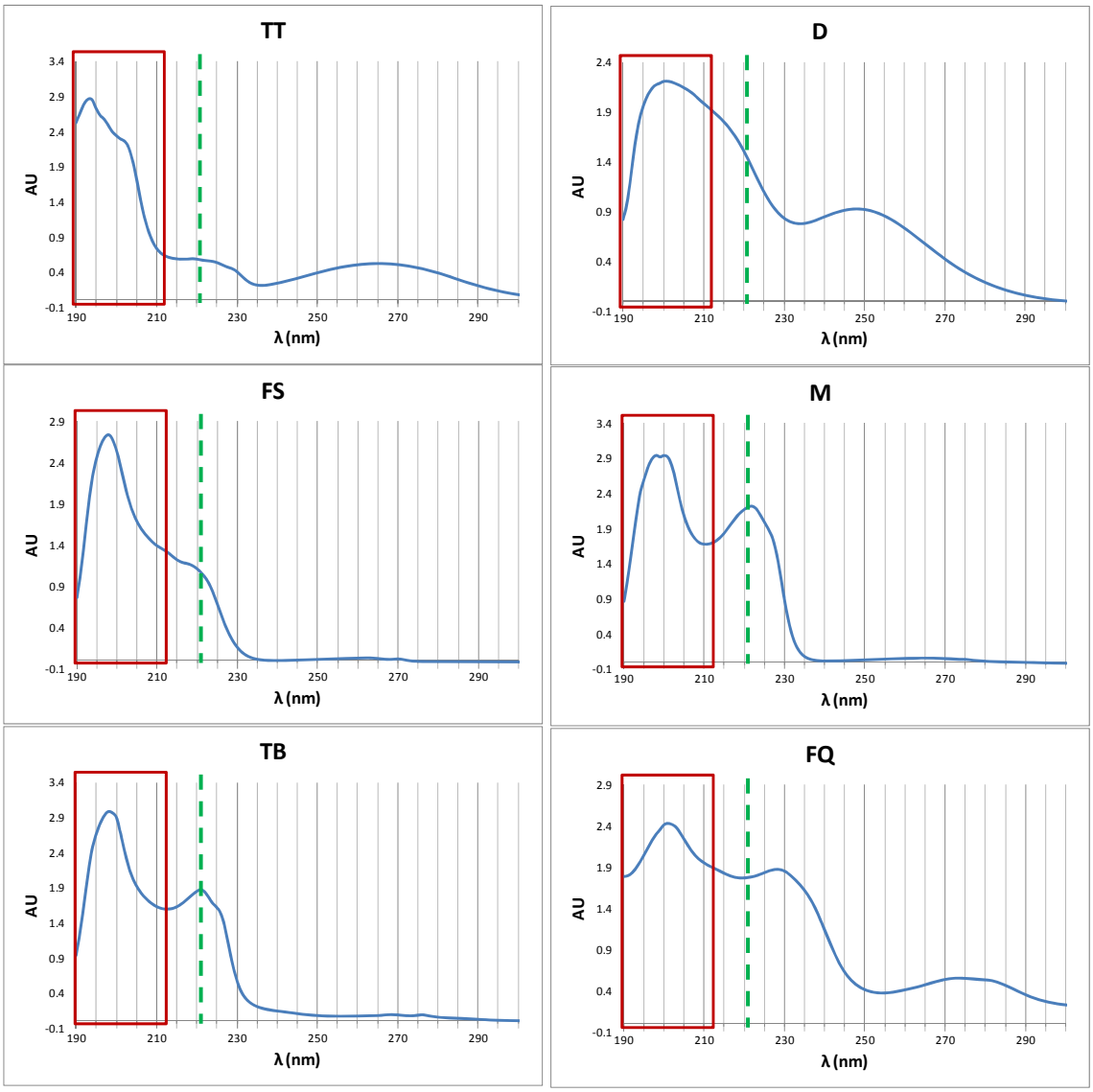

Figure 4.5. *UV spectra of each analyte. TT: tetraconazole; D: diniconazole; FS: flusilazole; M: myclobutanil; TB: tebuconazole; FQ: fluquinconazole.*

The chosen wavelength was 221 nm (marked in green discontinuous line in figure 4.5), where most of the analytes showed a second maximum or an inflexion point. The chromatograms and the determination from now on are the ones recorded

at 221 nm. Further studies took into account the areas obtained in this specific wavelength.

4.3.2 Chromatographic separation. Mobile phase

HPLC separation is based on affinity of analytes with the stationary (chromatographic column) and mobile phase (solvent mixture). Different parameters are involved when performing a liquid-chromatographic method: organic phase (e.g. methanol, ethanol, acetonitrile, etc.), flow rate, mobile phase composition (isocratic or gradient mixture) and buffer usage among others.

In this work because of the fiber coating some solvents are not suitable. Solvents such as methanol, can cause the swelling of the coating when is soaked by the solvent. If the fiber is retracted at that moment, the needle can damage the coating. Acetonitrile was considered a safe solvent for the fiber coating. Therefore, mixtures using acetonitrile and water solutions were considered for the chromatographic separation. Firstly, different mobile phase gradients were tried with a 1 mL min^{-1} flow. However, no successful gradient was found for the analytes separation. Chromatograms show strong or complete overlapping among different analytes (see figure 4.6).

Moreover, the flow of 1 mL min^{-1} was not suitable for the use of SPME-HPLC interface. The pressure formed in desorption chamber (60-80 bar) throw the fiber out when the injector was turned to the *"inject"* position. Therefore, a lower flow was considered for further studies. Flow changes during the whole separation were also considered; but, this option was rejected due to severe baseline drifts.

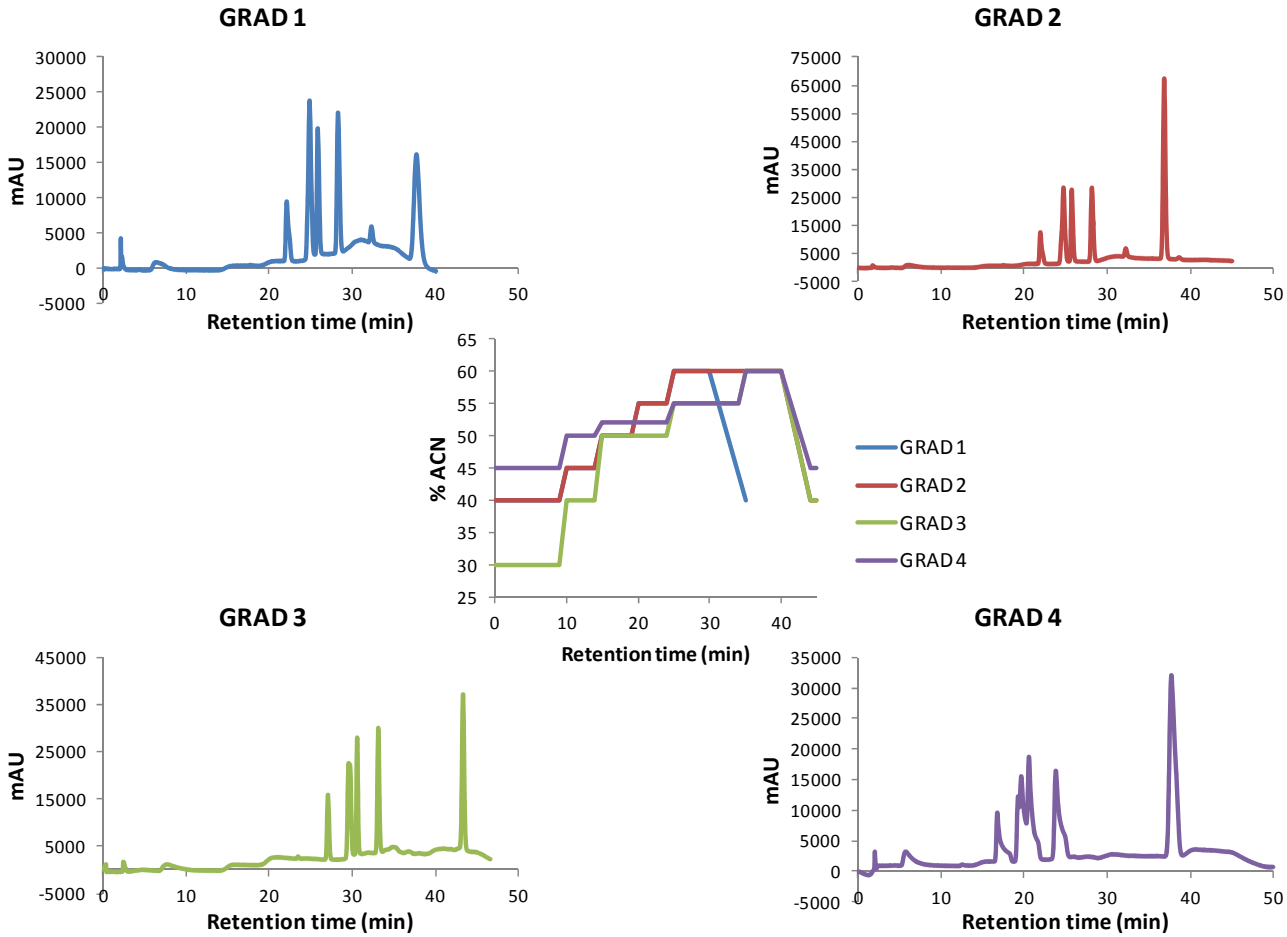

Figure 4.6. *Different gradients with acetonitrile (ACN) and water with 1 ml min^{-1} flow. Gradient composition is represented in the center of this figure.*

The isocratic flows were also tried. With low acetonitrile percentage long chromatographic times were needed and separations were not successful. If 78% acetonitrile was used a better separation of the six analytes was found as it can be seen in figure 4.7

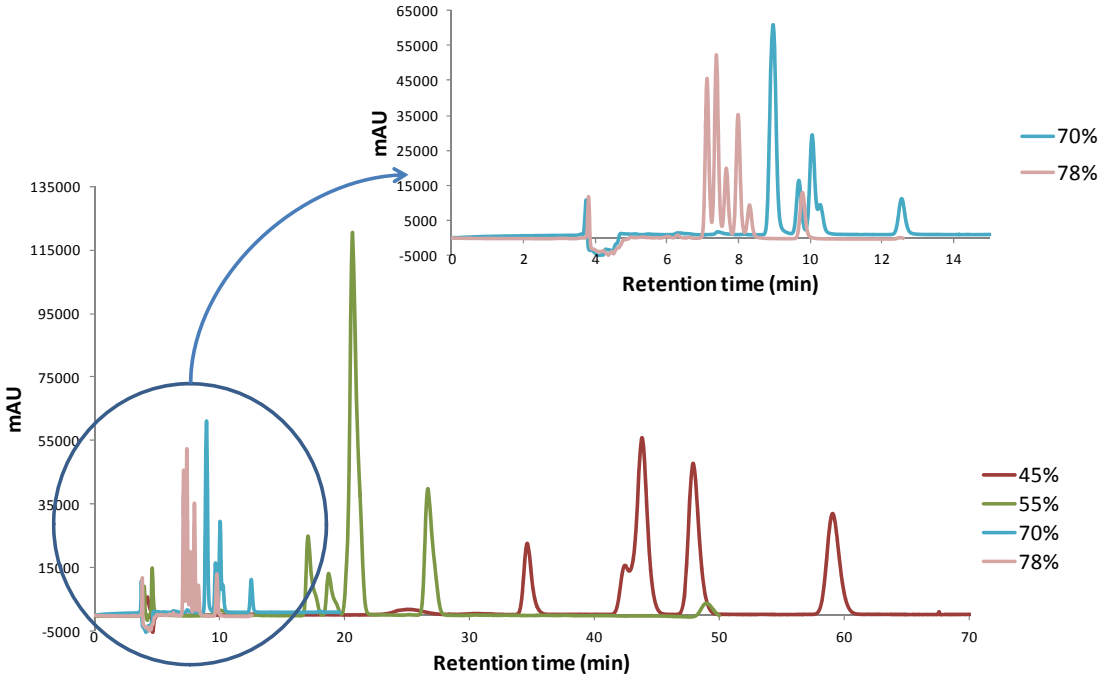

Figure 4.7. *Obtained chromatograms with different isocratic compositions with a 0.5 mL min^{-1} flow. Percentages show the acetonitrile content. Chromatogram in right/upper position shows the elution range for the 70% and 78% ACN.*

Also, the effect of the pH in the mobile phase was checked. Other conditions were kept constant and only the aqueous phase was changed. pH 4 buffer was made using 0.01 mol L^{-1} acetic acid solution adjusted with 0.01 mol L^{-1} sodium acetate and pH 7 buffer was obtained adjusting 0.01 mol L^{-1} potassium phosphate solution with 0.01 mol L^{-1} sodium hydroxide. The results are shown in figure 4.8. As it can be seen the broadening of the peaks occurs using only water, and therefore a stronger overlap is seen. Among pH 4 or 7, there was slight differences, but pH 4 buffer seems to give sharper peaks. Hence, pH 4 buffer was used to continue with the study.

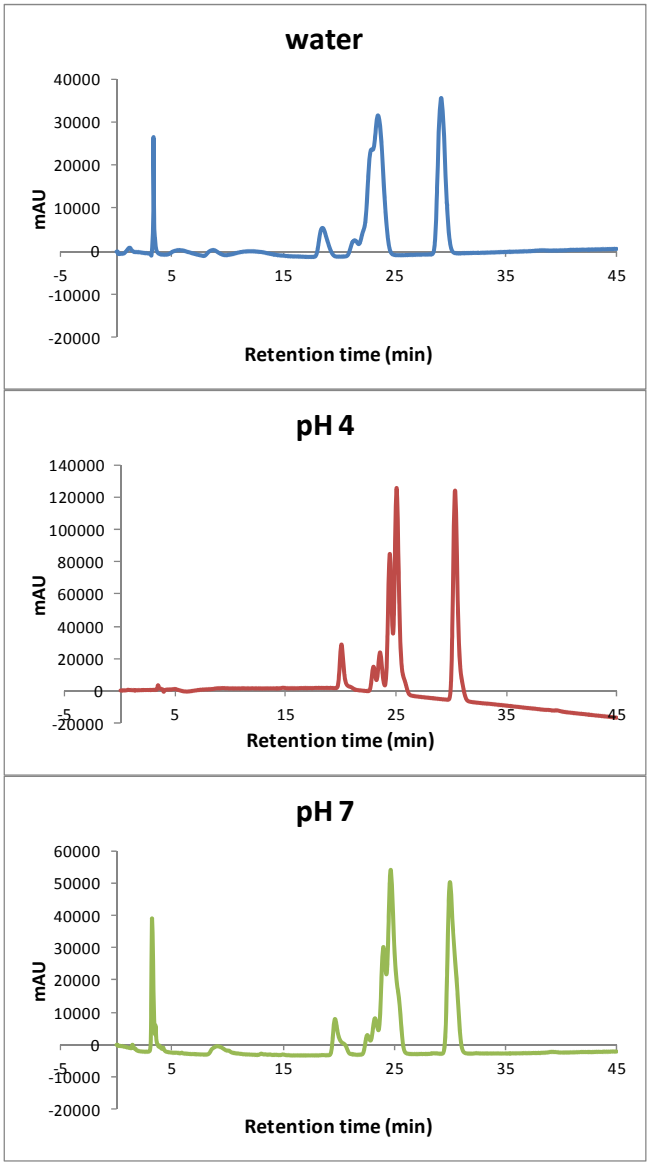

Figure 4.8. *Chromatograms showing differences using water, acetic/acetate pH4 buffer and phosphate pH 7 buffer. Other chromatographic conditions: 0.6 mL min^{-1} flow and an acetonitrile gradient starting at 50% (10 min) following with a ramp until reach the 60% at 40 min (3 min).*

Although a complete resolution of peaks was not achieved, the mobile phase chosen conditions confidently allowed to continue with the work. Those were: 0.5 mL min^{-1} flow with 78% acetonitrile and 22% of acetic/acetate buffer adjusted to pH 4. With this gradient analytes eluted as follows: tetraconazole (7.0 min), myclobutanil (7.3 min), flusilazole (7.7 min), fluquinconazole (8.0 min), tebuconazole (8.3 min) and diniconazole (9.7 min).

4.3.3 Experimental design

In order to obtain an appropriate method for SPME-HPLC for triazole compound determination it is important to find the best experimental conditions. There are different variables affecting the SPME process in immersion mode. Extraction time and temperature, desorption time and mode, salt addition, agitation, fiber type are some of the most important variables [Sánchez-Ortega *et al.*, 2005; Sagratini *et al.*, 2007; Viñas *et al.*, 2008; Parrilla-Vázquez *et al.*, 2008]. Hence, the importance of selecting variables and choosing levels to find the best experimental conditions should be considered. For this purpose screening and optimization designs were carried out. The concentration of analytes in the analysis vial to perform the screening and optimization designs were: tetraconazole and myclobutanil, 15.3 µg L^{-1}; flusilazole 15.0 µg L^{-1}, fluquinconazole and tebuconazole, 15.6 µg L^{-1}, and diniconazole 15.9 µg L^{-1}. The screening design was performed using four analytes: tetraconazole, myclobutanil, diniconazole and flusilazole and for the optimization step two other analytes were added: tebuconazole and fluquinconazole.

4.3.3.1 Screening. Fractional Factorial Design

The screening stage was performed to check the importance of some variables for the method. For this aim, a screening design including extraction time and temperature, salt addition, desorption mode and time variables were considered. Fiber coating type was not included in the study due to two reasons: first, the thickness of the fiber. Desorption chamber ferrule was too thin to some fiber thickness (for instance, 85 µm PA fibers). The other reason was the previous experience working with PDMS/DVB [Bordagaray *et al.*, 2011]. Therefore, PDMS/DVB (65 µm) was the chosen coating. Moreover, agitation was not considered for screening because of the same previous studies made by the authors.

Selected variables and its levels are shown in table 4.3. NaCl addition was considered between 0 and 300 g L^{-1} (almost saturation), extraction time ranged from 15 to 60 minutes, extraction temperature between 20 to 75^{o}C and desorption time

was considered between 1 and 10 minutes. Those were the quantitative variables, and the only qualitative variable was desorption type, in static or dynamic mode.

Table 4.3. *Considered variables and its levels in the 2^{5-1} fractional factorial design.*

Variables	Type	Levels	
		Low [-1]	High [+1]
NaCl addition [NaCl] (g L^{-1})	quantitative	0	300
Extraction time [Ext t] (min)	quantitative	15	60
Extraction temperature [Ext T] (ºC)	quantitative	20	75
Desorption time [Des t] (min)	quantitative	1	10
Desorption type [Des type]	qualitative	dynamic	static

In order to reduce the number of experiments a 2^{5-1} fractional factorial design was planned. This design reduced the experimental run number to a half. The 2^{5-1} fractional factorial design was completed with 20 runs (16 experiments from the fractional factorial design plus four central points). With this fractional factorial design a resolution V is obtained. This resolution does not confound the main effects neither the secondary nor its interactions.

Table 4.4 shows the randomly carried out experiments with its coded values [1, -1 and 0]. This codes were adjudicated to the highest value [+1], lowest value [-1] and the mean point [0] when the quantitative variables are considered. For the qualitative variables, [-1] code was dynamic desorption mode and [+1] was the static desorption mode.

Table 4.4. *Coded matrix of the 2^{5-1} fractional factorial design with the random order of experiments.*

Experiment	Variables				
	NaCl	**Ext t**	**Ext T**	**Des t**	**Des type**
15	-1	1	1	1	-1
10	1	-1	-1	1	1
19C	0	0	0	0	-1
3	-1	1	-1	-1	-1
8	1	1	1	-1	-1
18C	0	0	0	0	1
20C	0	0	0	0	1
11	-1	1	-1	1	1
4	1	1	-1	-1	1
7	-1	1	1	-1	1
14	1	-1	1	1	-1
5	-1	-1	1	-1	-1
1	-1	-1	-1	-1	1
17C	0	0	0	0	-1
2	1	-1	-1	-1	-1
12	1	1	-1	1	-1
13	-1	-1	1	1	1
6	1	-1	1	-1	1
16	1	1	1	1	1
9	-1	-1	-1	1	-1

C: Central point

The table 4.5 shows the chromatographic areas obtained at 221nm with the 2^{5-1} fractional factorial design previously described.

Table 4.5. *Experimental runs in random order and analytes responses in peak areas.*

Experiment	Areas			
	TT	M	FS	D
15	1009251	262602	1534399	1407174
10	160290	94232	157689	135772
19C	881696	413893	898617	841843
3	618213	265975	736652	646409
8	1836423	996330	1942310	1716966
18C	1067574	450505	1219639	1196115
20C	813659	393273	909096	876852
11	512600	212663	655795	581668
4	800047	475706	788667	684771
7	1002176	275045	1479492	1329768
14	427374	231582	477442	394468
5	452455	161643	595301	559350
1	253542	112883	310065	259544
17C	1205759	532174	1338776	1246088
2	216887	130938	223767	198424
12	950848	560102	911498	850604
13	311399	111900	423554	403351
6	602280	345218	693370	625422
16	2031256	1109806	2223222	2030059
9	144350	58300	174967	157720

C: Central point

Main effects were evaluated by analysis of variance (ANOVA) (table 4.6) and visualized using Pareto charts (Figures 4.9 and 4.10) where the significance of the different factors was evaluated by the *F-test*. Interactions were not considered in the model, therefore the table and the figures show only the principal effects in the process.

The *F*-coefficient was calculated for each variable dividing mean square effect by the error mean square effect and this coefficient is compared with *F*-distribution table, taking into account the degrees of freedom. If the calculated *F* is higher than the table-*F* means the variable is significant. Thus, the change of these variables affects the system. The *p*-value, shows the probability that a certain phenomena occurred by pure chance, consequently if this value is little means that the variation in the system was

not pure chance. The limit was established in p=0.05. Values lower than 0.05 are significant variables.

Table 4.6 shows the ANOVA results for each analyte. Numbers in red are the significant variables. Extraction time and temperature were important for all the analytes. Salt addition resulted significant for tetraconazole and myclobutanil but not for flusilazole and diniconazole. On the other hand, desorption was not significant for the process, both factors (desorption time and type) were not statically important.

Table 4.6. *ANOVA for the included analytes in the screening design.*

		TETRACONAZOLE				
		SS[a]	**df[b]**	**MS[c]**	***F*[f]**	***p*[g]**
Variables	**NaCl**	4.628826E+11	1	4.628826E+11	5.51815	0.034023
	Ext t	2.396487E+12	1	2.396487E+12	28.56917	0.000103
	Ext T	1.007934E+12	1	1.007934E+12	12.01585	0.003779
	Des t	3.441436E+09	1	3.441436E+09	0.04103	0.842401
	Des type	1.775350E+09	1	1.775350E+09	0.02116	0.886406
Error		1.174372E+12	14	8.388368E+10		
Total SS		5.046893E+12	19			

		MYCLOBUTANIL				
		SS[a]	**df[b]**	**MS[c]**	***F*[f]**	***p*[g]**
Variables	**NaCl**	3.853005E+11	1	3.853005E+11	12.55448	0.003244
	Ext t	5.298140E+11	1	5.298140E+11	17.26325	0.000972
	Ext T	1.566828E+11	1	1.566828E+11	5.10529	0.040325
	Des t	9.386717E+08	1	9.386717E+08	0.03059	0.863673
	Des type	5.219034E+07	1	5.219034E+07	0.00170	0.967689
Error		4.296638E+11	14	3.069027E+10		
Total SS		1.502452E+12	19			

Table 4.6 (continuation). *ANOVA for the included analytes in the screening design.*

		FLUSILAZOLE				
		SS[a]	df[b]	MS[c]	*F*[f]	*p*[g]
Variables	NaCl	1.420800E+11	1	1.420800E+11	1.71594	0.211304
	Ext t	3.254308E+12	1	3.254308E+12	39.30322	0.000021
	Ext T	1.829249E+12	1	1.829249E+12	22.09237	0.000341
	Des t	2.784092E+09	1	2.784092E+09	0.03362	0.857137
	Des type	3.607298E+07	1	3.607298E+07	0.00044	0.983642
Error		1.159201E+12	14	8.280004E+10		
Total SS		6.387658E+12	19			

		DINICONAZOLE				
		SS[a]	df[b]	MS[c]	*F*[f]	*p*[g]
Variables	NaCl	1.042486E+11	1	1.042486E+11	1.34915	0.264847
	Ext t	2.651498E+12	1	2.651498E+12	34.31485	0.000042
	Ext T	1.532425E+12	1	1.532425E+12	19.83216	0.000546
	Des t	2.237866E+08	1	2.237866E+08	0.00290	0.957842
	Des type	5.436742E+08	1	5.436742E+08	0.00704	0.934338
Error		1.081776E+12	14	7.726969E+10		
Total SS		5.370714E+12	19			

[a]SS: Sum of Squares
[b]df: degrees of freedom
[c]MS: Mean Square effect
[f]*F*: Fischer coefficient
[g]*p*: Probability

Pareto charts show the estimation of standardized effects of the variables. The line of the *p*-value drew in the charts separate the significant values at the right. The positive and negative sign in Pareto charts indicate that the response is enhanced or reduced, respectively, when passing a given factor from the lowest to the highest level.

Salt addition, extraction time and temperature were important factors for the method. Taking into account the bar lengths especially the extraction time was important for all the analytes. High extraction times were preferred as indicate the positive sign in the values after the bars. Temperature was also important for all the variables and also the extraction time, in both cases high values were preferred. Salt addition was significant in two analytes: tetraconazole and myclobutanil. Although it was not relevant for the other two analytes, all of them showed better responses with higher salt concentrations as indicated with the positive sign after the bar.

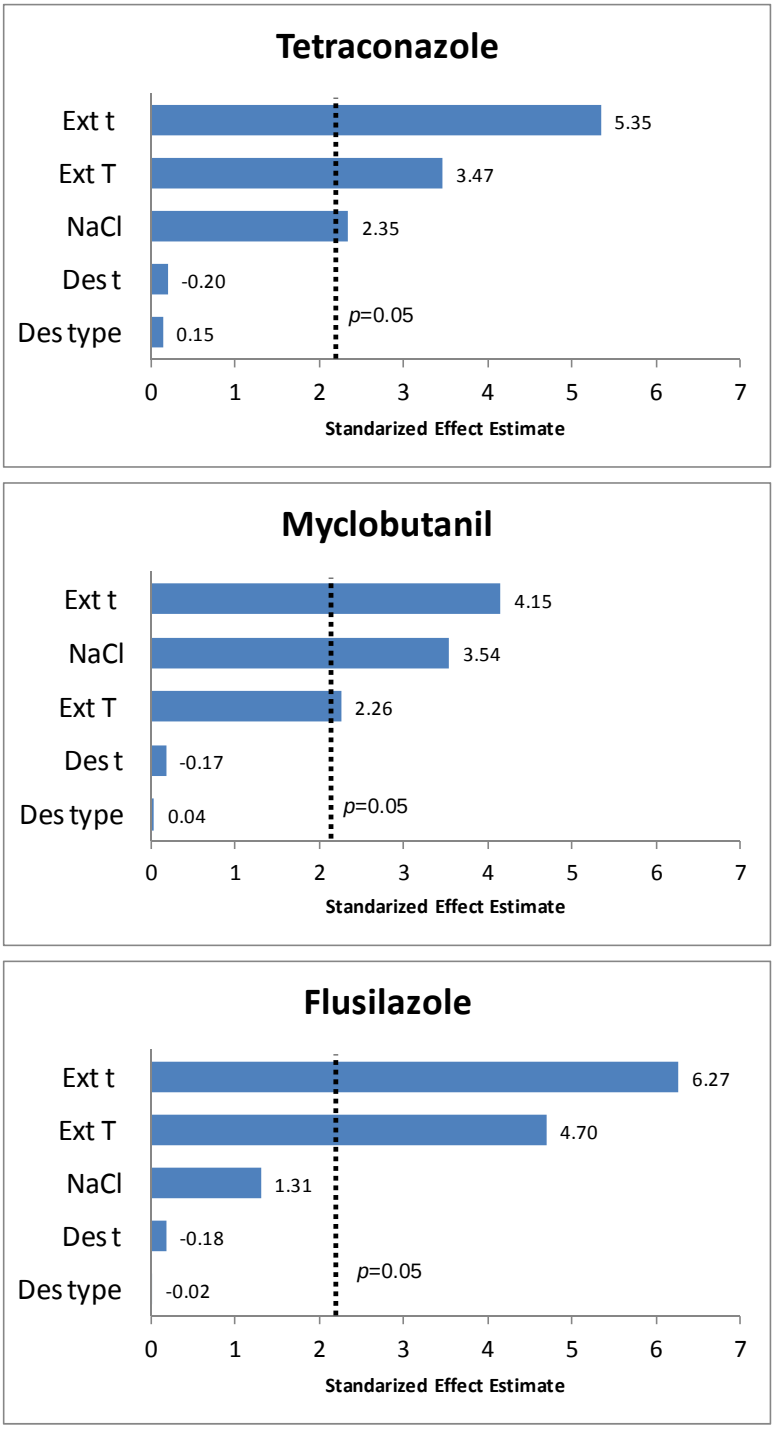

Figure 4.9. *Pareto charts for tetraconazole, myclobutanil and flusilazole analytes. Values after the bars indicate the absolute values of the main effects and the dotted line indicates 95% confidence level (p=0.05).*

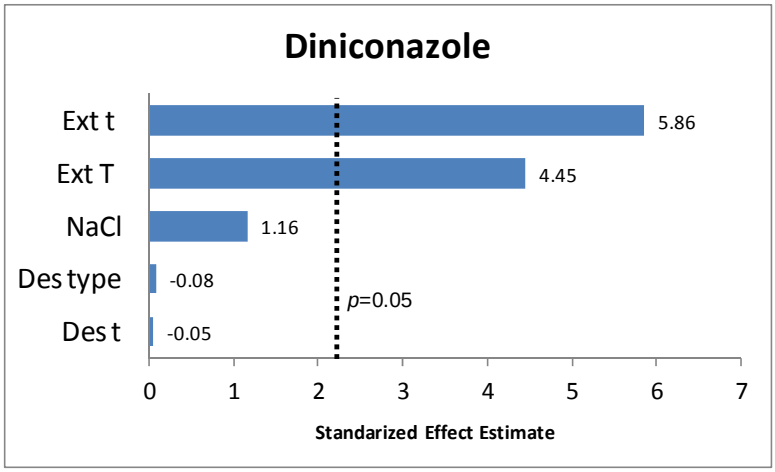

Figure 4.10. *Pareto chart for diniconazole analyte. Values after the bars indicate the absolute values of the main effects and the dotted line indicates 95% confidence level (p=0.05).*

Desorption conditions, time and type, were not significant. But due to the negative sign, low desorption time was preferred. For desorption type, the dynamic mode was slightly better for flusilazole and diniconazole; and static mode was slightly better for tetraconazole and myclobutanil. Since the dynamic desorption mode showed band broadening comparing with the static mode, the latter mode was chosen. As previously noted, a little dynamic desorption step was necessary because to swap the mobile phase from desorption chamber the circuit need to be closed. And the only way to do so, it was with the fiber inside the chamber. Therefore, desorption was done two minutes in the static mode followed by another minute in dynamic mode.

The ANOVA and Pareto charts results are summarized in the table 4.7. Underlined values mean significant variables for the analyte. The variables that were not significant (desorption time and type), were fixed in the preferred values as explained before. Other three variables, salt addition, extraction time and temperature (shaded variables in table 4.7) were taken for the following optimization step.

Table 4.7. *Results of the fractional factorial design.*

Variable	TT	M	FS	D	Action
NaCl	+1	+1	+1	+1	**To optimization**
Ext t	+1	+1	+1	+1	**To optimization**
Ext T	+1	+1	+1	+1	**To optimization**
Des t	-1	-1	-1	-1	Fixed: **2 min (+1 min)**
Des type	+1	+1	-1	-1	Fixed: **static (+ dyn)**

4.3.3.2 Optimization. Central Composite Design

The next step to know the best condition to perform a method is to optimize the important variables. In this research the screening design reduces the number of variables from five to three and those three showed real relevance in the method. Variations in those variables led to significant changes in the obtained chromatographic areas. Hence, factors will be optimized to obtain the maximum possible response. For this aim, a central composite design (CCD) was built to optimize extraction time and temperature and salt addition.

This design was formed by 2^3 factorial points plus [(2 x 3) + 1] star points. Three additional central points were added with a total number of eighteen assays. To set the axial distances rotatability or orthogonal conditions can be fulfilled. In this study the rotatability condition was considered and axial points were established at distances defined by $\alpha = \sqrt[4]{N_f} = 1.682$ ($N_f = 2^3$). The rotatability condition means that there is the same information about the response surface at the same distance in any direction from the design center [Dean and Voss, 1999] and the variance of its estimates depends only on the distance from the center point; that is, the precision of the predicted response is the same for all points situated around the center of design [Ferreira *et al.*, 2007]. Figure 4.11 shows the graph with the carried out experiments.

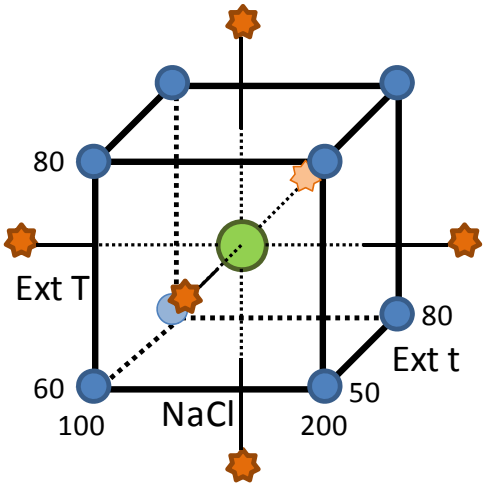

Figure 4.11. *Graphical representation of CCD experimental design with 3 factors.*

The variables and its values to optimization are shown in table 4.8. The ranges for salt addition were from 100 to 200 g L^{-1}, from 50 to 80 minutes for extraction time and from 60 to 80ºC for extraction temperature. The star points were located at 66 and 234 g L^{-1} for salt addition, 40 and 90 minutes for extraction time and 53 and 87ºC for extraction temperature.

Table 4.8. *Variables to optimize with the central composite design. Low and high levels and the star points (with rotatability condition).*

Variable	Low [-1]	High [+1]	[-α]	[+α]
NaCl (g L^{-1})	100	200	66	234
Ext t (min)	50	80	40	90
Ext T (ºC)	60	80	53	87

The experimental runs were planed as is shown in table 4.9. The assays were randomly carried out and the chromatographic areas obtained at 221 nm were considered as the response.

Table 4.9. *The randomly carried out experimental matrix with the coded variables and the chromatographic responses for the analytes.*

Experiment	Variables			Areas					
	NaCl	Ext t	Ext T	TT	M	FS	FQ	TB	D
12	0	0	α	196162	165585	274542	543940	238758	268189
7	-1	1	1	172001	159818	314318	569397	294397	312083
6	1	1	-1	202876	227340	308522	561060	271891	280600
10	0	α	0	205581	218204	345781	639414	289793	327074
3	-1	-1	1	130866	121461	244697	375531	215696	218234
15 C	0	0	0	170048	182518	270185	476358	247373	255041
2	1	-1	-1	128244	147308	182318	312500	192345	152312
5	-1	1	-1	172973	177578	294066	452834	251938	261574
18 C	0	0	0	162890	169833	259257	405322	219320	221292
14	α	0	0	162720	191191	244081	244164	231742	151083
8	1	1	1	197676	219920	311035	414209	294937	232205
17 C	0	0	0	162248	179704	262803	348761	223164	227471
13	-α	0	0	134190	146373	208276	257313	168731	154605
4	1	-1	1	134160	152450	187747	220669	163928	118614
11	0	0	-α	146991	158647	229311	236155	190929	166328
9	0	-α	0	131341	136189	188461	176331	153656	126748
1	-1	-1	-1	118868	121910	181880	170882	154649	112805
16 C	0	0	0	144513	162000	225044	225463	180106	138484

C: Central point

The obtained data were analyzed using STATISTICA software. A second-degree plynomial model was built taking into account main effects, interactions and quadratic components. This models are represented by response surfaces for each analyte. These plots represent the variation of the chromatographic areas according to two of the variables, the third variable needs to be fixed. The plots can be analyzed separately for each analyte, but can be difficult to make a decission when some different charts are involved.

Other plots, such as the figure 4.12, represent the predicted values and desirability for each analyte and variable and finally gives the optimum values to get a desirable results for all the analyte. Desirability function asigned values of 0 to the undesirable value (lowest chromatogaphic area values) and 1 to the most desirable value (maximum areas). First column represent extraction time for each analyte. Extraction time seems to be very meaningful, because desirability increase remarkably at higher times. On contrary, extraction temperature barely change at the studied range (second column). Salt addition, drawn in third column, shows the optimum value is reached when the added amount of NaCl is around 190 g L^{-1}.

As an overall result the last line in figure 4.12 shows the optimum variable values. Extraction time looks crucial in the method but temperature does not affect very much. Response increases with salt addition but the desirability does not change significantly when the values are higher than 180 g L^{-1}.

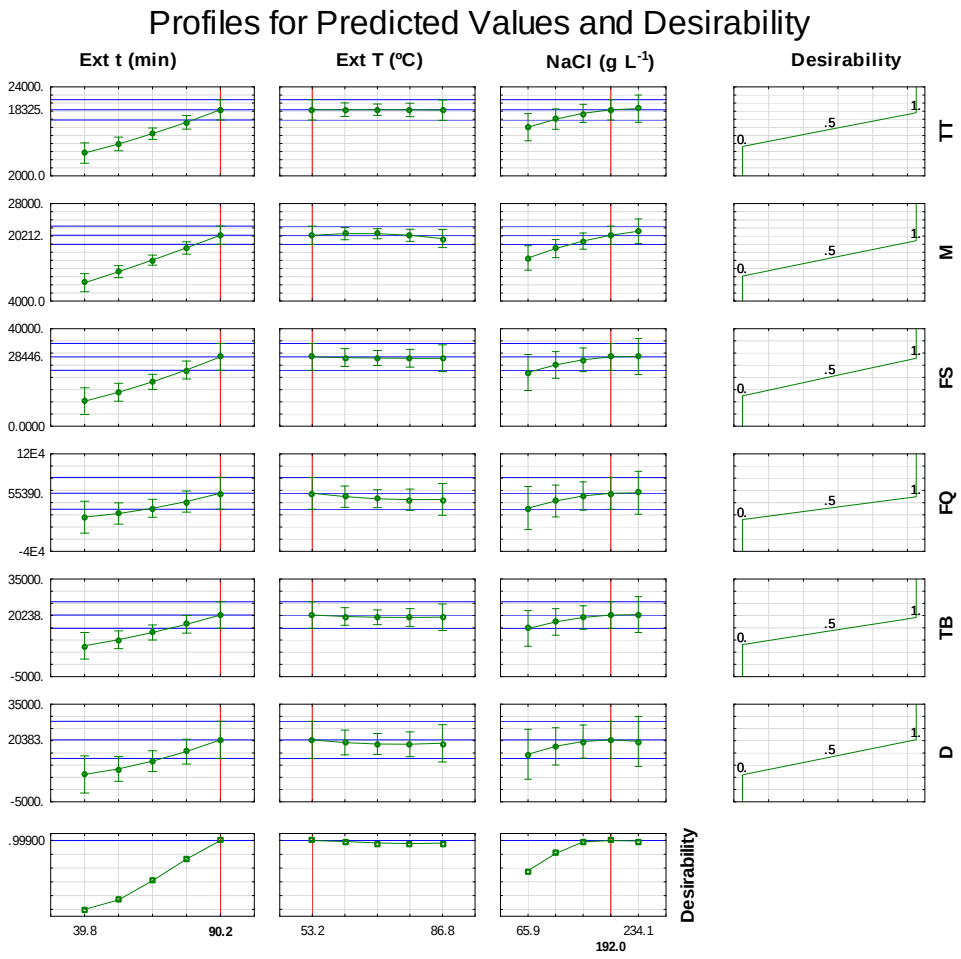

Figure 4.12. *Profiles for predicted values and desirability for the optimization in each analyte.*

Global desirability can be summarized in 3D graphs considering all the analytes and two of the variables. Each plot represents two of the involved variables. In this work because of the combination of variables three total plots are obtained (see figure 4.13).

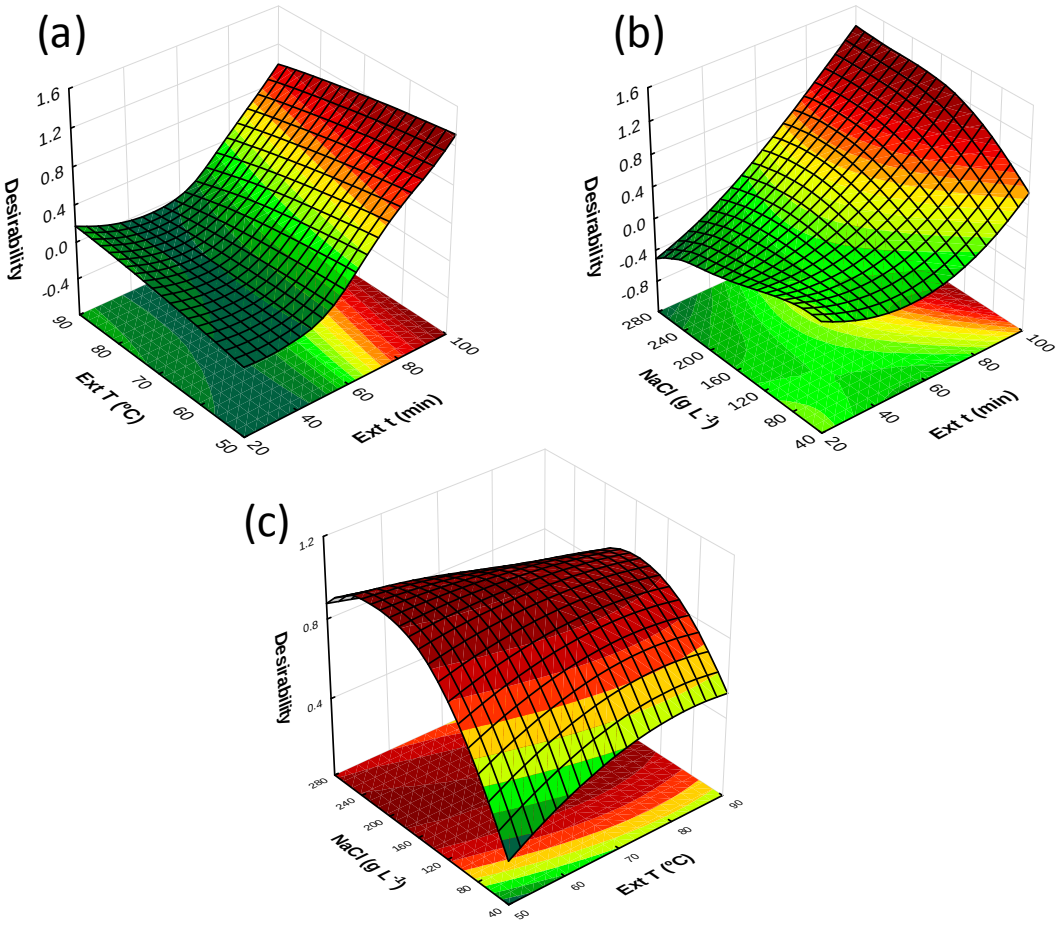

Figure 4.13. *Global desirability surface response plots taking into account (a) extraction time vs. extraction temperature; (b) extraction time vs. NaCl addition and (c) extraction temperature vs. NaCl addition.*

Color scale is the next: green for the undesirable response and intense red for the most desirable result. Looking at the extraction time in plots *(a)* and *(b)*, it can be seen that the response increases with the time. In *(a)* and *(c)* does not show big variation across the extraction temperature axis. Finally, salt addition in *(b)* and *(c)* graphs shows better results with higher salt concentrations.

Considering the above results the best experimental conditions were fixed: Extraction time, 90 minutes; extraction temperature, 60ºC and the salt addition 180 g L^{-1}. 60ºC was chosen instead of 53ºC because it was considered more manageable temperature and 180 g L^{-1} of NaCl addition was chosen because there was not

remarkable difference in desirability with the optimum value (192 g L^{-1}) and excessive salt can results in early fiber damage.

Therefore, after the screening and optimization steps the conditions were fixed as follows:

- **Extraction time: 90 min**
- **Extraction temperature: 60°C**
- **NaCl addition: 180 g L^{-1}**
- **Desorption time: 2 min (+ 1 min)**
- **Desorption type: static (+ dynamic)**

4.3.4 Analytical characteristics

After fixing the conditions for the analysis, the method needs to be validated. Validation of an analytical method includes testing of its important characteristics. The final aim is to be certain that the analysis process is reliable and precise, and variations remain under operator control. Many parameters are recommendable to include in validation process, depending on the time wanted to spend or the restrictive expected values for parameters. That is, if more restrictive parameters values are expected, method should be tested or "revalidated" more often [Konieczka and Namieśnik, 2009]. Some of the tested parameters are described below:

- Linear range and linearity: Linearity refers to a (usually) linear relationship between an analyte concentrations with a measurement used in the calibration step.
- Sensitivity: the capability of the apparatus for giving a certain signal for a given concentration. This parameter is related to the slope of the calibration.
- Precision: is the agreement between replicates of measurement given by the relative standard deviation (RSD %). Different precision values can be given: intra-day precision, inter-day precision, and inter-laboratory precision.
- Accuracy: the closeness of a measured value with the expected (real) value. Spiked samples are used for this parameter and are expressed as recovery (%).
- Limit of detection (LOD): the lowest amount to be detected in a method. There are several proposals to calculate limits of detection and the appropriate one should be chosen for each case.

As explained in section 4.3.2 the chromatographic separation was previously performed. Due to the similarities between analytes molecular structure, complete resolution between peaks was not achieved. The best separation was obtained using an isocratic 0.5 mL min^{-1} flow of acetonitrile and acetic/acetate buffer (v/v, 78/22). Each analyte can be determined but a slight overlap is remarkable. Chromatogram obtained under these conditions is shown in figure 4.14.

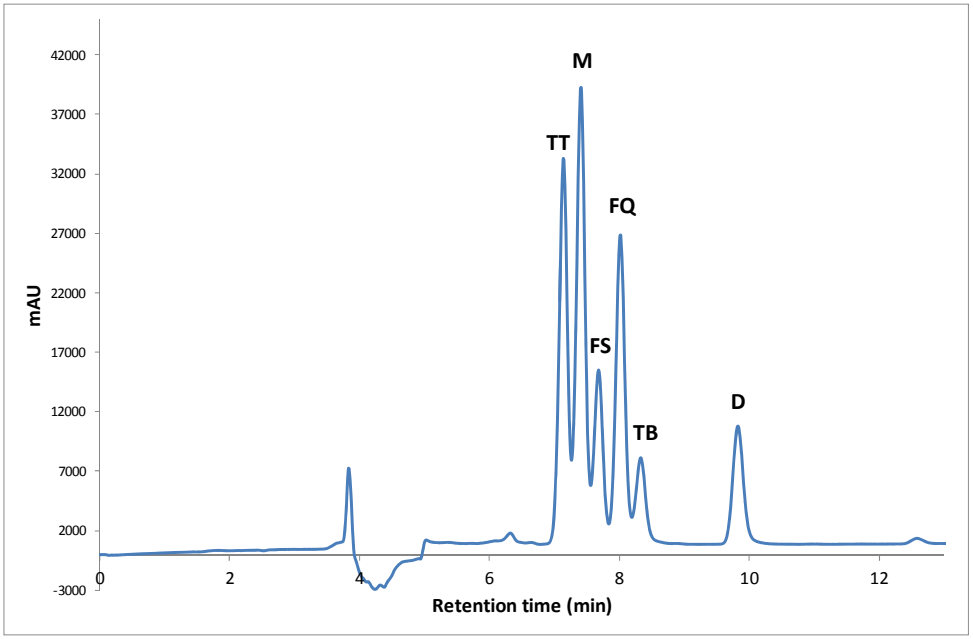

Figure 4.14. *Chromatographic separation of the analytes.*

The calibration ranges were established in similar values for the analytes. Within those range correlation coefficients higher than 0.9878 were obtained (see table 4.10).

When there is the possibility of measuring a serial of blank samples, the most known method for calculating the limit of detection uses several times the standard deviation signal of the blank. The value to multiply to the standard deviation depends on the *t* Student's distribution when data is considered normal distributed and homoscedastic [Rajaković *et al.*, 2012]. For instance, when the reported LOD is 99% confidence level and seven replicates are involved (thus, df = n-1; degrees of freedom equal to six) the *t* value is 3.14 [Kleinbaum *et al.*, 1988]. However, scientific community agreed to used three [Foley and Dorsey, 1984; Taverniers *et al.*, 2004] or three point three [ICH Harmonised Tripartite Guideline, 2005; Konieczka and Namieśnik, 2009] as a general coefficient.

Nevertheless, when the chromatographic data are considered two main problems arised: first, the long chromatographic times and second, the retention time shifts. If very long times are involved, the replicates of analysis can be very tedious to obtain (as in this case that complete analysis adds 90 minutes of extraction time plus 15 minutes of chromatographic run). Secondly, because of the retention time shifts integration of a definite range can be imprecise.

Among different ways of calculating limits of detection (LOD), in this work it was considered the standard deviation calculated from the deviation of the obtained calibration curve slope ($s_{y/x}$) [Konieczka and Namieśnik, 2009]. The formula used for calculating this deviation is shown in equation 4.1.

$$s_{y/x} = \sqrt{\frac{\sum(\hat{y}_i - y_{i,ref})^2}{I-2}} \quad (4.1)$$

The y axis residual is calculated; therefore the y value obtained from the calibration curve ($\hat{y}_i$) and the real y value ($y_{i,ref}$) are taken into account: the term $(\hat{y}_i - y_{i,ref})^2$ belongs to the residual of each sample (i) and the total residual is calculated with the summation (Σ). The I term is the number of total number of samples used for the calibration and (I-2) is used to calculate the degree of freedom.

The LOD is calculated using this value as the deviation standard divided by the slope of the calibration (b). Three was chosen as the coefficient for this case. The results are shown in table 4.10.

$$LOD = \frac{3 \cdot s_{y/x}}{b} \quad (4.2)$$

Table 4.10. *Calibration characteristics for the developed method.*

	Linear range (µg L^{-1})	R^2	Equation	LOD (µg L^{-1})
TT	5.5- 33.0	0.9980	y = 9296x – 1324.8	1.54
M	5.5- 33.0	0.9968	y = 8804x + 54275	1.94
FS	7.0- 42.0	0.9912	y = 19147x + 29828	4.15
FQ	5.4- 32.4	0.9906	y = 42897x + 33391	3.31
TB	8.4- 50.4	0.9878	y = 12033x + 67141	5.87
D	5.2- 31.2	0.9978	y = 24199x + 34394	1.53

Precision was given in order to evaluate the deviation of the measurements. For this aim, intra and inter-day precisions were calculated. For intra-day precision four measurements were done in the same day. On the other hand, for inter-day precision eight measurements obtained in four different days in two consecutive weeks were

considered. Each precision was calculated in two different concentrations (*ca*. 15 and 25 μg L^{-1}), the standards number 7 and 8 according to table 4.1. The values for intra-day precision for the 7th standard were ranged between 5.1 to 11.5% and for the 8th standard were from 2.8 to 5.4%. For inter-day precision RSDs between 5.1 and 13.1% were obtained for the 7th standard. For the 8th standard values were from 5.8 to 9.8%. The detailed results are shown in table 4.11.

The recovery studies were carried out in water. Standards 7 (15 μg L^{-1}) and 8 (25 μg L^{-1}) were spiked and analyzed to see the accuracy of the method. Each standard was measured two times and the values and its deviations are given in table 4.11. Recoveries between 95.3 and 108.3% were obtained for 7th standard and between 94.5 and 123.4% for the 8th standard.

Table 4.11. *Recovery and precision characteristics for the developed method.*

	Intra-day precision [n=4] (RSD %)		**Inter-day precision [n=8] (RSD %)**		**Recovery (%)**	
	15 μg L^{-1}	*25 μg L^{-1}*	*15 μg L^{-1}*	*25 μg L^{-1}*	*15 μg L^{-1}*	*25 μg L^{-1}*
TT	7.2	5.4	7.1	9.8	100.0 ± 5.7	101.8 ± 7.6
M	5.1	2.8	5.1	5.8	96.2 ± 14.8	123.4 ± 27.1
FS	11.5	5.0	8.3	8.7	104.1 ± 11.1	94.5 ± 0.3
FQ	8.2	3.3	9.5	7.8	95.3 ± 2.7	95.0 ± 0.4
TB	7.0	5.3	13.1	7.7	108.3 ± 11.3	97.9 ± 9.9
D	10.6	5.4	6.5	9.0	101.6 ± 1.1	107.2 ± 0.6

AOAC establishes some precision and recovery values to consider the method acceptable (see table 4.12) [AOAC International, 2012]. Taking into account both inter and intra-day precisions with two concentrations, the highest value was obtained for tebuconazole in the 7th standard (13.1%). This precision is considered more than acceptable according to table 4.12. On the other hand all recoveries are considered good enough except the one obtained for myclobutanil in standard number eight (123.4%).

Table 4.12. *AOAC expected precision and recovery values for a method.*

Analyte fraction	Unit	RSD (%)	Mean Recovery (%)
1	100 %	1.3	98-102
10^{-1}	10 %	1.9	98-102
10^{-2}	1 %	2.7	97-103
10^{-3}	0.1 %	3.7	95-105
10^{-4}	100 ppm	5.3	90-107
10^{-5}	10 ppm	7.3	80-110
10^{-6}	1 ppm	11	80-110
10^{-7}	100 ppb	15	80-110
10^{-8}	10 ppb	21	60-115
10^{-9}	1 ppb	30	40-120

Comparing with other methods used for triazole determination in liquid samples, the LODs obtained for tebuconazole are lower than LODs showed using HPLC/DAD [Ravelo-Pérez *et al.*, 2009] and similar to LODs indicated using solid-phase extraction-hollow fiber preconcentration followed by gas chromatography-flame ionization detection (SPE-HF-GC/FID) [Farajzadeh *et al.*, 2012] and using dispersive liquid-liquid microextraction (DLLME) followed by HPLC/DAD [Ravelo-Pérez *et al.*, 2009]. However, it is higher than the 0.012 μg L^{-1} value obtained using quick, easy, cheap, effective, rugged, safe (QuEChERS) sample preparation and the more sensible instrumentation GC/MS [Jiang *et al.*, 2009].

Referring to precision and recovery the obtained results are comparable to others with different extraction techniques. For diniconazole and tebuconazole in wine the recoveries from 83 to 110% and the RSDs were from 5 to 10% using QuEChERS coupled to GC/MS [Jiang *et al.*, 2009]. For tebuconazole in grape juices the RSD was 6.5% and the recoveries were from 87 to 117% using SPME-HF-GC/FID [Farajzadeh *et al.*, 2012]. For tebuconazole in water using the DLLME procedure and HPLC/DAD the given values were 93% for recovery and 2.5% for RSD [Ravelo-Pérez *et al.*, 2009]. Myclobutanil, tebuconazole, flusilazole and diniconazole were simultaneously determined using DI-SPME-GC/ToF/MS and the obtained RSDs were ranging between 4-20 %, while the recoveries were between 70-120% [Souza-Silva *et al.*, 2013]. Using other methodologies such as UAE and PLE coupled to GC/TQ/MS the obtained results for tebuconazole were 105-120% for recoveries and 5.8- 90.6% for RSD [Celeiro *et al.*, 2014].

4.3.5. Application to samples

The developed method was applied to real samples. Determination of the pesticides in samples within a pre-harvest period was carried out. Two commercial products containing myclobutanil and tebuconazole were applied to a vineyard and an apple tree and were applied according with the manufacturer instructions as it has been explained in section 4.2.2.

Pesticide residues were found only in peel samples in both fruits (apple and grape) but the pesticides were detected neither in pulp nor juice samples. This fact led to think that the products are superficial treatments and did not go to inner portion of the fruits. The results shown in table 4.13, are the mean values of three measurements and its standard deviation. Found values in peel were ranging from 4.8 to 18.9 mg kg^{-1} for myclobutanil in apple peel samples and 6.6 to 10.4 mg kg^{-1} for tebuconazol. On the other hand in grape peel samples, the found myclobutanil residues were ranging from 8.7 to 23.9 mg kg^{-1} and 15.9 to 37.2 mg kg^{-1} for tebuconazole.

Table 4.13 *Mean concentration ± s.d.[a] (mg kg^{-1}) of triazole fungicides in collected fruit samples.*

Fruit samples		Sample collecting days							
		Day 1		Day 4		Day 8		Day 15	
		M	TB	M	TB	M	TB	M	TB
Apple	Peel	12.5 ± 4.0	6.6 ± 0.3	9.6 ± 0.8	10.4 ± 0.1	18.9 ± 1.4	7.8 ± 1.8	4.8 ± 0.1	n. d.
	Pulp	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	Juice	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
Grape	Peel	18.4 ± 1.9	37.2 ± 5.5	23.9 ± 1.0	29.7 ± 2.6	8.7 ± 0.1	24.7 ± 4.4	13.9 ± 8.4	15.9 ± 4.1
	Pulp	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	Juice	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.

[a]s.d. refers to standard deviation (n=3)

n. d.: not detected; M, myclobutanil; TB, tebuconazole

Figure 4.15 shows one of the positive chromatograms obtained for an apple peel sample. Blue color indicates the apple peel sample while the green line corresponds to a blank sample. For the latter, samples were collected before the application of the product.

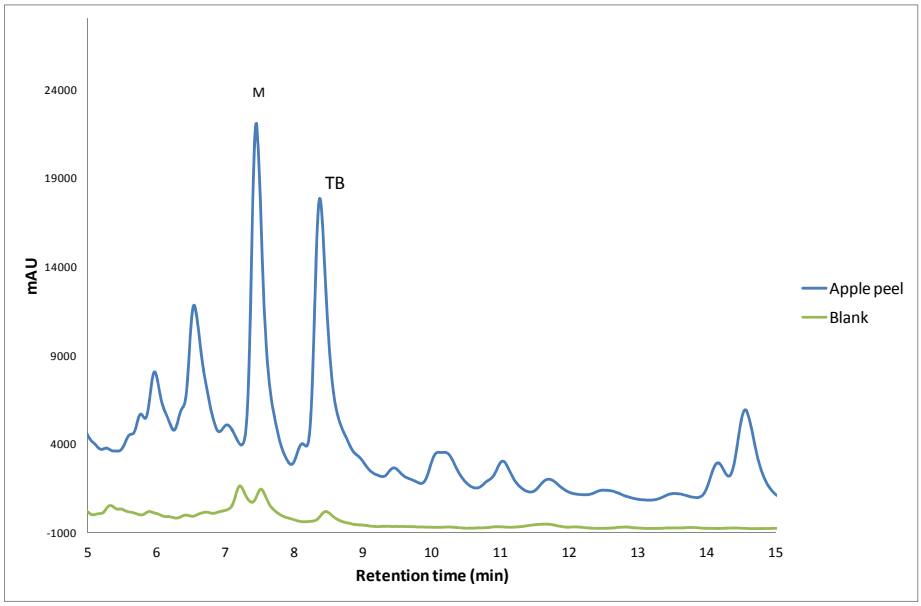

Figure 4.15. *Apple peel sample chromatogram (1st day) with the presence of analytes.*

As it can be seein in table 4.13 the pesticide content is diminished after the application day (figure 4.16). Even though fruit pieces were taken from different spots of trees, the treatment may not equally reach to every part and therefore more heterogeneous dispersion would happen when working with solid samples. Raining days (simulated and the real) are also marked in the chart.

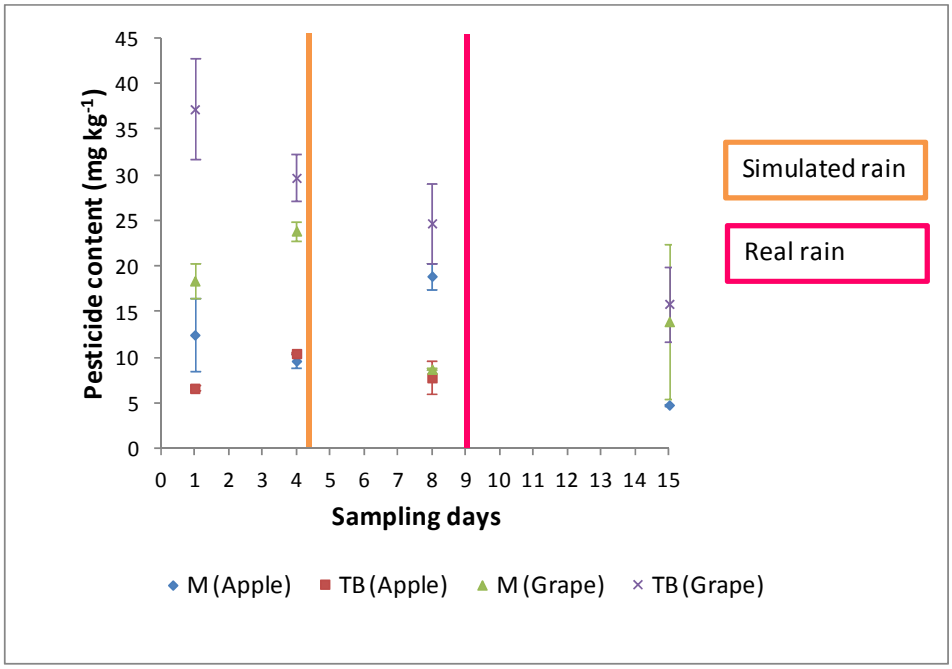

Figure 4.16. *Determination levels of myclobutanil and tebuconazole during the pre-harvest period.*

Rain samples were also analyzed collecting the water under the trees. Both samples gave positive analysis in both analytes the first collecting day (4th day); 253.1 and 114.6 µg L^{-1} for myclobutanil and 71.8 and 14.1 µg L^{-1} for tebuconazole. The 9th day the tebuconazole residues were not detected and myclobutanil residues were found for water samples collected under apple tree and vineyard, 42.2 and 34.4 µg L^{-1} respectively. The results are shown in table 4.14.As indicated in figure 4.16, looks like rain carried over the analytes from the fruit surfaces.

Table 4.14 *Mean concentration ± s.d.[a] (µg L^{-1}) of triazole fungicides in collected rain samples.*

Rain samples	Sample collecting days			
	Day 4 (simulated rain)		Day 9 (real rain)	
	M	TB	M	TB
Apple	253.1 ± 4.8	71.8 ± 8.1	42.2 ± 0.5	n. d.
Grape	114.6 ± 7.0	14.1 ± 0.2	34.4 ± 2.5	n. d.

[a]s.d. refers to standard deviation (n=3)
n. d.: not detected; M, myclobutanil; TB, tebuconazole

The chromatogram of the sample collected under the vineyard at the day 4 is shown in figure 4.17. As it can be seen there are eluted two peaks where myclobutanil and tebuconazole eluted (blue color line). In the same figure is included the chromatogram of blank rain sample (green line).

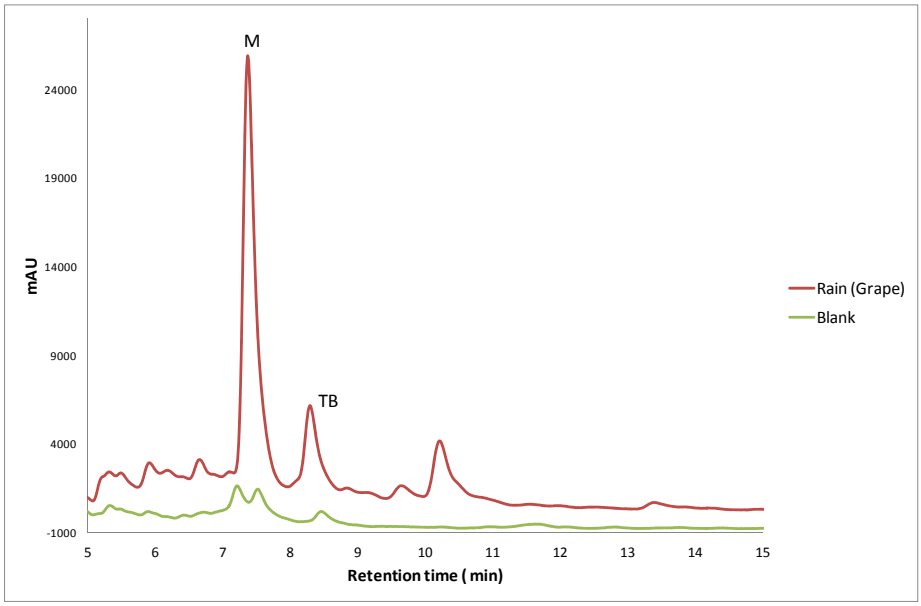

Figure 4.17. *Rain sample chromatogram collected under vineyard with the presence of analytes. Sample collected at day 4.*

The results obtained were compared with the MRLs established by the European Regulations. The MRLs for the studied analytes and fruits are shown in table 4.15. These regulations are changing from time to time and limits are narrowing to assure security in their consumption. The first regulation for this group of fungicides was published in 2008 including flusilazole, fluquinconazole, tetraconazole, myclobutanil and diniconazole [Regulation EC 149/2008]. For tebuconazole the first regulation was published in 2010 [Regulation EU 750/2010]. Nevertheless the allowed maximum residue levels are changing to more severe values and thus these regulations are updated.

Table 4.15. *MRLs allowed for apple and wine grapes according to European Regulations.*

Analyte	Fruit	MRL (mg kg^{-1})	Regulation	Regulation Date
FQ	**Apple**	0.1	Reg. (EC) No 149/2008	29 January 2008
	Wine Grape	0.5		
M	**Apple**	0.5	Reg. (EC) No 149/2008	29 January 2008
	Wine Grape	1		
FS	**Apple**	0.02	Reg. (EC) No 459/2010	27 May 2010
	Wine Grape	0.2		
TT	**Apple**	0.3	Reg. (EU) No 34/2013	16 January 2013
	Wine Grape	0.5		
D	**Apple**	0.01	Reg. (EU) No 1317/2013	16 December 2013
	Wine Grape	0.01		
TB	**Apple**	0.3	Reg. (EU) No 61/2014	24 January 2014
	Wine Grape	1		

The obtained results in the work were above from the MRLs shown in the table 4.15. For triazole fungicides the limits are established between 0.01 and 0.5 mg kg^{-1} for apples, while the limits are from 0.01 to 1 mg kg^{-1} in wine grapes. However, it should be considered that these MRL values are for the whole pieces. The results given in table 4.13 are referred only to the weight of the peel which is only a part of the entire fruit.

4.4 Conclusions

A reliable method using SPME and HPLC simple equipment has been developed for the determination of triazole fungicides in liquid and fruit samples. The best experimental conditions for SPME extraction were obtained using an experimental design with two steps. Good results were obtained regarding limits of detections, inter and intra-day precisions, and recoveries. The proposed method could be easily incorporated to more sensible and confirmatory techniques such as LC-MS.

Although the obtained results for figures of merit were considered satisfactory, the chromatographic resolution was the main drawback. Even though the six analytes were determinate, no complete resolution was obtained separately. This led to consider multivariate analyses in further studies.

Due to the use of triazole fungicides in grape and apple trees, the peel, pulp and the juices of the samples were analyzed. It was concluded, that the treatment was superficial because there were found no detectable levels in pulp and juice samples within the pre-harvest period established by the commercial product. However, some residues of analytes were found in the peel; hence, the fruit cleaning before the consumption is advisable. Finally, there were also found some analites concentration in rain samples. Being a superficial treatment, the rain would clean the fruits sweeping the analytes.

4.5 References

Allen, J.W., Wolf, D.C., George, M.H., Hester, S.D., Sun, G., Thai, S., Delker, D.A., Moore, T., Jones, C., Nelson, G., Roop, B.C., Leavitt, S., Winkfield, E., Ward, W.O. and Nesnow, S.; *Toxicity Profiles in Mice Treated with Hepatotumorigenic and Non-Hepatotumorigenic Triazole Conazole Fungicides: Propiconazole, Triadimefon, and Myclobutanil.* **2006.** Toxicologic Pathology, 34(7), 853-862.

AOAC INTERNATIONAL, **2012.** AOAC Official Methods of Analysis, Guidelines for standard method performance requirements. Appendix F, .

Araoud, M., Douki, W., Rhim, A., Najjar, M.F. and Gazzah, N.; *Multiresidue analysis of pesticides in fruits and vegetables by gas chromatography-mass spectrometry.* **2007.** Journal of Environmental Science and Health, Part B, 42(2), 179-187.

Aulakh, J.S., Malik, A.K., Kaur, V. and Schmitt-Kopplin, P.; *A Review on Solid Phase Micro Extraction—High Performance Liquid Chromatography (SPME-HPLC) Analysis of Pesticides.* **2005.** Critical Reviews in Analytical Chemistry, 35(1), 71-85.

Bordagaray, A., García-Arrona, R. and Millán, E.; *Optimization of Solid-Phase Microextraction Procedure Coupled to GC-ECD for Triazole Fungicides Determination in Juice Samples.* **2011.** Food Analytical Methods, 4(3), 293-299.

Burgess, C. and Frost T.; *Standards and best practice in absorption spectrometry.* **1999.** 1 edn. Blackwell Science Ltd. Oxford (UK).

Celeiro, M., Llompart, M., Lamas, J.P., Lores, M., Garcia-Jares, C. and Dagnac, T.; *Determination of fungicides in white grape bagasse by pressurized liquid extraction and gas chromatography tandem mass spectrometry.* **2014.** Journal of Chromatography A, 1343, 18-25.

Dean, A. and Voss, D.; *Design and analysis of experiments.* **1999.** Springer. New York (USA).

Ekman, D.R., Keun, H.C., Eads, C.D., Furnish, C.M., Murrell, R.N., Rockett, J.C. and Dix, D.J.; *Metabolomic evaluation of rat liver and testis to characterize the toxicity of triazole fungicides.* **2006.** Metabolomics, 2(2), 63-73.

Farajzadeh, M.A., Khoshmaram, L. and Mogaddam, M.R.A.; *Combination of solid-phase extraction-hollow fiber for ultra-preconcentration of some triazole pesticides followed by gas chromatography-flame ionization detection.* **2012.** Journal of Separation Science, 35(1), 121-127.

Ferreira, S.L.C., Bruns, R.E., da Silva, E.G.P., dos Santos, W.N.L., Quintella, C.M., David, J.M., de Andrade, J.B., Breitkreitz, M.C., Jardim, I.C.S.F. and Neto, B.B.; *Statistical*

designs and response surface techniques for the optimization of chromatographic systems. **2007.** Journal of Chromatography A, 1158(1-2), 2-14.

Foley, J.P. and Dorsey, J.G.; *Clarification of the limit of detection in chromatography.* **1984.** Chromatographia, 18(9), 503-511.

González-Barreiro, C., Lores, M., Casais, M.C. and Cela, R.; *Optimisation of alachlor solid-phase microextraction from water samples using experimental design.* **2000.** Journal of Chromatography A, 896(1–2), 373-379.

ICH HARMONISED TRIPARTITE GUIDELINE, **2005.** Validation of analytical procedures: Text and methodology Q2 (R1), .

Jiang, Y., Li, X., Xu, J., Pan, C., Zhang, J. and Niu, W.; *Multiresidue method for the determination of 77 pesticides in wine using QuEChERS sample preparation and gas chromatography with mass spectrometry.* **2009.** Food Additives & Contaminants: Part A, 26(6), 859-866.

Kleinbaum, D.G., Kupper, L.L. and Muller, K.E.; *Applied regression analysis and other multivariable methods.* **1988.** PWS-KENT. Boston (USA).

Kmellár, B., Abrankó, L., Fodor, P. and Lehotay, S.J.; *Routine approach to qualitatively screening 300 pesticides and quantification of those frequently detected in fruit and vegetables using liquid chromatography tandem mass spectrometry (LC-MS/MS).* **2010.** Food Additives & Contaminants: Part A, 27(10), 1415-1430.

Konieczka, P. and Namieśnik, J.; *Quality Assurance and Quality Control in the Analytical Chemical Laboratory.* **2009.** 1 edn. CRC Press, Taylor & Francis Group. Boca Raton (USA).

Parrilla-Vázquez, P., Mughari, A.R. and Martínez-Galera, M.; *Solid-phase microextraction (SPME) for the determination of pyrethroids in cucumber and watermelon using liquid chromatography combined with post-column photochemically induced fluorimetry derivatization and fluorescence detection.* **2008.** Analytica Chimica Acta, 607(1), 74-82.

Pawliszyn, J.; *Applications of solid phase microextraction.* **1999.** The Royal Society of Chemistry. Cambridge (UK).

Picó, Y., Fernández, M., Ruiz, M.J. and Font, G.; *Current trends in solid-phase-based extraction techniques for the determination of pesticides in food and environment.* **2007.** Journal of Biochemical and Biophysical Methods, 70(2), 117-131.

Rajaković, L.V., Marković, D.D., Rajaković-Ognjanović, V.N. and Antanasijević, D.Z.; *Review: The approaches for estimation of limit of detection for ICP-MS trace analysis of arsenic.* **2012.** Talanta, 102, 79-87.

Ravelo-Pérez, L.M., Hernández-Borges, J., Asensio-Ramos, M. and Rodríguez-Delgado, M.A.; *Ionic liquid based dispersive liquid–liquid microextraction for the extraction of pesticides from bananas.* **2009.** Journal of Chromatography A, 1216(43), 7336-7345.

Regulation EC 149/2008 of 29 January 2008 amending Regulation (EC) No 396/2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto. Official Journal of the European Union. **(1.3.2008)** L 58/1-398. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EC 459/2010 of 27 May 2010 amending Annexes II, III and IV to Regulation (EC) No 396/2005 of hte European Parliament and of the council as regards maximum residue levels for certain pesticides in or on certain products. Official Journal of the European Union. **(28.5.2010)** L 129/3-49. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 750/2010 of 7 July 2010 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for certain pesticides in or on certain products. Official Journal of the European Union. **(21.8.2010)** L 220/1-56. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 34/2013 of 16 January 2013 amending Annexes II, III and IV to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residuel levels for 2-phenylphenol, ametoctradin, *Aureobasidium pullulans* strains DSM 14940 and DSM 14941, cyproconazole, difenoconazole, dithiocarbamates, folpet, propamocarb, spinosad, spirodiclofen, tebufenpyrad and tetraconazole in or on certain products. Official Journal of the European Union. **(26.1.2013)** L 25/1-48. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 1317/2013 of 16 December 2013 amending Annexes II, III and V to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for 2,4-D, beflubutamid, cyclanilide, diniconazole, florasulam, metolachlor and S-metolachlor, and milbemectin in or on certain products. Official Journal of the European Union. **(17.12.2013)** L 339/1-43. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 61/2014 of 24 January 2014 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for cyromazine, fenopropidin, formetanate, oxamyl and tebuconazole in or on certain products. Official Journal of the European Union. **(24.1.2014)** L 22/1-32. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Sagratini, G., Mañes, J., Giardiná, D., Damiani, P. and Picó, Y.; *Analysis of carbamate and phenylurea pesticide residues in fruit juices by solid-phase microextraction*

and liquid chromatography–mass spectrometry. **2007.** Journal of Chromatography A, 1147(2), 135-143.

Sánchez-Ortega, A., Sampedro, M.C., Unceta, N., Goicolea, M.A. and Barrio, R.J.; *Solid-phase microextraction coupled with high performance liquid chromatography using on-line diode-array and electrochemical detection for the determination of fenitrothion and its main metabolites in environmental water samples.* **2005.** Journal of Chromatography A, 1094(1-2), 70-76.

Souza-Silva, E.A., Lopez-Avila, V. and Pawliszyn, J.; *Fast and robust direct immersion solid phase microextraction coupled with gas chromatography–time-of-flight mass spectrometry method employing a matrix compatible fiber for determination of triazole fungicides in fruits.* **2013.** Journal of Chromatography A, 1313, 139-146.

Tankiewicz, M., Fenik, J. and Biziuk, M.; *Solventless and solvent-minimized sample preparation techniques for determining currently used pesticides in water samples: A review.* **2011.** Talanta, 86, 8-22.

Taverniers, I., De Loose, M. and Van Bockstaele, E.; *Trends in quality in the analytical laboratory. I. Traceability and measurement uncertainty of analytical results.* **2004.** Trends in Analytical Chemistry, 23(7), 480-490.

Taxvig, C., Hass, U., Axelstad, M., Dalgaard, M., Boberg, J., Andeasen, H.R. and Vinggaard, A.M.; *Endocrine-Disrupting Activities In Vivo of the Fungicides Tebuconazole and Epoxiconazole.* **2007.** Toxicological Sciences, 100(2), 464-473.

Viñas, P., Campillo, N., Hernández-Pérez, M. and Hernández-Córdoba, M.; *A comparison of solid-phase microextraction and stir bar sorptive extraction coupled to liquid chromatography for the rapid analysis of resveratrol isomers in wines, musts and fruit juices.* **2008.** Analytica Chimica Acta, 611(1), 119-125.

Walorczyk, S., *Application of gas chromatography/tandem quadrupole mass spectrometry to the multi-residue analysis of pesticides in green leafy vegetables.* **2008.** Rapid Communications in Mass Spectrometry, 22(23), 3791-3801.

Zambonin, C.G., Cilenti, A. and Palmisano, F.; *Solid-phase microextraction and gas chromatography–mass spectrometry for the rapid screening of triazole residues in wine and strawberries.* **2002.** Journal of Chromatography A, 967(2), 255-260.

Zou, T., Wang, D., Guo, H., Zhu, Y., Luo, X., Liu, F. and Ling, W.; *Optimization of Microwave-Assisted Extraction of Anthocyanins from Mulberry and Identification of Anthocyanins in Extract Using HPLC-ESI-MS.* **2012.** Journal of Food Science, 77(1), C46-C50.

5. Development of a method for triazole determination in liquid samples with Ultrasound Assisted Emulsification Microextraction with Solidification of Floating Organic Droplet coupled to HPLC/DAD

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Determination of Triazole Fungicides in Liquid Samples Using Ultrasound-Assisted Emulsification Microextraction with Solidification of Floating Organic Droplet Followed by High-Performance Liquid Chromatography

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Abstract An ultrasound-assisted emulsification microextraction with solidification of organic droplet method followed by high-performance liquid chromatography with diode array detection for six triazole fungicide determination (diniconazole, fluquinconazole, flusilazole, myclobutanil, tebuconazole, and tetraconazole) was developed. After some preliminary experiments, undecanol was chosen as extracting solvent using 50 μL for 10 mL of liquid sample. A central composite design was performed to obtain the best experimental conditions for the following variables NaCl concentration (250 g L^{-1}), extraction time (18 min), and temperature (30 °C) in ultrasonic bath. After the ultrasound-assisted extraction, two steps considering centrifugation (4,200 rpm, 10 min) and solidification (5 min, 3 °C) were done. Following these conditions, the method showed linearity higher than 0.9930 with the concentration ranged from 20 to 890 μg L^{-1}. The limits of detection obtained using calibration curves were from 10.9 to 17.2 μg L^{-1} and the intra- and inter-day repeatability at two levels showed RSD values between 1.9 and 10.6 %. The enrichment factors for the studied triazoles were between 226 (flusilazole) and 255 (tebuconazole). Recovery studies at two spiked levels in apple and grape juices gave values from 64 to 112 %.

Keywords Triazole fungicides · Ultrasound-assisted emulsification microextraction · Solidification of floating organic droplet · Fruit juice · Central composite design · High-performance liquid chromatography

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Introduction

Triazole fungicides are systemic pesticides widely used to control fungus diseases in field crops. These triazole compounds inhibit ergosterol biosynthesis needed for membrane structure and function (Tomlin 2000). The widespread use of triazoles has increased the concern about the detrimental effects in ecosystems and human health. Some toxicological studies in rats have shown tumorigenic (Wolf et al. 2006) and endocrine disrupting (Goetz et al. 2007) effects. Since the fungicide residues have been found on food for human consumption, the accurate determination of the residue levels is necessary for food safety monitoring and regulatory purposes. In order to protect the health of consumers, maximum residue levels (MRLs) in raw products have been established. European Union has fixed MRLs ranging from 0.01 to 2 mg kg^{-1} in apples and grapes for diniconazole, fluquinconazole, flusilazole, myclobutanil, tebuconazole, and tetraconazole (EC Commission Regulation 149/2008 of 29 January 2008).

The sample preparation is a critical step in the analytical process for determination of pesticides residues. In the previous years, considerable efforts have been made in trying to develop new sample preparation techniques that save time and require less amount of hazardous organic solvent, which allow improving the quality and sensitivity of the analytical procedures (Tankiewicz et al. 2011). Sorptive extraction techniques mainly include solid-

phase extraction (SPE), solid-phase microextraction (SPME), and stir bar sorptive extraction (SBSE). SPE is a widely used sample preparation technique; and modification and development of new adsorbent are a major goal to improve the extraction efficiency. The main drawbacks of SPE are the cost of cartridges and the relatively high consumption of sample and solvent that can be reduced using miniaturized SPE. SPME and SBSE are both solvent-free extraction techniques and show some other advantages such as the high sensitivity and wide range of compound application. The main drawbacks are due to the long time required for a single extraction, the difficulties in variables optimization and reliable calibration, and the cost of the fibers or the magnetic stirrers (Hyötyläinen 2009).

Liquid-phase microextraction (LPME) avoids at least the great problem of the solvent consumption associated with conventional liquid-liquid extraction. LPME can be done in several modes, where the extraction is performed by using small amount (drop) of water immiscible solvent suspended in a sample or the extraction is done via a membrane which can be a selective barrier between two phases (Stocka et al. 2011). The dispersive liquid-liquid microextraction (DLLME), with dispersion of very fine droplets of organic solvents into the aqueous phase, has emerged as a valuable alternative for the traditional liquid-liquid extraction. DLLME employs a ternary component solvent system composed of an aqueous solution containing the analytes, a water immiscible solvent, and a water–miscible disperser solvent. The extraction solvent is generally collected at the bottom of the tube after centrifugation. DLLME is fast, cheap, simple, requires minute amounts of organic solvent, and provides high enrichment factors (Bosch Ojeda and Sánchez Rojas 2011). However, the extraction solvents used in DLLME were generally highly toxic. A valuable LPME method based on solidification of a floating organic solvent droplet (SFO) was presented by Khalili Zanjani et al. (2007). In this method, a small volume of an organic solvent with a melting point near room temperature is floated on the surface of the agitated aqueous sample. Later, the sample vial is placed in an ice bath and the solidified organic droplet is collected and immediately melted for analyte determination. The technique is cheap, quick, and sensitive; but the rate of extraction is slightly slow. A posterior modification considering DLLME methodology (DLLME-SFO) achieved faster mass transfer and better extraction times (Leong and Huang 2008). As previously mentioned, one of the main disadvantages of DLLME is the use of relatively high volumes of disperser organic solvents (Bosch Ojeda and Sánchez Rojas 2011). With the goal of avoiding the environmentally unfriendly disperser solvents, the ultrasound-assisted emulsification microextraction (USAEME) has emerged to assist the dispersion of extraction solvent in the aqueous solution (Regueiro et al. 2008).

Some of the abovementioned extraction techniques have been used for the determination of triazoles residues in different types of water and liquid fruit juices. After the treatment step, the procedures mostly use gas chromatography or liquid chromatography (LC) with diode array detectors (DAD) or mass spectrometry (MS). A meliorated SPE procedure was used for simultaneous multiclass pollutants, including triazoles, in water (Baugros et al. 2008). A graphene-based magnetic nanoparticles was showed as adsorbent for triazole fungicides in environmental water (Wang et al. 2012). Other sorptive techniques were also used: SPME in liquid samples (Bordagaray et al. 2013) and SBSE combined with DLLME in aqueous samples (Farajzadeh et al. 2010). Working with DLLME, other alternatives have been showed; addition of ionic liquids (Ravelo-Pérez et al. 2009), use of a narrow-bore tube (Farajzadeh et al. 2012), and dispersion with methanol followed by SFO (Wang et al. 2011a). Until now, there is no application of ultrasound-assisted emulsification microextraction with solidification of organic droplet (USAEME-SFO) without disperser solvent for the triazole fungicides determination in water and fruit juice samples.

There are several variables affecting the extraction procedure, among them are the type and volume of extraction solvent, salt addition, stirring rate, sample solution temperature, and extraction time (Ghambarian et al. 2013). Experimental designs that take into account simultaneously several variables and its interaction effects seem to be an appropriate way to find the convenient experimental conditions with a reduced number of experiments. Response surface designs, including central composite design (CCD), are used during method optimization to determine optimal conditions for the factors that have the most influence on the interest response (Tranter 2000; Stalikas et al. 2009). This approach was presented in developing an IL-DLLME-HPLC-DAD for pesticide determination (Ravelo-Pérez et al. 2009), and used with DLLME for simultaneous determination of carbamates and organophosphorus pesticides in water (Sousa et al. 2013).

The aim of the work was to develop an appropriate green method for the determination of triazoles fungicides in water and fruit juices samples using USAEME-SFO followed by high-performance liquid chromatography with diode array detection (HPLC-DAD). The method only used a minute amount of alcohol organic solvent and avoided the use of the hazardous disperser solvent. After selection of some important extraction variables (among them type and volume of extraction solvent and sample liquid volume), the optimization of time and temperature extraction conditions and sodium chloride amount was performed using a CCD approach. The

method was validated and used in the analysis of different spiked apple and grape juice samples.

Experimental

Chemicals and Samples

Diniconazole (99.8 %, Pestanal) and tebuconazole (99.6 %, Pestanal) were acquired in Sigma-Aldrich (Madrid, Spain), flusilazole (99.3 %) and myclobutanil (99.4 %) were supplied by LGC Standards (Barcelona, Spain), and tetraconazole (97.5 %) and fluquinconazole (98.5 %) from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Individual stock solutions of triazoles were prepared in methanol (SpS) supplied by Teknokroma (Barcelona, Spain) at a concentration of 6, 000 mg L^{-1}. Working standard solutions were prepared in a range from 20 to 890 μg L^{-1} by appropriate dilutions in salted water. HPLC-grade methanol was from Teknokroma and sodium acetate (PA grade), and acetic acid used for buffer were supplied by Panreac (Barcelona, Spain). The buffer made adjusting with 0.01 mol L^{-1} acetic acid/sodium acetate solution to pH 4 was stored at 4 °C. 1-undecanol (purity 99 %), hexadecane (purity 99 %), and 1-bromohexadecane (purity 97 %) were acquired from Sigma-Aldrich (Madrid, Spain), while 1-dodecanol (purity 98 %) and sodium chloride (PA) were acquired from Panreac (Barcelona, Spain).

Homemade and commercial apple and grape juices were used. Commercial products were acquired in a store, while the homemade juice was made by squeezing the apples and filtering the liquid.

Equipment

HPLC analyses were performed on a LC-20AD system equipped with a SPD-M20A DAD (Shimadzu Corporation, Duisburg, Germany). Data were collected and processed using LC solution 2.1 version software. Separations were carried out using XDB-C18 column (250 mm×4.6 mm, 5 μm) from Agilent (Wilmington, DW, USA). The analysis was done at ambient temperature, and the injection volume was 20 μL. A binary mobile-phase gradient with methanol and sodium acetate/acetic acid buffer solution was used at a flow rate of 0.5 mL min^{-1}. The initial mobile phase was held for 1 min with 78 % methanol, followed by a decrease to 71.5 % methanol from 1 to 13 min, then raised to 85 % methanol from 13 to 14 min, and kept for 10 min. The system was re-equilibrated at the initial conditions (78 % methanol) from 24 to 30 min. In order to homogenize faster the circuit, the flow rate was increased to 1.5 mL min^{-1} in the last 2.5 min. With these conditions, the triazole sequence and elution time was myclobutanil (9.9 min), fluquinconazole (10.6 min), tetraconazole (TT) (11.2 min), flusilazole (FS) (12.2 min), tebuconazole (15.4 min), and diniconazole (D) (23.0 min). The UV–Vis spectra were recorded from 190 to 500 nm, using 249 nm as a working wavelength for diniconazole and 221 nm for the other 5 analytes.

The extraction was carried out in a Bandelin Sonorex Digitec DT100H ultrasound bath (ALLPAX GmbH & Co. KG, Papenburg, Germany) with 35 kHz ultrasound frequency. The cooling bath used was a Julabo F26 from GmbH (Augsburg, Germany). Experimental design was performed, and results were evaluated using Statistica software (StatSoft, Tulsa, USA).

USAEME-SFO Procedure

Ten milliliter of the 250 g L^{-1} NaCl solution was placed in a 40 mL screw cap glass vial and a mixed solution of triazole standards was spiked. Then, 50 μL of 1-undecanol as a extraction solvent was added to the solution, shacked by hand, placed into ultrasound bath previously heated to 30±1 °C, and maintained at 18 min. After, the vial was first placed into a centrifuge for 10 min at 4,200 rpm and later into the cooling thermostatic bath at 3 °C for 5 min. The formed solidified organic drop was carefully collected with a spatula and transferred to an Eppendorf vial where it melted rapidly at room temperature. Twenty-five microliter of the melted solution was collected and mixed with 20 μL of methanol before the injection into the HPLC.

For the fruit juices, 0.5 mL of the filtrate sample was added to 9.5 mL salted water before spiking the solution of analytes and the extracting solvent. All the rest of the procedure followed the above indicated conditions. Several previous tries with 10 mL of salted sample and lower dilutions (i.e., 5 mL sample/5 mL water, 2.5 mL sample/7.5 mL water) were done, but the drop did not properly form and was hardly managed.

Enrichment Factor

Enrichment factor (EF) was calculated as the relation between the concentration of each analyte in the initial aqueous sample (C_0) and the final concentration in the extracting phase (C_{final}).

$$EF = \frac{C_{final}}{C_0}$$

The initial concentration (C_0) is the concentration spiked into the aqueous solution before the extraction. The extracting phase concentration was calculated after the extraction process (C_{final}), taking into account an external calibration made by injecting directly standards into the HPLC-DAD.

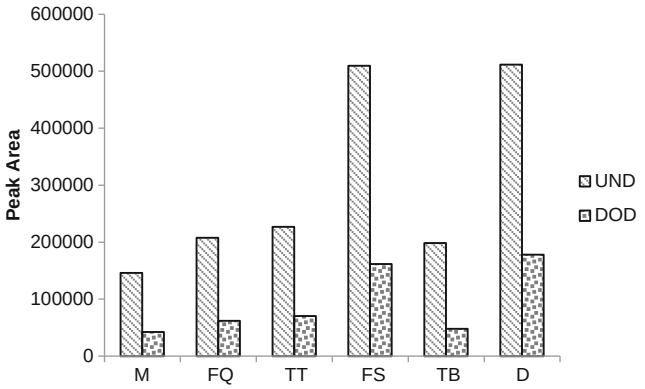

Fig. 1 Effect of 1-undecanol (*UND*) and 1-dodecanol (*DOD*) extracting solvent on the extraction efficiency following LPME-SFO method. Extraction conditions: triazole concentration, 80 μg L^{-1}; sample volume, 10 mL; volume of extracting solvent, 15 μL; extraction time, 30 min; extraction temperature, 60 °C; no salt addition; agitation, 500 rpm. Solidification conditions: time, 10 min; cooling temperature, 3 °C. Triazole abbreviations: *D* diniconazole, *FQ* fluquinconazole, *FS* flusilazole, *M* myclobutanil, *TB* tebuconazole, *TT* tetraconazole

Results and Discussion

Selection of Extracting Solvent

For extraction process, it is important to choose an extracting solvent that fits well with the analytes and experimental working conditions. The solvent should be immiscible in water and present a melting point (MP) near to the room temperature (Ganjali et al. 2010). Taking into account these characteristics, 1-bromohexadecane (BRHEX, MP 17 °C), n-hexadecane (HEX, MP 18 °C), 1-dodecanol (DOD, MP 22 °C), and 1-undecanol (UND, MP 14 °C) were chosen as extracting solvents.

Firstly, the compatibility of the extracting solvent with the HPLC mobile phase was checked. After several assays, with different proportions of methanol with water, UND and DOD showed better solubility than BRHEX and HEX. Hence, only the alcohols were considered in the following experiments. UND showed better miscibility than DOD with less proportion of organic solvent, and it was considered miscible from the proportion above 60 %. In the comparison of extraction efficiency for triazole analytes, UND showed better results than DOD as it can be seen in Fig. 1. Therefore, further experiments were carried out considering UND as the extracting solvent.

Comparison with LPME-SFO

USAEME was compared with LPME both followed by SFO. In all the runs, the following extractions conditions were considered: concentration of triazoles, 80 μg L^{-1}; sample volume, 10 mL; volume of extraction solvent, 20 μL; extraction temperature, 25 °C; and salt addition, NaCl 180 g L^{-1}. The solidification conditions (3 °C for cooling temperature during 5 min) were also similar. For LPME, 500 rpm agitation during extraction was used. And, for USAEME, extraction followed by centrifugation (4,200 rpm, 10 min) was done. Different extraction times, up to 60 min for LPME and up to 15 min for USAEME, were considered to compare the extraction efficiency. As it can be seen in Fig. 2, the extraction efficiency for the

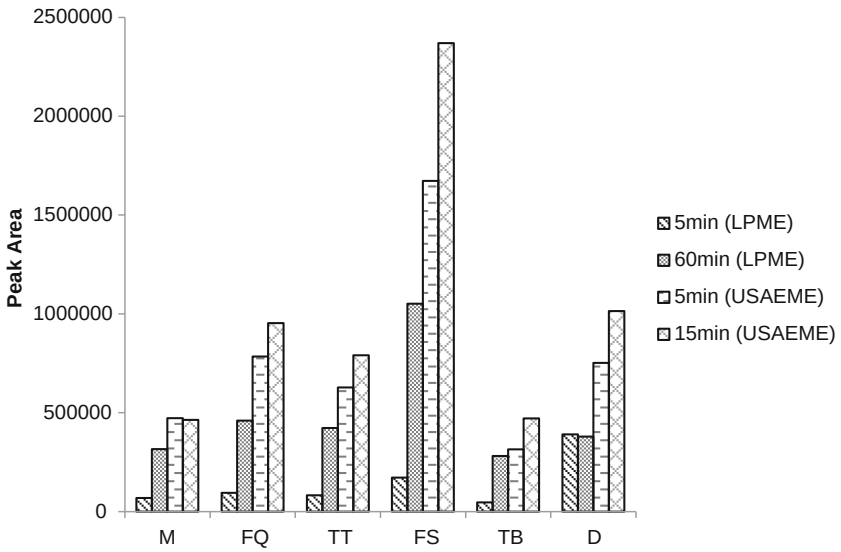

Fig. 2 Effect of incorporation of ultrasound assistance on extraction efficiency in LPME-SFO method. Extraction conditions: concentration of triazoles, 80 μg L^{-1}; sample volume, 10 mL; volume of extraction solvent, 20 μL, extraction temperature, 25 °C; salt addition, NaCl 180 g L^{-1}. Solidification conditions: time, 5 min, and cooling temperature, 3 °C. For LPME, agitation during extraction 500 rpm. For USAEME, extraction followed by centrifugation (4,200 rpm, 10 min). Triazole abbreviations: *D* diniconazole, *FQ* fluquinconazole, *FS* flusilazole, *M* myclobutanil, *TB* tebuconazole, *TT* tetraconazole

Table 1 Experimental variables, levels, matrix of central composite design (CCD), and results in peak areas for the triazole fungicides determination using USAEME-SFO-HPLC-DAD method

Variable			Code	Level					
				Low	Central	High			
Extraction temperature (°C)			T_{ext}	25	30	35			
Extraction time (min)			t_{ext}	5	10	15			
NaCl concentration (g L^{-1})			NaCl	130	180	230			
Run	T_{ext}	t_{ext}	NaCl	M	FQ	TT	FS	TB	D
1	25	5	130	785,479	805,805	2,115,226	1,606,017	555,994	1,039,442
2	25	5	230	758,541	764,908	2,065,959	1,601,087	535,944	1,020,278
3	25	15	130	766,836	736,297	2,003,889	1,582,637	579,061	1,113,181
4	25	15	230	763,899	750,416	2,195,582	1,634,068	598,419	1,484,520
5	35	5	130	719,030	758,690	2,016,598	1,557,274	531,821	1,022,966
6	35	5	230	700,240	690,240	1,984,585	1,476,899	542,672	994,878
7	35	15	130	697,082	717,831	2,054,310	1,596,952	583,388	1,143,580
8	35	15	230	758,817	741,773	2,156,140	1,613,883	593,318	1,145,262
9 C	30	10	180	785,767	792,603	2,155,597	1,634,932	555,707	1,311,018
10	21	10	180	887,740	837,642	2,350,107	1,827,153	656,042	1,243,770
11	38	10	180	757,699	768,782	2,133,107	1,794,588	554,879	1,092,962
12	30	2	180	705,895	693,373	1,977,589	1,472,447	552,769	1,042,229
13	30	18	180	735,103	728,034	2,097,653	1,549,233	571,112	1,494,757
14	30	10	96	768,034	773,762	2,085,880	1,638,853	547,073	965,614
15	30	10	264	767,111	725,774	2,131,325	1,542,750	568,866	1,360,141
16 C	30	10	180	737,504	760,393	2,061,979	1,589,878	532,765	1,004,423
17	25	5	130	819,439	828,122	2,211,654	1,805,352	582,469	1,461,784
18	25	5	230	797,267	730,267	2,119,212	1,530,695	568,035	1,343,438
19	25	15	130	910,073	751,868	2,079,796	1,580,599	590,643	1,153,723
20	25	15	230	749,174	753,634	2,120,660	1,628,680	601,069	1,498,336
21	35	5	130	690,373	707,533	1,992,041	1,587,163	577,538	1,412,607
22	35	5	230	766,548	734,046	2,082,176	1,525,272	588,531	1,424,109
23	35	15	130	720,236	719,484	2,008,127	1,609,721	572,064	1,513,036
24	35	15	230	800,072	786,484	2,280,209	1,788,034	606,155	1,431,937
25 C	30	10	180	782,065	738,729	2,114,198	1,583,286	610,491	1,389,217
26	21	10	180	896,170	841,231	2,176,186	1,679,985	568,574	1,155,942
27	38	10	180	778,136	759,934	2,075,546	1,526,284	589,957	1,530,291
28	30	2	180	771,531	711,050	2,015,644	1,536,705	585,771	1,746,323
29	30	18	180	1,009,943	847,876	2,264,475	1,984,968	584,182	1,616,679
30	30	10	96	781,349	722,576	2,037,206	1,584,125	589,798	1,430,800
31	30	10	264	915,935	763,095	2,129,599	1,515,268	588,882	1,441,173
32 C	30	10	180	796,205	711,458	2,059,134	1,543,403	584,884	1,134,552
33 C	30	10	180	761,538	687,025	1,930,512	1,499,539	536,061	1,051,115

C central point, *D* diniconazole, *FQ* fluquinconazole, *FS* flusilazole, *M* myclobutanil, *TB* tebuconazole, *TT* tetraconazole

six triazole analytes is better using ultrasound assistance. Even in the comparison of 1-h extraction time with LPME with 5 min in USAEME, the results are higher using the ultrasound-assisted microextraction. The application of ultrasonic energy facilitates the emulsification phenomenon and accelerates the mass-transfer process that leads to an increment in the extraction efficiency in a very short time (Ghambarian et al. 2013).

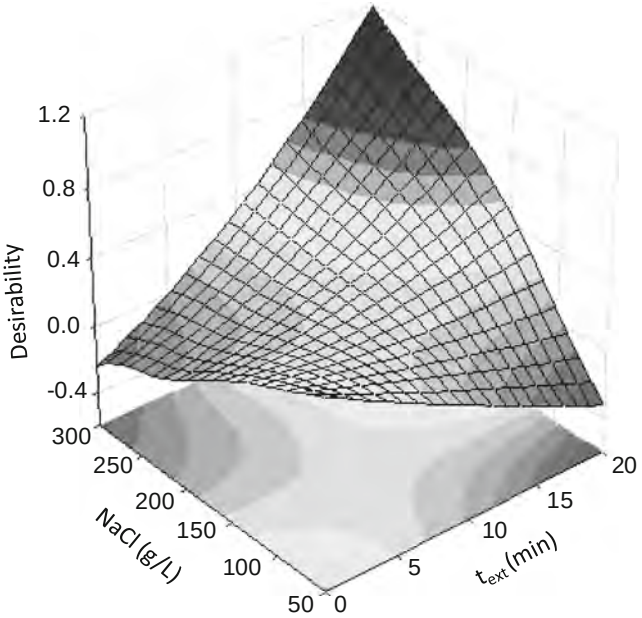

Fig. 3 Response surface for global desirability estimated from central composite design considering NaCl concentration and extraction time variables

Central Composite Design: Application in Selection of Experimental Conditions for Influent Extraction Factors

The experimental conditions for some of the variables that affect the extraction process were selected according to working characteristics. For example, drop volume was big enough to collect sufficient volume before injecting to HPLC, but as small as possible to avoid the dilution of analytes in the drop. Centrifugation speed and time values were chosen as minimum to recollect the cloudy solution into a drop, and changes with longer time or different speed did not affect in extraction efficiency. The cooling step conditions were fixed in order to freeze the drop in a good way to collect with the spatula. Thus, the drop was cooled at 3 °C for 5 min with agitation, and the changes of conditions in cooling step did not show differences.

Other variables are not so easy to fix in a reduced amount of experiments. Response surface methodologies, including CCD, are used to optimize simultaneously some variables following established series of experiments that considerably reduce the number of the experiments comparing with the approach if each variable is taken in an univariate way. Taking into account the variables reported in previous works (Leong and Huang 2008; Wang et al. 2011b; Ghambarian et al. 2013), in this study, three variables were selected for CCD design. Those were NaCl concentration, extraction time, and temperature in ultrasonic bath. The variables and its low, medium, and high levels are shown in Table 1.

The CCD done was based in a factorial design (2^3) increased by a [(2×3)+1] star design considering the rotatability conditions ($\alpha=\pm1.682$). Each experimental point was performed twice, except the central point that was run out five times. In total, the matrix of CCD design consisted of 33 experimental runs that are presented in Table 1. Due the large amount of experiments, the design was randomly carried out in 2 days according to blocks. The obtained values (in units of peak areas) for each studied triazole are also shown in Table 1.

The most common way to present the results of a CCD is with a response surface. This can be done in 3D plots representing results and selecting two independent variables (i.e., temperature and time). Instead of the independent response surfaces for each studied triazoles, the global desirability surface was chosen, since it can provide an overall view of the considered analytes. The desirability function for each dependent variable was fixed by assigning desirability values of 0.0 (for undesirable, lowest area), 0.5 (for medium), and 1.0 (for very desirable, highest area). Therefore, the conditions are better when the desirability surface shows values closer to 1. Otherwise, the conditions are less favorable when the graph shows the desirability values close to 0. The response surface for global desirability considering NaCl concentration and extraction time is showed in Fig. 3. The extraction temperature variable for this response surface was fixed at 30 °C. As it can be seen, in the studied experimental domain, the best responses were obtained working with high NaCl

Table 2 Analytical characteristics of the USAEME-SFO-HPLC-DAD method for the studied triazole fungicides

		M	FQ	TT	FS	TB	D
Linear range (μg L^{-1})		20.7–830	19.0–762	20.5–819	20.9–835	22.2–887	20.6–824
R^2		0.9971	0.9960	0.9971	0.9931	0.9947	0.9948
LOD (μg L^{-1})		11.2	11.9	10.9	17.2	16.0	14.8
Intraday repeatability (RSD %) ($n=6$)	50.0 μg L^{-1}	6.0	4.1	4.4	3.1	5.1	5.4
	400 μg L^{-1}	3.1	4.6	3.0	1.9	2.6	3.7
Interday repeatability (RSD %) ($n=15$)	50.0 μg L^{-1}	9.4	8.6	5.7	10.6	6.7	10.2
	400 μg L^{-1}	6.1	5.2	4.3	4.3	3.1	6.1
Enrichment factor (EF) ($n=6$)	80.0 μg L^{-1}	246±5	253±5	240±2	226±4	255±1	249±1

D diniconazole, *FQ* fluquinconazole, *FS* flusilazole, *M* myclobutanil, *TB* tebuconazole, *TT* tetraconazole

Table 3 Average recoveries of the studied triazole compounds from juice samples using USAEME-SFO-HPLC-DAD method

	Recovery (%) ± standard deviation (n=4)						
	Spiked	M	FQ	TT	FS	TB	D
Commercial apple juice	80.0 μg L^{-1}	111±10	110±8	95±3	112±6	103±2	101±3
	400 μg L^{-1}	109±4	109±2	98±2	109±2	108±2	101±4
Homemade apple juice	80.0 μg L^{-1}	84±4	84±7	82±6	82±8	84±6	83±5
	400 μg L^{-1}	91±9	93±10	91±10	94±9	91±10	88±6
Grape juice	80.0 μg L^{-1}	92±11	81±12	74±11	79±11	78±11	64±14
	400 μg L^{-1}	112±8	94±12	88±10	88±12	88±10	68±15

D diniconazole, *FQ* fluquinconazole, *FS* flusilazole, *M* myclobutanil, *TB* tebuconazole, *TT* tetraconazole

concentrations and extraction times. The extraction temperature showed a similar behavior with the best responses at high levels. Hence, 250 g L^{-1} NaCl, 18 min extraction time, and 30 °C extraction temperatures were selected as the experimental conditions.

Method Performance

Important analytical parameters such as linearity, limit of detection (LOD), precision, EF, and recovery were determined to evaluate the performance of the USAEME-SFO method following the previously detailed experimental conditions. The obtained data for the analytical characteristics are summarized in Table 2. All analytes showed a good linearity between 20 and 890 μg L^{-1} with R^2 correlation coefficients ranging from 0.9931 (flusilazole) to 0.9971 (myclobutanil, tetraconazole).

There are several methods to calculate LODs (Konieczka and Namiesnik 2009). In chromatographic analysis, the simplest and widely applied way is based considering the lowest concentration of an analyte that yields a signal to noise ratio of 3. This method is applicable when analyte concentration is measurable for blank sample or for a sample with a very low analyte concentration. The method used in this work follows the next equation and take into consideration the calibration equation and its properties. LOD=3 $s_{y/x}/b$; where b is the slope of calibration curve and $s_{y/x}$ is the residual standard deviation of the calibration curve. The LODs obtained were between 10.9 μg L^{-1} (TT) and 17.2 μg L^{-1} (FS).

The precision of the method was evaluated considering the repeatability of the measurements and expressed as relative standard deviation (RSD) in percentages. The repeatability was run out at two different analyte concentrations (50 and 400 μg L^{-1}). For intraday repeatability, six experiments were carried out in the same day under same conditions. For interday repeatability, 15 runs were performed in 3 days in two different weeks. The RSD obtained ranged from 1.9 to 6.0 % for intraday repeatability and was from 3.1 to 10.6 % for interday repeatability.

Taking into account the selected experimental conditions, the EF of USAEME-SFO for the studied triazole fungicides were from 226 (flusilazole) to 255 (tebuconazole). The EFs were calculated as an average of six independent samples with 80 μg L^{-1} of each analyte, and the data are shown in Table 2.

The recovery studies were carried out in commercial and homemade apple juice and commercial grape juice. The juice

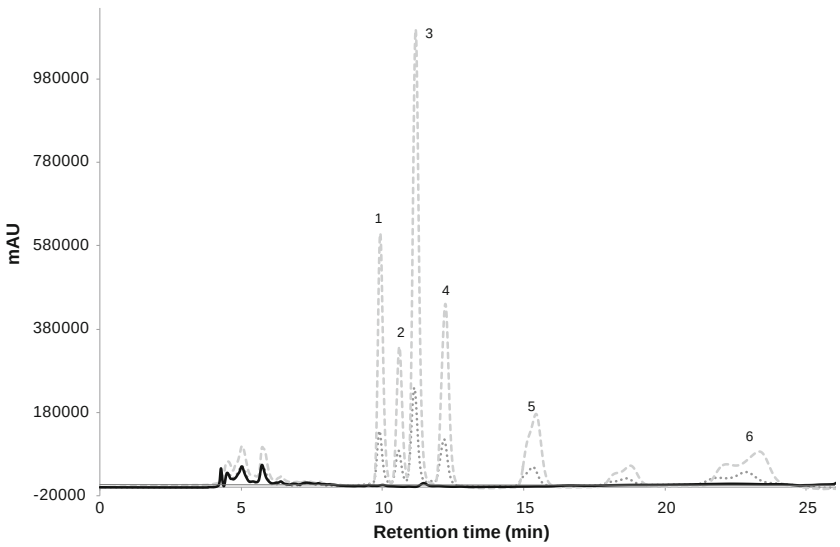

Fig. 4 HPLC-DAD chromatograms (λ 221 nm) obtained from a commercial apple juice spiked with 80 μg L^{-1} (*dotted line*) and 400 μg L^{-1} (*discontinuous line*) of the six triazole compounds following the USAEME-SFO optimized procedure. *Continuous line* belongs to the blank of apple juice analysis. Peak assignment (*1*) myclobutanil, (*2*) fluquinconazole, (*3*) tetraconazole, (*4*) flusilazole, (*5*) tebuconazole, and (*6*) diniconazole

Table 4 Comparison of the USAEME-SFO method with other sample extraction techniques for the triazole fungicides determination in liquid samples

Method	Matrix	Analyte	Linear range (μg L^{-1})	LOD[a] (μg L^{-1})	RSD[b](%)	EF[c]	Recovery (%)	Reference
SPE-HPLC-UV	Water various	M-TB	0.2–80	0.004–0.007*	3.0–5.9	–	83.8–105.0	Zhou et al. 2007
SPE-HPLC-MS-MS	Water various	FQ-TB-TT		0.0005–0.004*	5.1–12.0	–	95.0–118.0	Baugros et al. 2008
IL-DLLME-HPLC-DAD	Water	TB	122–6,830	3.9*	2.5	–	93	Ravelo-Pérez et al. 2009
IL-DLLME-HPLC-DAD	Wine	D	50–2,000	9.7*	5.2–9.2	–	74.6–83.5	Wang et al. 2011b
SBSE-DLLME-GC-FID	Water various, juices	D-TB	10–50,000	1.3–1.6*	–	690–710	72–108	Farajzadeh et al. 2010
SVE-DLLME-GC-FID	Water various, juices	TB	0.5–2,000	0.14*	3.4	482	86–103	Farajzadeh et al. 2011
SPME-GC-ECD	Grape-apple juices	D-M-TT-TB	0.4–106	0.2–7.5**	3.3–18.0	–	93.6–112.1	Bordagaray et al. 2011
SPME-HPLC-DAD	Water, grape-apple juices	D-M-FQ-FS-TB-TT	5.5–50.4	1.5–5.9**	2.8–13.1	–	94.5–123.4	Bordagaray et al. 2013
CPE-HPLC-UV	Water	D-TB	0.05–20	0.02–0.03*	4.2–5.3	> 60	93–95	Tang et al. 2010
DLLME-SFO-HPLC-DAD	Water	M-TB	0.5–200	0.08–0.1*	4.3–5.7	190–289	86–110	Wang et al. 2011a
HF-LPME-GC-MS	Water various, grape juice	D	1–5,000	0.4*	9.0	173.9	83.1–118.9	Sarafraz-Yazdi et al. 2012
MSPE-HPLC-UV	Water various	M-TB	0.05–50	0.005–0.01*	4.2–6.6	3,600–5,824	86–100.8	Wang et al. 2012
DLLME-GC- FID(MS)	Water, grape juice	D-TB	2–5,000	0.5–2.0*	3.4–5.2	306–380	74–91	Farajzadeh et al. 2012
USAEME-SFO-HPLC-DAD	Water, grape-apple juice	D-M-FQ-FS-TB-TT	20–890	10.9–17.2**	1.9–10.6	226–255	64–112	This method

[a] *LOD* limit of detection, * based on S/N=3,** based on calibration line

[b] *RSD* relative standard deviation

[c] *EF* enrichment factor

CPE-HPLC-UV cloud point extraction–high-performance liquid chromatography-ultraviolet detection, *DLLME-GC-FID* (*MS*) dispersive liquid-liquid microextraction-gas chromatography-flame ionization detector (mass spectrometry), *DLLME-SFO-HPLC-DAD* dispersive liquid-liquid microextraction-solidification of floating organic drop-high performance liquid chromatography-diode array detector, *HF-LPME-GC-MS* hollow fiber liquid-phase microextraction-gas chromatography–mass spectrometry, *IL-DLLME-HPLC-DAD* ionic liquid-dispersive liquid-liquid microextraction-high-performance liquid chromatography-diode array detector, *MSPE-HPLC-UV* magnetic solid-phase extraction-high-performance liquid chromatography-ultraviolet detection, *SBSE-DLLME-GC-FID* Stir bar sorptive extraction-dispersive liquid-liquid microextraction-gas chromatography-flame ionization detector, *SPE-HPLC-MS-MS* solid-phase extraction-high-performance liquid chromatography-tandem mass spectrometry, *SPE-HPLC-UV* (multiwalled carbon nanotubes) solid-phase extraction-high-performance liquid chromatography-ultraviolet detection, *SPME-GC-ECD* solid-phase microextraction-gas chromatography-electron capture detector, *SPME-HPLC-DAD* solid-phase microextraction-high-performance liquid chromatography-diode array detector, *SVE-DLLME-GC-FID* silylated extraction vessel-dispersive liquid-liquid microextraction-gas chromatography-flame ionization detector, *USAEME-SFO-HPLC-DAD* ultrasound-assisted emulsification microextraction–solidification of floating organic drop-high-performance liquid chromatography-diode array detector

D diniconazole, *FQ* fluquinconazole, *FS* flusilazole, *M* myclobutanil, *TB* tebuconazole, *TT* tetraconazole

samples were spiked with the standards of six triazoles at two concentrations (80 μg L^{-1} and with 400 μg L^{-1}), and each analysis was done with four independent samples. The average recoveries of the studied triazoles are shown in Table 3. The recovery values were from 82 to 112 % in apple juices and ranged from 64 to 112 % in grape juice. Figure 4 shows the HPLC-DAD chromatograms that belong to the non-spiked commercial apple sample and the spiked sample with 80 and 400 μg L^{-1} of triazole standards.

The performance of the proposed USAEME-SFO method was compared with other reported extraction methods for triazoles in liquid matrices that are listed in Table 4. The best LODs were obtained with SPE followed by HPLC using UV or MS detectors (Zhou et al., Baugros et al. 2008). Working with different extraction techniques (IL-DLLME, DLLME-SFO, SPME) and HPLC-DAD, the LODs ranged from around 0.1 to 10 μg L^{-1}. The obtained values with the proposed method are slightly higher. However, it has to be taken into consideration that LODs were evaluated considering the calibration curve instead of the signal to noise relation of three. The LODs estimated based on calibration curve are more realistic but gave higher results.

The RSDs obtained with the proposed procedure were less than 11 % and were in the order of the other listed procedures. The enrichment factors calculated in this work agreed with most of the studies where the EFs were showed. The results of recovery studies taken from consulted references were made both in water and in liquid juices. The comparison of obtained recovery data with results of similar juices was satisfactory.

Conclusions

A reliable method based on USAEME-SFO was developed. The extracting solution was later injected in a HPLC-DAD in order to separate and determine six triazole fungicides. The method provided good linearity, repeatability, and enrichment factors. The LODs are in the order of a few micrograms per liter. Also, satisfactory recoveries were achieved in fruit juice samples.

Compared to other extraction processes, this method provides the advantages of simplicity and low cost (regarding chemicals and equipment). Also, it is relatively fast since the extraction is made in 18 min. Furthermore, it can be considered an environmentally friendly method due to the use of only 1-undecanol as organic solvent in the extraction process.

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Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

Conflict of Interest Ane Bordagaray has no conflict of interest. Rosa Garcia-Arrona has no conflict of interest. Esmeralda Millán has no conflict of interest.

References

Baugros JB, Giroud B, Dessalces G, Grenier-Loustalot MF, Cren-Olivé C (2008) Anal Chim Acta 607:191
Bordagaray A, Garcia-Arrona R, Millán E (2011) Food Anal Methods 4: 293
Bordagaray A, Garcia-Arrona R, Millán E (2013) Anal Methods 5:2565
Bosch Ojeda C, Sánchez Rojas F (2011) Chromatographia 74:651
EC Commission Regulation 149/2008 of 29 January 2008 amending regulation (EC) No 396/2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto. OJ L 58, 1.3.2008, p.1. MRLs available at: http://ec.europa.eu/sanco_pesticides
Farajzadeh MA, Djozan D, Nouri N, Bamorowat M, Shalamzhari MS (2010) J Sep Sci 33:1816
Farajzadeh MA, Bahram M, Jafary F, Bamorowat M (2011) Chromatographia 73:393
Farajzadeh MA, Djozan D, Khorram P (2012) Anal Chim Acta 713:70
Ganjali MR, Sobhi HR, Farahani H, Norouzi P, Dinarvand R, Kashtiaray A (2010) J Chromatogr A 1217:2337
Ghambarian M, Yamini Y, Esrafili A (2013) Michrochim Acta 180:519
Goetz AK, Ren H, Schmid JE, Blystone CR, Thillainadarajah L, Best DS, Nichols HP, Strader LF, Wolf DC, Narotsky MG, Rockett JC, Dix DJ (2007) Toxicol Sci 95:227
Hyötyläinen T (2009) Anal Bioanal Chem 394:743
Khalili Zanjani MR, Yamini Y, Shariati S, Jönsson JA (2007) Anal Chim Acta 585:286
Konieczka P, Namiesnik J (2009) Quality assurance and quality control in the analytical chemical laboratory. CRC Press, Boca Raton, USA
Leong MI, Huang SD (2008) J Chromatogr A 1211:8
Ravelo-Pérez LM, Hernández-Borges J, Asensio-Ramos M, Rodríguez-Delgado MA (2009) J Chromatogr A 1216:7336
Regueiro J, Llompart M, Garcia-Jares C, Garcia-Monteagudo JC, Cela R (2008) J Chromatogr A 1190:27
Sarafraz-Yazdi A, Assadi H, Ibrahim WAW (2012) Ind Eng Chem Res 51:3101
Sousa R, Homem V, Moreira JL, Madeira LM, Alves A (2013) Anal Methods 5:2736
Stalikas C, Fiamegos Y, Sakkas V, Albanis T (2009) J Chromatogr A 1216:175
Stocka J, Tankiewicz M, Biziuk M, Namiesnik J (2011) Int J Mol Sci 12: 7785
Tang T, Quian K, Shi T, Wang F, Li J, Cao Y (2010) Anal Chim Acta 680:26
Tankiewicz M, Fenik J, Biziuk M (2011) Talanta 86:8
Tomlin CDS (2000) The pesticide manual, 12th edn. British Crop Protection Council, Farnham, UK
Tranter RL (ed) (2000) Design and analysis in chemical research. Sheffield Academic Press Ltd, Sheffield, England
Wang C, Wu Q, Wu C, Wang Z (2011a) J Hazard Mater 185:71
Wang S, Ren L, Xu Y, Liu F (2011b) Microchim Acta 173:453
Wang W, Ma X, Wu Q, Wang C, Zang X, Wang Z (2012) J Sep Sci 35: 2266
Wolf DC, Allen JW, George MH, Hester SD, Sun GB, Moore T, Thai SF, Delker D, Winkfield E, Leavitt S, Nelson G, Roop BC, Jones C, Thibodeaux J, Nesnow S (2006) Toxico Pathol 34:895
Zhou Q, Xiao J, Ding Y (2007) Anal Chim Acta 602:223

6. Multivariate calibration of partially overlapped chromatographic peaks with PARALIND

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MODELLING HIGHLY CO-ELUTED PEAKS OF ANALYTES WITH HIGH SPECTRAL SIMILARITY.

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Summary

Modelling co-eluted peaks has always been a main keystone in chemometric applications on chromatographic data. This interest is nowadays increased due to the higher capability of modern chromatographic devices coupled with multichannel detectors and obtaining complex data structures. Techniques like Multivariate Curve Resolution (MCR) or Parallel Factors Analysis 2 (PARAFAC2) have been widely used as curve resolution methods to solve the co-elution problem (among others). The main advantage of these curve resolution techniques is that they profit the property of uniqueness of the spectrum for each co-eluted analyte. Nevertheless, there are cases where these curve resolution approaches may fail. That is when the same analyte gives two co-eluted peaks or when two analytes have highly correlated spectra, leading to a rank deficiency/co-linearity problem. In this paper we put forward the usefulness of a recent multi-way technique, parallel factor analysis with linear dependence (PARALIND) that is able to handle overlapping peaks when the spectral profile of the analytes are highly correlated. To illustrate the problem, two different situations are thoughtfully studied here: first, the co-elution of two peaks, belonging to L-proline produced in a wrong derivatization step and; second, an overlapped mixture of triazole fungicides with a high similarity in their spectra.

Keywords: PARALIND; chromatography; co-elution; enantiomers; three-way; PARAFAC2; MCR

1. Introduction

The use of multi-channel detectors in chromatographic instruments (e.g. high performance liquid chromatography with diode array detector, HPLC-DAD, or gas chromatography coupled to a mass detector, GC–MS) generates a vast amount of information for a single sample (one complete spectral pattern for each elution time). To treat this data, powerful mathematical tools have been generated to extract the desirable information from the datasets and for solving the typical problems found in chromatographic runs[1, 2]. Attending to the curve resolution methods commonly used with hyphenated chromatographic data[1], it is worth highlighting the important role of multi-way and multi-set techniques (such as Parallel Factor Analysis 2 -PARAFAC2[3-6] and multivariate curve resolution -MCR[7-10]). They have enormously demonstrated their benefits in solving/modelling problems like elution time shifts between samples, baseline drifts, or what is more relevant, co-elution problems.

The general target of multi-way and multi-set curve resolution methods is the extraction of the underlying chemical model of the dataset by using the linear relationship between the spectral intensity and the relative concentration of the analytes. The success of the abovementioned techniques is based on two resolution theorems provided by Manne[11] and highlighted by de Juan et al.[7]:

- Theorem 1: The correct elution profile of a analyte can be recovered when all the analytes inside its elution window are also present outside.
- Theorem 2: The correct spectrum of a analyte can be recovered if its related elution window is not completely embedded inside the elution window of a different analyte.

In an ideal overlapping situation where both theorems are fulfilled, the application of curve resolution methods will succeed in revealing the individual elution and spectral profile of all the analytes. This is graphically depicted in Figure 1a.

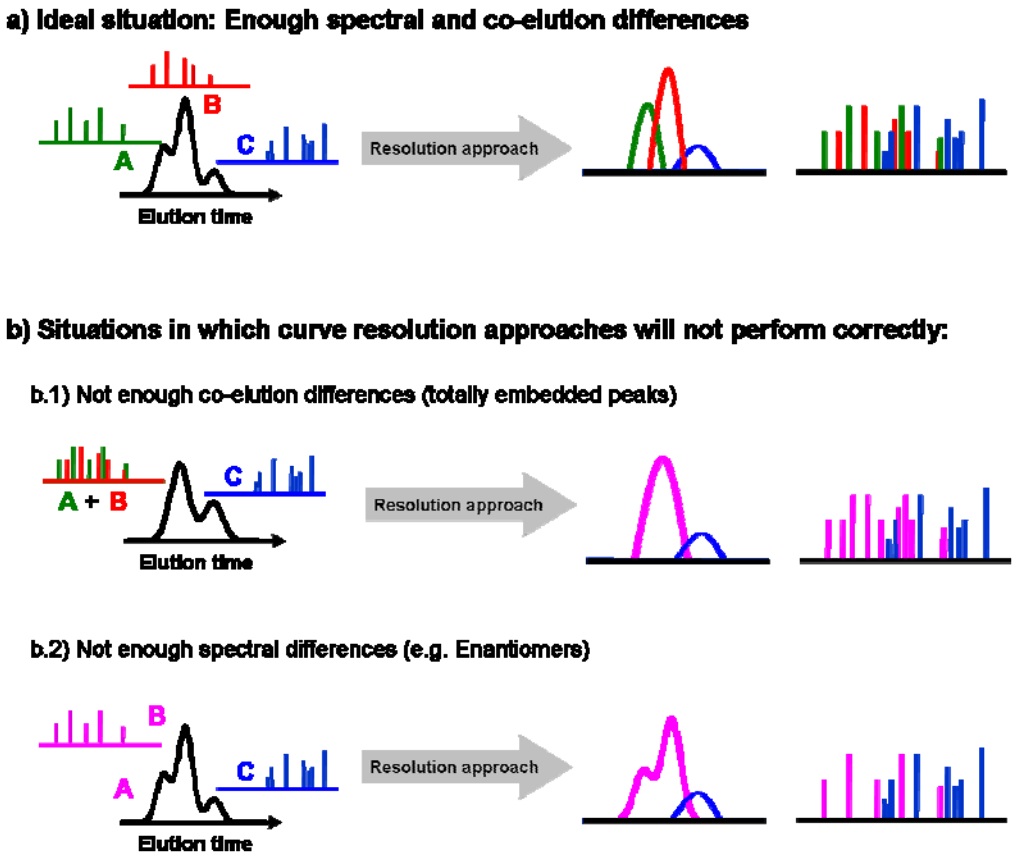

Figure 1: Graphical interpretation of theorems proposed by Manne[10]. a) Ideal situation in which there is enough spectral and elution differences between analytes. In this case, resolution methods will work. b) Situations in which curve resolution approaches may not offer a good solution: b.1) Peaks of A and B are totally embedded. Therefore, they will be model as a unique peak. b.2) Peaks A and B have a highly similar spectrum (e.g. enantiomers). Therefore, there are no enough differences between their spectra.

Nevertheless, complex situations arise during the chromatographic performance because of the inherent chemical nature of the samples (especially with biological samples [12]) and the sample preparation steps, putting forward situations in which the two theorems are not totally fulfilled, and, thus, the resolution of the correct elution and spectral profiles may not be an easy task. For instance, the case shown in Figure 1b.1 is related to the total co-elution of two analytes having different structure (and, consequently, different spectral pattern). The situation in Figure 1b.2 is directly related to the differences in the spectral profile of two co-eluting analytes. In a normal case, it will be possible modelling several co-eluting analytes, since their spectral profile is different. However, there are several situations where it is possible to find two co-eluted, though not totally embedded, peaks for the same analyte (thus, having the same spectral pattern):

a) Mixtures of components with highly similar spectral pattern: This particular issue may be handled by using chiral chromatographic columns. Nevertheless, in complex samples with dozens of analytes it is difficult to find a general chromatographic setup that allows the complete chromatographic resolution of all peaks.

b) It has been demonstrated that in several cases the reaction between analytes and derivatization reagents can generate more than one unique peak for the same analyte if the chromatographic conditions are not the proper ones[13, 14]. Many molecules have different binding sites in which the derivatization agent can link. Therefore, a mixture of the same analyte with different derivatization sites may occur, allowing their slight separation in the column, but not giving a clearly different spectral profile.

In terms of model performance, these situations are directly associated to a rank deficiency problem that hampers the correct resolution of the chromatographic profiles. Taking as an example the case shown in Figure 1b.2 it seems obvious that there are three elution profiles, corresponding to three analytes. Nevertheless, since the spectra of A and B are highly similar (or even the same), there will be two spectral influences on the system (A-B and C).

Despite the fact that many methods have been proposed for assessing the number of components in chromatographic intervals (e.g. Principal Component Analysis or evolving factor analysis) [1, 8, 15, 16,], none of them is actually able to detect rank deficiency problems, being more important the experience of the operator or the previous knowledge of the studied interval. Nevertheless, once detected, it should be possible to have independent effects in the chromatographic profiles yet nonetheless be linearly dependent in the spectral profile [17].

PARALIND (parallel factor analysis with linear dependence) is a recently developed three-way method specially designed for solving problems in rank deficient systems. PARALIND is a PARAFAC-type model constrained in one of the modes to be rank deficient respect to the other two modes. It usefulness has been previously demonstrated in flow injection analysis and multi-way fluorescence[17, 18] and even in chromatography when several analytes share the same concentration profile[19]. This makes PARALIND a perfect method to handle rank deficiency situations like the one exposed in the Figure 1, where two or more analytes have an extremely similar spectral profile.

In this paper, we put forward the application of PARALIND in situations where the spectral profiles of several analytes are highly correlated (or even the same). Results obtained by PARALIND are also compared to those obtained by more common multi-way and multi-set techniques (PARAFAC2 and MCR), showing a better understanding of the problem and better fitting to the data. For this aim two different datasets were used. The first case belongs to a dataset created from GC-MS chromatograms of biological samples, where a problem in the derivatization process generates two peaks with an extremely similar spectral pattern. The second case was a dataset from HPLC-DAD of a mixture of five fungicides. The fungicides belong to the same pesticide family and, therefore, they have very similar spectra that can lead to resolution problems. The comparison between PARALIND, PARAFAC2 and MCR has been performed under the same constraint conditions.

2. A brief introduction to PARALIND

PARAFAC2 and MCR methods are well known methodologies and have been described before in a chromatographic framework [1, 3, 4, 7, 9, 20, 21]. Therefore, here we will focus on a brief introduction to the PARALIND method, encouraging the readers to find more information about the algorithm in the supplied references [17, 18].

When a chromatographic run is monitored with a multi-channel detector, a bidimensional data matrix $\mathbf{X}$ (I x J) is obtained for each sample, containing I elution times and J spectral channels. Consequently, the straightforward way of arranging an experiment with a set of samples is the generation of a three-way data array $\underline{\mathbf{X}}$ (I x J x K), in which K denotes the number of samples. Assuming that $\underline{\mathbf{X}}$ (I x J x K) is given for which an F-component PARAFAC model holds, this model can be written as follow:

$$\mathbf{X}^{(I \times JK)} = \tilde{\mathbf{A}}(\mathbf{C} \odot \mathbf{B})^{T} + \mathbf{E} \qquad (1)$$

where the operator $\odot$ is the Khatri-Rao product. $\tilde{\mathbf{A}}$ (IxF), $\mathbf{C}$ (JxF) and $\mathbf{B}$ (KxF) are the corresponding spectral, elution and concentration loadings; whereas $\mathbf{E}$ (IJxK) is the residual matrix. Considering the example depicted in Figure 1.b2 involving three analytes where two of them have

highly similar spectra, a three-component model should be valid considering that the spectral loadings matrix **A** has rank 2 because two of the loadings profiles are identical:

$$\mathbf{A} = [\tilde{\mathbf{a}}_1\ \tilde{\mathbf{a}}_2] \qquad (2)$$

With this assumption, a new matrix **H** can be introduced containing the inner relationship between the full rank (columns) and the rank deficiency (rows) components. This matrix is also called the dependency matrix, and collects the interactions between the different components in a particular column. In our example it will contain 2 rows and 3 columns indicating the dependency between two components in the full rank system (two last columns) with one component in the rank deficient system (second row):

$$H = \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 1 \end{bmatrix} \qquad (3)$$

A new set of spectral loadings $\tilde{\mathbf{A}}$ can be defined considering the dependency matrix:

$$\tilde{\mathbf{A}} = \mathbf{AH} = [\mathbf{a}_1\ \mathbf{a}_2\ \mathbf{a}_2]^*\mathbf{H} = [\tilde{\mathbf{a}}_1\ \tilde{\mathbf{a}}_2\ \tilde{\mathbf{a}}_3] \qquad (4)$$

Accordingly to Equation 4, the second and third columns of $\tilde{\mathbf{A}}$ are forced to be identical. Thus, the new matrix $\tilde{\mathbf{A}}$ can be easily calculated by the PARAFAC model with the introduction of the dependency matrix. Graphically speaking (Figure 2) PARALIND will calculate three concentration and elution profiles and only two spectral profiles.

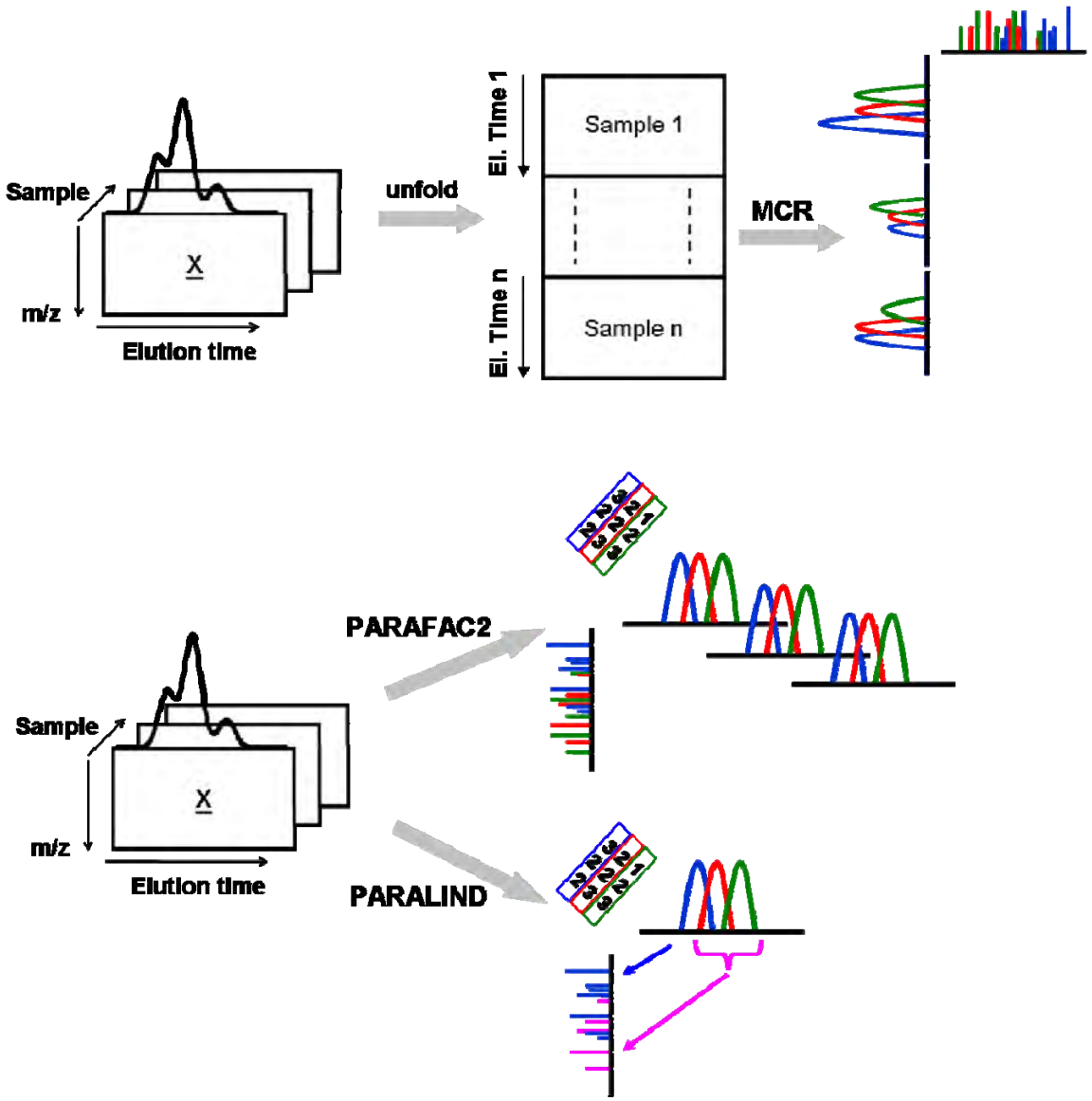

Figure 2: Graphical description of MCR, PARAFAC2 and PARALIND decomposition for a simulated system with three components. In PARALIND representation, the red and green elution profiles are constrained to have the same spectral profile (pink). Figure partially adapted from figure 22 in reference [1] with permission of ACS publications.

3. Experimental setup and software

3.1. Case 1: L-proline derivatization

3.1.1. Chromatographic system setup

A total of eighteen samples of biological origin were used. Due to confidentiality reasons further information cannot be given. Nevertheless, relevant experimental details are given herein. N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was used as derivatization reagent from freshly opened 1-mL bottles. Reagents and solvents were stored in a desiccator in the darkness. Derivatizations are carried out in thermoshakers which are set to 45°C reaction step. GC-MS

analysis was carried out on a quadrupole mass spectrometer equipped with autosampler and electron impact ionization (low bleeding injector septa or septum free injector systems were prerequisite). The column used was a 30 m long, 0.32 mm I.D. and 0.25 μm (35%-Phenyl)-methylpolysiloxane column. The oven was temperature programmable up to 360 °C. An amount of 1 μL of sample was injected into the column. Injection temperature was set to 230°C. The temperature ramp used was as follow: Start conditions at 80°C, 2 min isothermal, ramp with 5°C/min up to 330°C, cool down to initial conditions. The ion source should be turned off during the solvent delay. More specifications about the method can be found elsewhere[22].

3.1.2. Models development

PARALIND, PARAFAC2 and MCR models were constrained to be nonnegative in both spectral and elution time dimensions. The performance of the models and the assessment of the final results were made by comparing the spectral profiles obtained with the mass spectrum found in the 'NIST MS Search 2.0' software (NIST/EPA/NIH Mass Spectral Library, NIST Scientific and Technical Databases, Gaithersburg, MD 20899-8380). The concentration profiles obtained for each model were compared with the peak intensity found in the maximum of both peaks in the raw data. The comparison was performed by using the Pearson's correlation coefficient.

3.2. Case 2: Triazole fungicides

3.2.1. Experimental procedure

Five triazole fungicides (tetraconazole [TT], myclobutanil [M], flusilazole [FS], fluquinconazole [FQ] and tebuconazole [TB]) chromatographic determination was carried out after a preconcentration step with Solid Phase Microextraction (SPME) fibers. The fiber used for extraction was a polydimethylsiloxane/divinylbenzene (PDMS/DVB) acquired in Supelco (Bellefonte, PA, USA). Before starting the analysis fibers were conditioned during 30 min in the desorption chamber of the HPLC. A thermostatic bath was heated to 60 ºC and the magnetic stirrer was fixed in 500 rpm.

Stock solutions were prepared in the range between 5 and 40 mg/L (approximately) and these analyte solutions were added (20 μL) to the vial containing 20 mL of salted water, resulting concentrations between 5 to 40 μg/L. The solutions were exposed to the fiber in immersion mode and after 90 min for exposition time the fiber was withdrawn and put into the SPME-HPLC

interface to desorb the analytes. The desorption mode was the static mode (2 min), following by another dynamic mode step of 1 min to swept all the mobile phase with the analytes of the desorption chamber. After each analysis the fiber was maintained in stirred water for 15 min as cleaning step and trying to avoid the crystallization of the salt.

HPLC analysis was performed in a LC- 20AD liquid chromatographer coupled with a SPD- M20A diode array detector (Shimadzu Corporation, Duisburg, Germany). Data were collected and processed by LC Solution software (1.2. version). An interface with a Rheodyne® Valve (Supelco) with 60 ± 10 µL chamber volume was used for desorption. All separations were carried out with a XDB- C18 column (4.6 mm x 250 mm, 5 µm) (Agilent, Wilmington, DW, USA). The mobile phase was a mixture of sodium acetate buffer (0.01 M, pH 4) and acetonitrile (22:78, v/v) running at 0.5 mL/min in isocratic mode. UV-Vis absorption spectra were recorded from 190 to 300 nm. A total of 12 analyses were carried out for calibration (calibration set) and other 6 for validation (validation set)

3.2.2. Models development

Data present an important misalignment, thus, for PARALIND alignment with COW [23,24] was performed (segment length of 15 points and slack size of 4 points). Alignment was not necessary for MCR and PARAFAC2. Non-negativity and unimodality constraints were used in order to achieve the best model in PARALIND, PARAFAC2 and MCR.

3.3. Software

MCR and PARAFAC2 models were performed by using PLS-Toolbox v. 3.5[25]. PARALIND model was performed by using the algorithm freely available on the web[26] (last accessed July 2014). PLS-Toolbox and PARALIND work under MATLAB environment[27].

4. Results and discussion

4.1.1 Case 1: L-proline derivatization

4.1.1. First approach: PARAFAC2 and MCR

The raw total ion chromatograms (TICs) and the intensity at each maximum of the peaks are shown in Figure 3. At first sight, a high level of co-elution can be observed between the two main peaks of the raw data figure (red and green lines). Moreover, there is a slight inverse correlation between the intensity profiles of both peaks (Figure 3, right).

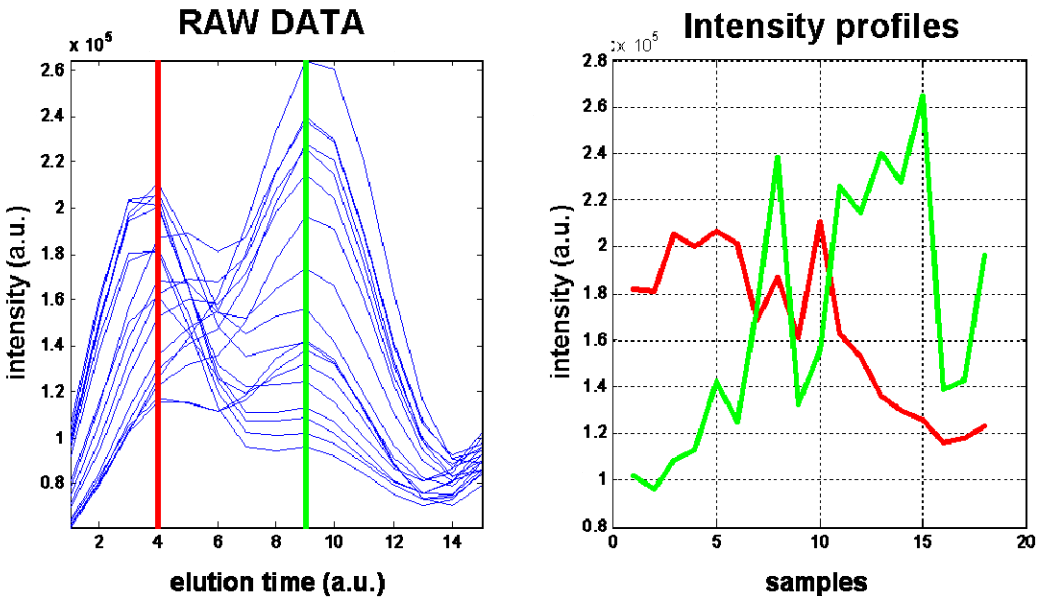

Figure 3: Left, raw TIC data obtained for the eighteen samples. Right, the evolution of the intensity in both maxima indicated in the raw data with the red and green line.

Principal Component Analysis (PCA) and Evolving Factor Analysis (EFA)[16,28] models were performed to assess the number of components (results not shown) finding that it was not clear whether a model with 3 or 4 components could be suitable. Therefore, PARAFAC2 and MCR models were built by using 3 and 4 components. The best results for PARAFAC2 and MCR models were obtained with four components, achieving an explained variance of 99.6 and 99.8%, respectively. A comprehensive study of these results will be done in further sections (see Figure 6 and Table 1). Nevertheless, we would like to highlight in this point the results obtained for the elution profiles (Figure 4):

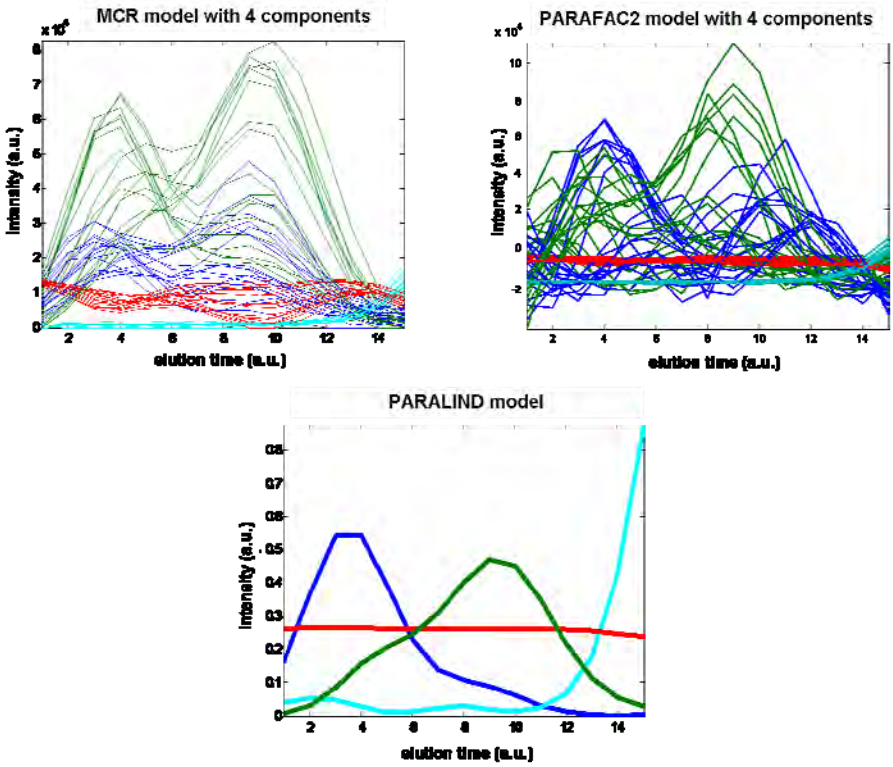

Figure 4: Elution time profiles obtained for MCR, PARAFAC2 and PARALIND models.

The results obtained for PARAFAC2 and MCR in the elution profile mode were surprising. First of all, the baseline and a third peak contributions were perfectly modelled (red and cyan profiles in Figure 4, for PARAFAC2 and MCR, respectively). These are two more contributions in the dateset. The first one is the tail of a very intense third peak clearly visible from elution time 13 in Figure 3a. Moreover, the contribution of baseline is high enough to be considered as a new component. Nevertheless, it was not possible to obtain two perfectly separated contributions for the two main peaks (blue and green lines in Figure 4).

4.1.2. PARALIND results and comparison with PARAFAC2 and MCR

Considering the fact that nor MCR neither PARAFAC2 were able to offer a satisfactory solution in the elution profiles, a new look at the raw data was mandatory. Comparing the mass spectra found in the two maxima of the peaks of interest for all the samples (Figure 5) the problem can be better understood. High correlation value was found between both mass spectra (values from 95% to 99% for all the samples, in terms of correlation coefficient). This leads to the conclusion that both peaks belong to the same analyte or, at least, both analytes have a highly similar structure. By using the NIST library, the spectra were found to be associated to proline aminoacid (Figure 5).

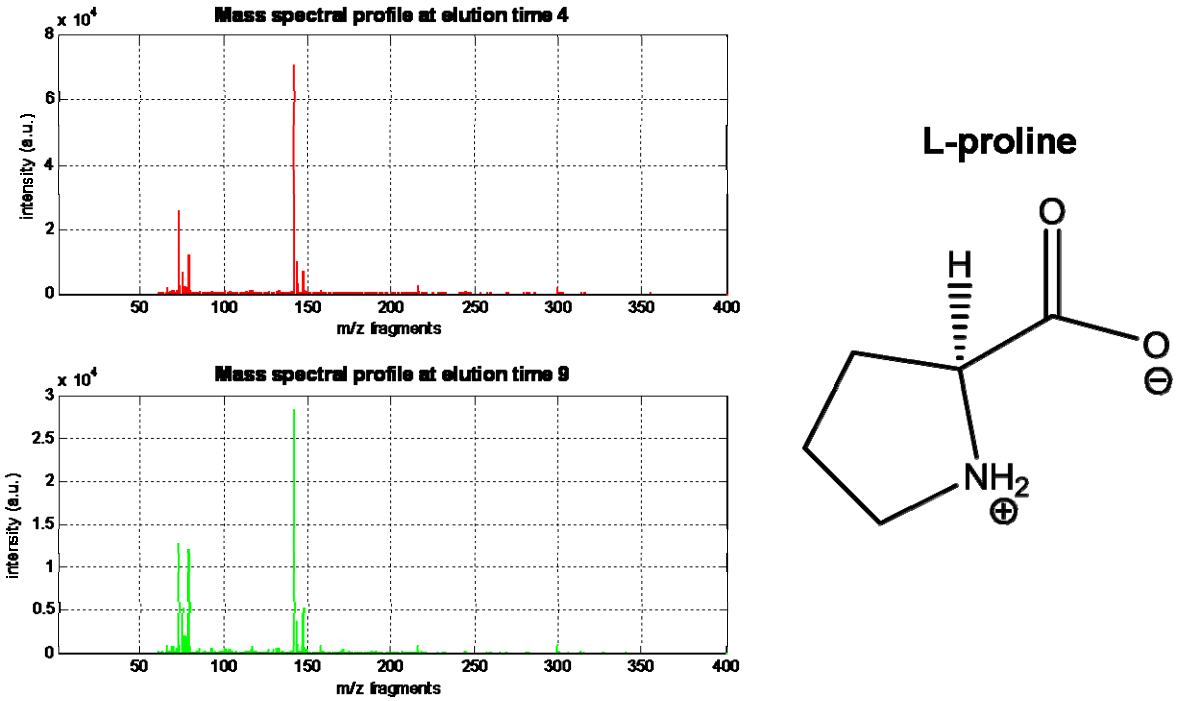

Figure 5: Mass spectra obtained for sample number 7 in the elution time 4 (red) and elution time 9 (red) and structure of L-proline aminoacid.

This highly similarity of the spectral patterns of both peaks makes the chromatographic interval rank deficient in the spectral mode. Thus, considering this, a PARALIND model was performed to handle the rank deficiency. The dependency matrix (**H**) was constructed considering a full rank system of 4 components, but a deficiency rank mode of 3 components. **H** will contain therefore four columns and three rows as is indicated in Equation 5:

$$\mathrm{H} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 1 \end{bmatrix} \qquad (5)$$

In this case, **H** (3 x 4) indicates that there are four main contributions, nonetheless the third and the fourth spectral profiles are constrained to be identical. It is important to enhance that since PARALIND is a PARAFAC-based solution, alignment of the peaks prior modelling may be advisable [27, 28]. In this example, this step was not necessary because the interval (Figure 3) did not present any misalignment.

Elution profiles

The explained variance of the best PARALIND model was 98.2%. This is slightly lower than the one obtained by PARAFAC2 or MCR. This may be due to the fact that PARALIND is a PARAFAC-based solution. Therefore, slight deviations from trilinearity may occur and, therefore, the model will not capture all the expected variance. Nevertheless, the results obtained for the elution mode by PARALIND shown in Figure 4 demonstrated that the model was able to model not only the baseline and the interfering peak, but also both main peaks, giving a better overview of the chromatographic pattern.

Mass spectral profiles

Comparing the mass spectral profiles obtained for each model with the pure mass spectrum of proline (Table 1) it can be seen that 2 of the components found by MCR and PARAFAC2 are clearly related to proline; whereas there is only one component of the PARALIND model highly correlated with proline. This is an expected result, since PARALIND model was constrained to have only three spectral profiles.

Table 1: Comparison between the results obtained by MCR, PARAFAC2 and PARALIND and the raw data.

	MCR (%)	PARAFAC2 (%)	PARALIND (%)
Mass spectrum of proline*	90-91	90-93	90
Concentration profile at time 4**	50	95	92
Concentration profile at time 9**	85	98	99

* Percentage of matching between the mass spectral profiles obtained for each model and the pure spectrum of proline found in the NIST library. For MCR and PARAFAC2 there are two values because the spectral mode was full rank. Therefore, the models found two spectral profiles very similar to proline

** Percentage of matching between the concentration profiles obtained for each model and the evolution of the intensity in the two main peaks. This comparison was done by using the Pearson's correlation coefficient.

Concentration profiles

Since no absolute concentrations for both peaks were available, the intensity in the maximum for each peak was used as a reference value and compared to the concentration loadings obtained for the different models. Results of this comparison can be seen in Figure 6 and Table 1.

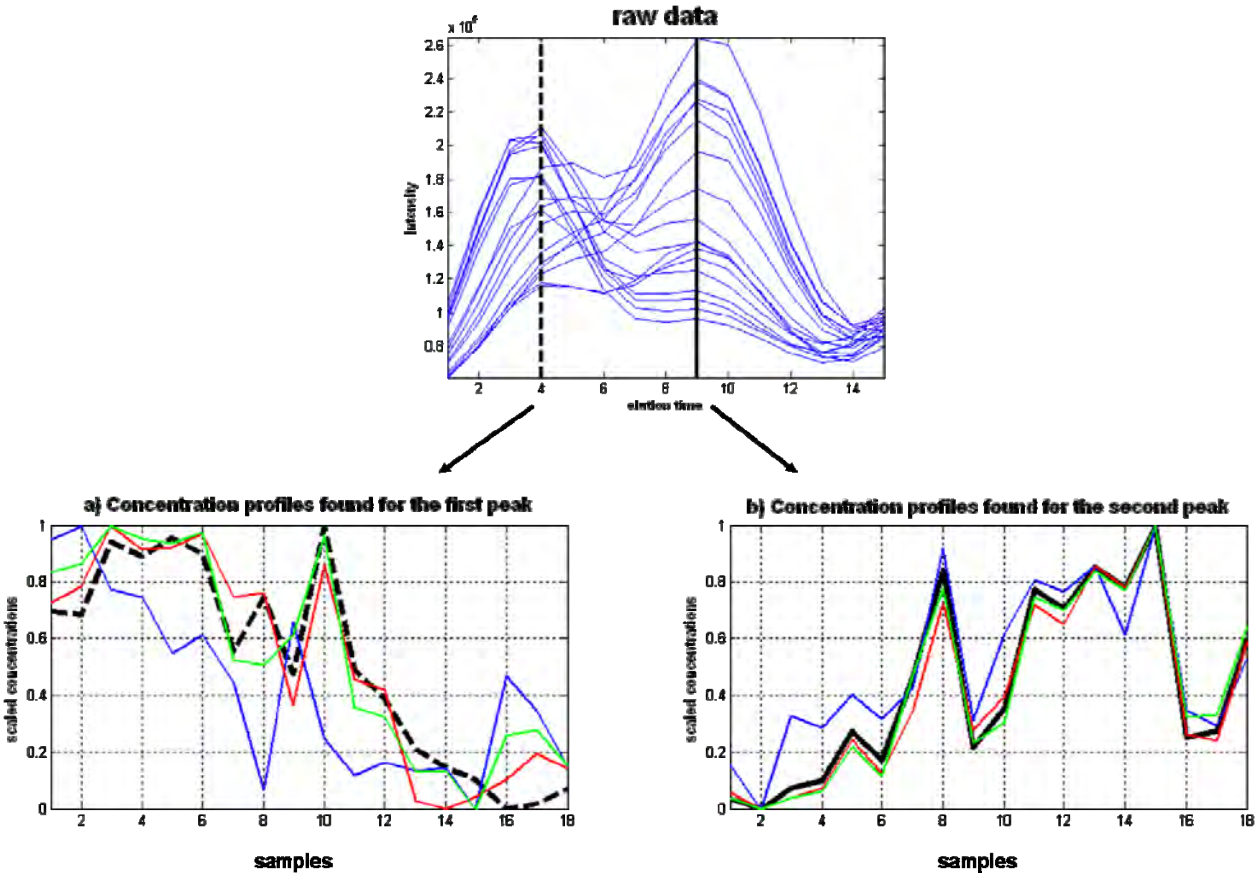

Figure 6: Comparison between the evolution of the intensity of both main peaks (dashed, elution times 4; solid, elution time 9) depicted in black and the concentration loadings obtained by MCR (blue), PARAFAC2 (red) and PARALIND (green).

In this case, the highest correlations between the concentration profiles obtained by the different models and the raw data were obtained for PARALIND and PARAFAC2. This denoted that three-way models were able to explain better the behaviour of the main peaks, especially in the second peak, in which the correlation between the concentration loadings and the intensity were found to be 98 and 99 for PARAFAC2 and PARALIND, respectively.

4.1.3. Chemistry of the problem

To validate the good performance of the PARALIND model, a sound chemical reason must be found. As said before, both major peaks were associated to proline. Nevertheless, the possibility of coexistence of both enantiomers (L and D) has to be rejected since D-aminoacids are scarcely found in biological samples. Furthermore, the different proportions between peaks could not be explained since transformation of L-proline into D-proline (and vice versa) is not thermodynamically possible in the experimental conditions. The most coherent source of the co-existence of both peaks must be found in the sample treatment and derivatization reaction. The main target of the analysis was not the determination of aminoacids, therefore, N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was used as derivatization reagent. MSTFA is a well known reagent, widely used in gas chromatographic analysis of organic and biochemical analytes [11, 12]. However, it is also well known that TFA-based agents are not the most suitable for the derivatization of aminoacids. The main reason is that the aminoacid may be silylated in the amino and carboxyl group in a single step. Kanani et al.[12] described that the kinetic of the reaction with the silylation groups varies accordingly to the specific groups. –OH and –COOH groups react faster and almost simultaneously, while the hydrogen atoms of the $–NH_2$ group react slower. Thus, if this silylation step is not conducted in the proper conditions, it may lead to the appearing of two peaks in the chromatographic profile belonging to the same aminoacid and, consequently, having highly similar spectral pattern.

4.2. Case 2: Triazole fungicides

4.2.1. Raw data

The chromatographic profiles obtained for the second dataset under study are shown in Figure 7. Chromatograms show overlapped five peaks, improvement of the separation was complicated because of the limitation of mobile phase: it was not possible to use other organic solvents that can damage the PDMS/DVB fiber.

The elution order in the chromatogram is as follow: TT (7.1 min), M (7.4 min), FS (7.6 min), FQ (8.0 min) and TB (8.4 min). Besides, an important misalignment between samples is observed. This misalignment was corrected by using COW [23] algorithm. The chosen parameters were 15 points as segment length and 4points as slack size. Figure 7 shows the data before and after alignment. It must be remarked, though, that PARAFAC2 and MCR do not need this previous step of alignment.

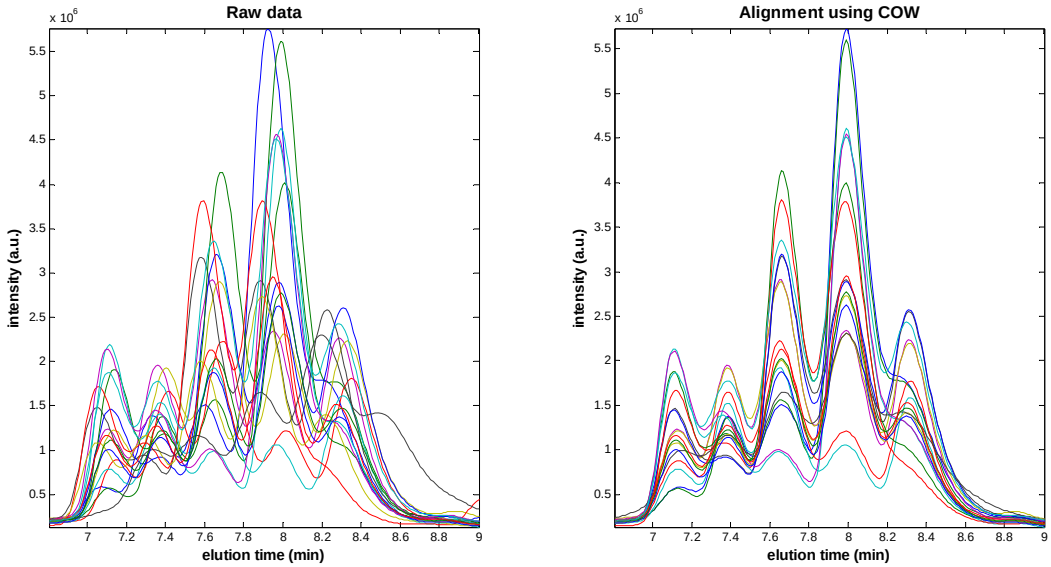

Figure 7: Misalignment presented with the raw data and the corrections made by COSHIFT (20 maximum shift corrections in data points) and COW (segment length = 15; slack size = 4).

4.4.2. Results and comparison between PARALIND, PARAFAC2 and MCR

Five components were expected due to the presence of five analytes, not having a strong influence of baseline drifts. Models with 4, 5 and 6 components were performed, but 5 was the best option for PARAFAC2 and MCR, as expected. Different constraints were applied to the models: non-negativity was applied in every mode (elution time, UV spectra and samples), except in the first mode for PARAFAC2. Unimodality was also applied to MCR model. The best models performance is highlighted in Table 2.

Table 2: Explained variance of the models PARAFAC2, MCR and PARALIND.

Explained variance (%)	
PARAFAC2 [(a)]	99.974
MCR [(a)]	99.980
PARALIND [(a)]	99.480
PARALIND [(b)]	99.443

(a): non-negativity constraints in every mode (except retention time mode in PARAFAC2)

(b): non-negativity constraints in every mode and unimodality in retention time mode

Looking at the elution profiles obtained for PARAFAC2 and MCR models (Fig. 8) it can be clearly observed that nor PARAFAC2 neither MCR offered satisfactory results in elution profiles when describing the second and the fifth analyte (myclobutanil and tebuconazole); while the rest of analytes were well described.

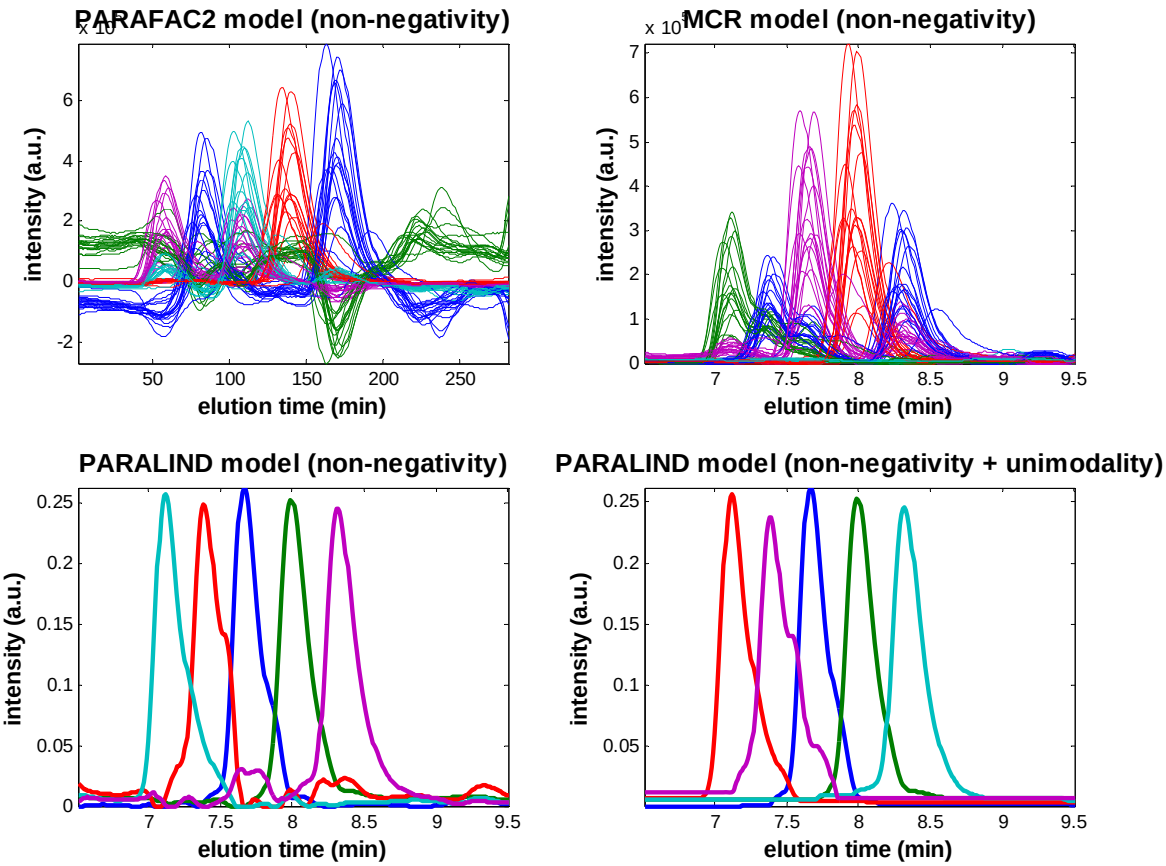

Figure 8: Comparison between the elution time loadings of the models: PARAFAC2, MCR and PARALIND (with only nonnegativity constraints and with nonnegativity and unimodality constraints)

Looking back at the raw data it can be seen that these two analytes have very similar UV spectra (between 97.06 and 98.53% in terms of Pearson's correlation coefficient in different samples). As in the previous case, this makes the dataset rank deficient in the elution time mode. Therefore, PARAFAC2 and MCR may have difficulties in resolving the correct elution profiles under the used constraints. Knowing this fact, PARALIND model was constructed with 5 components for elution time and concentrations modes; whereas, 4 components in the spectral mode was imposed. In this case **H** matrix will contain 5 columns and 4 rows:

$$H_{(4x5)} = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 1 & 1 \end{bmatrix} \quad (6)$$

Moreover, unimodality was also included in elution time mode for PARALIND. PARALIND results can be seen in Figure 8. Each component was perfectly modelled in the PARALIND model constrained with non-negativity and unimodality. The explained variance and other figures of merit showed in Table 2 and Table 3 where, a comparison between MCR, PARAFAC2 and PARALIND is shown.

4.2.3 Calibration and validation models

In order to study the predictive capability of the performed models, calibration models were constructed for the five analytes, being validated with the use of an external set of samples. Different parameters were calculated to compare the models: Mean recovery values (calculated from the predicted values of the calibration and validation sets), Root Mean Square Error of Predictions (RMSEP) (eq. 7) and Relative Errors ($RE_{cal/val}$) (eq. 8):

$$RMSEP = \sqrt{\frac{1}{N}\sum(\hat{x}_i - x_{i,ref})} \quad (7)$$

$$RE_{(cal/val)}(\%) = 100 \cdot \sqrt{\frac{\sum(\hat{x}_i - x_{i,ref})}{\sum x_{i,ref}^2}} \quad (8)$$

Where N is the number of samples, $\hat{x}_i$ is the predicted value of the i sample and the $x_{i,ref}$ is the reference value of the i sample. Univariate limits of detections (eq. 9) were calculated using the deviation of the regression ($s_{y/x}$) (eq.10):

$$LOD = \frac{3 \cdot s_{y/x}}{slope} \quad (9)$$

$$s_{y/x} = \sqrt{\frac{\sum(\hat{y}_i - y_{i,ref})}{n-2}} \quad (10)$$

Where $\hat{y}_i$ is the predicted y value calculated with the regression, $y_{i,ref}$ is the reference value of y and n is the number of samples to calculate the degrees of freedom (n-2). Table 3 shows the results

obtained for all the models applied to the five analytes. It is remarkable that the best results obtained in terms of accuracy and precision where those obtained by PARALIND for all the analytes.

Table 3: Table of results obtained with PARAFAC2, MCR and PARALIND [(a),.(b)] models for five fungicides: tetraconazole (TT), myclobutanil (M), flusilazole (FS), fluquinconazole (FQ) and tebuconazole (TB).

			TT		M		FS		FQ		TB	
Calibration range (µg L^{-1})			5.5-33.0		5.5-33.0		7.0-42.0		5.4-32.40		8.4-50.4	
Converging time		**PARAFAC2** [(a)]	8 h									
		MCR [(a)]	3 min									
		PARALIND [(a)]	10 min (+ alignment)									
		PARALIND [(b)]	13 min (+ alignment)									
	R^2	**PARAFAC2** [(a)]	0.8742		0.1347		0.9690		0.9937		0.9167	
		MCR [(a)]	0.8419		0.2085		0.7703		0.9847		0.7997	
		PARALIND [(a)]	0.9920		0.9830		0.9805		0.9908		0.9830	
		PARALIND [(b)]	0.9933		0.9883		0.9921		0.9904		0.9864	
CALIBRATION	**Mean calibration Recoveries (%)**		*mean*	*std*	*mean*	*std*	*mean*	*std*	*mean*	*std*	*mean*	*std*
		PARAFAC2 [(a)]	95.2	22.9	157.2	329.2	101.7	14.0	100.4	5.0	98.0	16.3
		MCR [(a)]	96.6	27.8	33.3	238.8	97.3	29.5	100.8	9.2	100.1	26.7
		PARALIND [(a)]	99.9	5.1	102.2	13.4	101.0	7.4	99.0	9.2	101.0	14.1
		PARALIND [(b)]	99.8	4.6	101.7	9.8	101.1	6.8	99.0	9.2	100.1	13.0
	RMSEC	**PARAFAC2** [(a)]	3.6		23.8		2.1		0.7		4.3	
		MCR [(a)]	4.1		18.3		6.5		1.1		7.2	
		PARALIND [(a)]	0.8		1.2		1.7		0.9		1.9	
		PARALIND [(b)]	0.8		1.0		1.1		0.9		1.7	
	REcal (%)	**PARAFAC2** [(a)]	5.3		35.7		2.5		1.1		4.2	
		MCR [(a)]	6.1		27.4		7.7		1.8		7.0	
		PARALIND [(a)]	1.3		1.9		2.0		1.4		1.9	
		PARALIND [(b)]	1.2		1.5		1.3		1.4		1.7	
VALIDATION	**Mean validation Recoveries (%)**		*mean*	*std*	*mean*	*std*	*mean*	*std*	*mean*	*std*	*mean*	*std*
		PARAFAC2 [(a)]	84.6	14.8	37.2	79.3	94.6	16.9	89.2	9.9	81.4	22.0
		MCR [(a)]	85.2	25.9	68.8	49.9	104.6	19.8	88.2	4.3	88.4	16.7
		PARALIND [(a)]	96.2	6.7	103.1	6.3	96.0	7.2	94.7	3.8	97.5	5.1
		PARALIND [(b)]	97.1	5.8	103.2	6.4	96.7	6.5	94.7	3.8	97.6	5.0

	RMSEP	PARAFAC2 [(a)]	3.6	17.0	3.5	2.4	7.0
		MCR [(a)]	4.8	10.9	4.6	2.1	5.5
		PARALIND [(a)]	1.2	1.1	1.6	1.1	1.5
		PARALIND [(b)]	1.0	1.2	1.4	1.1	1.4
	REval (%)	PARAFAC2 [(a)]	8.0	37.8	6.2	5.4	10.2
		MCR [(a)]	10.6	24.2	8.0	4.8	8.0
		PARALIND [(a)]	2.8	2.5	2.8	2.4	2.1
		PARALIND [(b)]	2.2	2.6	2.5	2.4	2.1
	LOD (μg L^{-1})	PARAFAC2 [(a)]	11.7*	78.2*	7.0*	2.4	14.2*
		MCR [(a)]	13.4*	60.2*	21.5*	3.8	23.6*
		PARALIND [(a)]	2.8	4.1	5.5	2.9	6.2
		PARALIND [(b)]	2.5	3.4	3.5	3.0	5.5

(a): non-negativity constraints in every mode (except retention time mode in PARAFAC2).
(b): non-negativity constraints in every mode and unimodality in retention time mode.
(*): LODs higher than the minimum concentration in the calibration range.

Regarding the linearity of the calibration range, PARALIND models (both models with different constraints) give the best results (R^2>0.98). PARAFAC2 and MCR models give linearity between 0.8742-0.9937 and 0.7997-0.9847, respectively.

Errors given by the models were observed using RMSEC and RMSEP values. In each case (calibration and validation) the figures obtained by PARALIND models are much lower than PARAFAC2 and MCR models. These results show that PARAFAC and MCR models give higher errors specially in M analyte, where it is demonstrated with the Figure 8 that those models are not able to identify separately from the TB analyte.

Models accuracy and precision was evaluated with recoveries. Best recovery values with its standard deviations were obtained with PARALIND models for both calibration and validation sets. Recovery values for the calibration set were in the range of 99.8-102.2% with standard deviations of 4.6-14.1 for PARALIND and the values for PARAFAC2 and MCR were 95.2-101.7% with standard deviations of 5.0-29.5 excluding again the problematic analyte (myclobutanil). For the validation set the trend is similar, PARALIND results are better in recovery values as well as in their standard deviations as it is shown in Table 3.

Another important parameter is the Limit of Detection. When the LOD is calculated as in above mentioned equations, sometimes happen that LOD values can be inside the calibration range if the data is not properly modelled. Indeed in this data set this is happening when data is modelled by PARAFAC2 and MCR, while LODs obtained using PARALIND are lower than the lowest concentration of the calibration therefore acceptable for the data set. This makes the method more reliable comparing with other models with PARAFAC2 and MCR.

Comparing PARAFAC2 and MCR models with PARALIND models (the two models using different constraints), PARALIND provide much better results in all the parameters. There are significant differences between PARAFAC2 and MCR and two PARALIND models, but there are not big differences between the two PARALIND models. Although the model with unimodality is slightly better regarding all the parameters, the difference is not remarkable and both models with PARALIND are satisfactories.

Although for PARALIND is necessary an alignment that is time consuming, overall time is much higher in PARAFAC2. MCR is faster, but the results are not as satisfactory as in PARALIND.

5. Conclusions

The main aim of this paper is to highlight the benefits that a methodology like PARALIND can have to solve rank deficiency problems in chromatographic datasets. In the cases shown here, this rank deficiency arises from peaks having highly similar spectral patterns. The results are compared with the application of more established curve resolution methods like PARAFAFAC2 and MCR, showing that PARALIND is a more suitable method when rank deficiency appears in a chromatographic datasets.

The obtained results in the first case showed that while the results obtained by PARAFAC2 and MCR provided some information but not a clear picture of the chemical situation, PARALIND was able to extract the exact underlying chemical behaviour of the studied interval, giving a more precise chemical understanding and, consequently, a better recovery of the latent structure of the data. In the second case, quantitative accurate results were obtained for all the analytes with PARALIND. Nevertheless, PARAFAC2 and MCR models gave quantitative satisfactory results for

three of the analytes; while the results for tebuconazol and myclobutanil were not as good, in terms of quantitative results, as the ones obtained by PARALIND.

In the context of chromatography, PARALIND can be used in some other situations apart from the one shown in this work. For instance, PARALIND may be useful in the modelling of enantiomeric mixtures or modelling analytes that have a highly correlated concentration profile. The rank deficiency problems can be much more complex, involving rank deficiency is some of the modes or more complicated cross-relationships between the components. This can easily be handled by tuning the dependency matrix (**H**) to the problem, allowing the establishment of the needed relationships between different analytes.

To conclude this paper, and as a matter of additional insight into the theorems formulated by Manne, it can be concluded that the full-rank resolution methodologies applied to datasets with more than one sample (multi-way or multi-set structures) will work only if there is enough difference in the behaviour of the components/analytes in the three modes (elution time, spectral channel and concentration profiles). Otherwise, the system must be considered as a rank deficiency one, and another solution must be adopted to solve it. For example, a more constrained MCR and PARAFAC2 models (e.g. selectivity constraint applied to the elution profile of the components). The main drawback of a more constrained model is that much clear knowledge of the dataset is necessary to apply correctly the constraints. PARALIND model directly constraints two profiles to be same without needed elaborated selectivity constraints. The main drawback of PARALIND, though, is that is a PARAFAC-based decomposition. Therefore, tri-linearity must be assumed. In terms of chromatography, this means that peaks must be aligned and baseline must be stable in order to obtain a sensible decomposition of the dataset.

6. Aknowledgements

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7. Reference List

[1] J.M. Amigo,T. Skov, R. Bro, ChroMATHography: Solving chromatographic issues with mathematical models and intuitive graphics, Chem. Rev..110 (2010) 4582-4605.

[2] T. Skov, R. Bro, Solving fundamental problems in chromatographic analyisis, Anal. Bioanal. Chem. 390 (2008) 281-285.

[3] J.M. Amigo, M.J. Popielarz, R.M Callejon, M.L Morales, A.M. Troncoso, M.A Petersen, T.B. Toldam-Andersen, Comprehensive analyisis of chromatographic data by using PARAFAC2 and principal components analyisis, J. Chromatogr. A 1217 (2010) 4422-4429.

[4] J.M. Amigo, T. Skov, J. Coello, S. Maspoch, R. Bro, Solving GC-MS problems with PARAFAC2, TrAC 27 (2008) 714-725.

[5] R. Bro, C.A. Andersson, H.A.L. Kiers, PARAFAC2: Part II. Modeling chormatographic data with retention time shifts, J. Chemometr. 13 (1999) 295-309.

[6] H.A.L. Kiers, J.M.F. Ten Berge, R. Bro, R. Parafac2: Part I: A direct fitting algortihm for the PARAFAC2 model, J. Chemometr. 13 (1999) 275-294.

[7] A.de Juan, R. Tauler, Factor analysis of hyphenated chromatographic data: Exploration, resolution and quantification of multicomponent systems, J. Chromatogr. A 1158 (2007) 184-195.

[8] de Juan, A.; Tauler, Multivariate curve resolution (MCR) from 2000: progress in concepts and applications, R. Crit. Rev. Anal. Chem. 36 (2006) 163-176.

[9] S. Mas, A. Carbo, S. Lacorte, A. de Juan, R. Tauler, Comprehensive description of the photodegradation of bromophenols using chromatographic monitoring and chemometric tools, Talanta 83 (2011) 1134-1146.

[10] F. Marini, A. D'Aloise, R. Bucci, F. Buiarelli, A.L. Magrí, A.D. Magrì, Fast analysis of 4 phenolic acids in olive oil by HPLC-DAD and chemometrics, Chemometr Intell Lab, 106 (2011) 142-149.

[11] R. Manne, B.V. Grande, Resolution of two-way data form hypheanted chromatography by means of elementary matrix trasformations, Chemometr. Intell. Lab. 50 (2000) 35-46.

[12] B. Khakimov, J.M. Amigo, S. Bak, S.B. Engelsen, Plant metabolomics: Resolution and quantification of elusive peaks in liquid chromatography-mass spectrometry profiles of complex plant extracts using multi-way decomposition methods, J Chrom A 1266 (2012) 84-94.

[13] I. Molnar-Perl, Z.F. Katona, GC-MS of amino acids as their trimethylsilyl/*t*-butyldimethylsilyl derivatives: In model solutions III, Chromatographia 51 (2000) S228-S236.

[14] H. Kanani, P.K. Chrysanthopoulos, M.I. Klapa, Standardizing GC–MS metabolomics, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 871 (2008) 191-201.

546 [15] L.G. Johnsen, J.M. Amigo, T. Skov, R. Bro, Automated resolution of overlapping peaks in
547 chromatographic data, J Chemometr 28 (2014) 71-82.
548 [16] M. Maeder, Evolving factor analyisis for the resolution of overlapping chromatographic peaks,
549 Anal. Chem. 59 (1987) 527-530.
550 [17] R. Bro, R.A. Harshman, N.D. Sidiropoulos, M.E. Lundy, Modeling multi-way data with
551 linearly dependent loadings,. J. Chemometr. 23 (2009) 324-40.
552 [18] M. Bahram, R. Bro, A novel strategy for solving matrix effect in three-way data using parallel
553 profiles with linear dependencies, Anal. Chim. Acta 584 (2007) 397-402.
554 [19] T. Khayamian, G.H. Tan, A. Sirhan, Y.F. Siew, S.M. Sajjadi, Comparison of three multi-way
555 models for resolving and quantifying bifethrin and tetramethrin using gas chromatography-mass
556 spectrometry, Chemometr, Intell, Lab, 96 (2009) 149-158.
557 [20] A. de Juan, S.C. Rutan, R. Tauler, D.L. Massart, D.L. Comparison between the direct trilinear
558 decomposition and the multivariate curve resolution-alternating least squares methods for the
559 resolution of three-way data sets Chemometr, Intell. Lab. 40 (1998) 19-32.
560 [21] I. Garcia, M.C. Ortiz, L. Sarabia, J.M. Aldama, Validation of an analytical method to
561 determine sulfamides in kidney by HPLC-DAD and PARAFAC2 with first-order derivative
562 chromatograms, Anal. Chim. Acta 578 (2007) 222-234.
563 [22] O. Fiehn, T. Kind, Metabolite profiling in blood plasma, Methods Mol. Biol. 358 (2007) 3-17.
564 [23] G. Tomasi, F. van der Berg, C. Andersson, Correlation optimized warping and dynamic time
565 warping as preprocessing methods for chromatographic data, J. Chemometr. 18 (2004) 231-241
566 [24] T. Skov, F. van der Berg, G. Tomasi, R. Bro, Automated alignment of chromatographic data, J.
567 Chemometr. 20 (2006) 484-497
568 [25] Eigenvector Research, 2011.
569 [26] www.models.life.ku.dk, Q & T group (2011).
570 [27] The Mathworks, MATLAB (2011).
571 [28] M. Maeder, A.D. Zuberbuehler, The resolution of overlapping chromatographic peaks by
572 evolving factor analysis, Anal. Chim. Acta 181 (1986) 287-291.
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7. General discussion

7. General discussion

The experimental part of this work has been based on SPME and USAEME microextraction techniques that allow preconcentration and isolation of analytes for its matrices. In the last years, the use of microextraction techniques has been greatly increased. In early 90's SPME started as an innovative technique allowing preconcentration and analyte isolation in a single step [Arthur and Pawliszyn, 1990]. But in some years, to overcome some SPME problems, liquid microextraction techniques started to develop [Jeannot and Cantwell, 1996]. However, the real increase of the use of different LPME techniques began in 2000 [Psillakis and Kalogerakis, 2003; Lambropoulou and Albanis, 2007; Pinto *et al.*, 2010; Sarafraz-Yazdi and Amiri, 2010]. Figure 7.1 shows the rising trend in microextraction publications in the last decade. It can be seen that the liquid microextraction techniques continue the trend of rising, while the increase in SPME publications is not as notorious as in liquid microextraction.

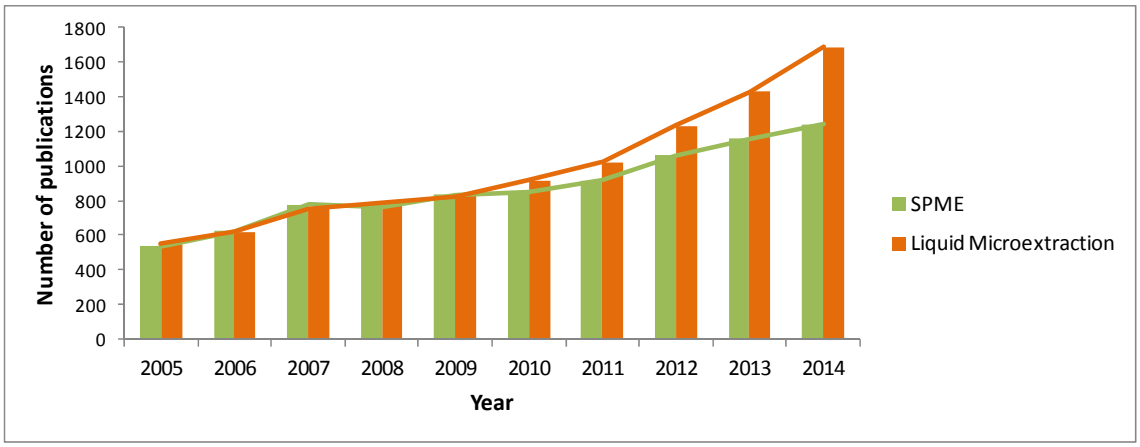


***Figure 7.1**. The increasing trend of SPME and liquid microextraction techniques during the last decade (data source Science Direct seeker).*

Firstly, in this chapter, an overview of different analytical characteristics and practical aspects of the developed microextraction methods have been remarked. Secondly, the comparison of analytical characteristics obtained in this work and other

published procedures, considering several liquid matrices and fruit samples have been considered.

7.1 Overview about the characteristics of the developed microextraction methods

Some features related to the extraction step have been taken into account to compare the SPME and USAEME techniques. Extractant material and some of the obtained results were compared, as well as some other features associated with the procedure handling (see tables 7.1, 7.2 and 7.3).

The analytical characteristics for the triazole determination of the three methods are indicated in table 7.1. Reproducibilities, calculated as RSD %, are quite similar in the three procedures. The better results are obtained with USAEME (1.9 - 10.6%). One astonishing result is the linear range observed when SPME-GC/ECD is used. The explanation is that for tebuconazole the method is not very sensitive, and consequently its range (190 – 2560 μg L^{-1}) and detection limits (162 μg L^{-1}) are much higher than the obtained with other analytes. Disregarding this analyte characteristic, it can be said that limits of detection are a bit higher when using USAEME. Nevertheless, it is remarkable that the linear range is wider when using USAEME. The reason could be that SPME fibers coatings can be saturated if high concentrations are used, and hence the linearity is lost. The recovery studies performed on the three methods gave quite similar behavior, although in general USAEME results were lower than the obtained results with SPME. The reason behind this might be the difficulty to form the drop when complex matrices are involved. If the drop is not formed in a proper way after centrifugation, the lost of the extractant results in lower recovery values.

Table 7.1. *Analytical characteristics of the triazole determination with the methods developed in this work.*

Method	Matrix	Analytes	Linearity ($\mu g\ L^{-1}$)	LOD[a] ($\mu g\ L^{-1}$)	RSD[b] (%)	EF[c]	Recovery (%)	Reference
SPME-GC/ECD	Grape and apple juices	D-M-TT-TB	0.4-2560	0.2-162	3.3-18.0	-	93.6-112.1	[Bordagaray *et al.*, 2011]
SPME-HPLC/DAD	Water, fruit samples	D-M-FQ-FS-TB-TT	5.5-50.4	1.5-5.9	2.8-13.1	-	94.5-123.4	[Bordagaray *et al.*, 2013]
USAEME-SFO-HPLC/DAD	Water, grape and apple samples	D-M-FQ-FS-TB-TT	20-890	10.9-17.2	1.9-10.6	226-255	82-112	[Bordagaray *et al.*, 2014]

SPME-GC/ECD: Solid Phase Microextraction coupled to Gas Chromatography with Electron Capture Detector.
SPME-HPLC/DAD: Solid Phase Microextraction coupled to High Performance Liquid Chromatography with Diode Array Detector.
USAEME-HPLC/DAD: Ultrasound Assisted Emulsification Microextraction coupled to High Performance Liquid Chromatography with Diode Array Detector.
Analytes: D: diniconazole; FS: flusilazole; FQ: fluquinconazole; M: myclobutanil; TB: tebuconazole; TT: tetraconazole.
[a] LOD: Limit of detection; based on calibration line.
[b] RSD: Relative standard deviation.
[c] EF: Enrichment factor.

The last experimental chapter of this work have included chemometric for solving of overlapping peaks. There was found a partial overlap between five peaks in the SPME with HPLC/DAD developed method. A better separation was not possible because the restriction of certain solvent when SPME was used. Consequently, the approach of chemometrics could help in the resolution of peaks and its quantification.

Some analytical characteristics obtained with univariate calibration and multivariate calibration using PARALIND are shown in table 7.2. It can be seen that, although the PARALIND are slightly better, the results are similar in almost all characteristics.

Table 7.2. *Comparison of the analytical characteristics of the data resolved by univariate calibration and multivariate calibration by PARALIND.*

Feature	Univariate calibration (221nm)	PARALIND calibration
RSD (%)	2.8 – 13.1	4.6 - 13.0
Recovery (%)	94.5 - 123.4	94.7 – 103.2
LOD (μg L^{-1})	1.5 - 5.9	2.5 – 5.5

The important point is that PARALIND provided much better results than PARAFAC2 and MCR. Because of the characteristics of the obtained spectra, it was impossible to solve with the most common chemometric methods for second order data such as PARAFAC2 and MCR. The similarity between two spectra led to rank deficiency problems and PARAFAC2 and MCR were not able to handle this kind of data. Therefore, alternative PARALIND was used. PARALIND provided much better results than PARAFAC2 and MCR, especially for myclobutanil and tebuconazole as it can be seen in chapter 6. PARAFAC2 and MCR were not able to solve in a proper way the peaks of myclobutanil and tebuconazole because its spectra were very similar.

Some practical aspects are also taken into account (see table 7.3). If the extraction time is considered, the extraction with SPME lasts between 45 and 90 minutes while USAEME spends 18 minutes; hence, five times less than with the use of SPME-HPLC procedure. Due to the dispersion in USAEME, there is a high increase in the contact surface between the two phases and therefore the transfer rate also increases.

Other aspect to evaluate is the automation possibility. In the developed procedures there was not automation; but if the technique is taken to a bigger scale, this feature has to be considered. SPME can be easily automatized with an appropriate injector interface. Unfortunately, USAEME technique needs more hand-work and it is difficult to automatize, because more steps are involved in the process. Nevertheless several efforts have been done to automatize similar LPME techniques [Lee and Lee, 2011].

If the work is doing manually, USAEME method is much easier to handle. There might be some failure analysis, but each extraction starts with a new droplet and that supposes to began again this specific analysis. However, a problem with a SPME fiber could be more complicated. The fiber is easily broken if the analyst is not careful enough in the work. If the fiber is broken completely or even partially, a new fiber is needed and if many errors are done in process the method will result very expensive. Apart from the fact that the introduction of a new fiber means to include batch-to-batch differences in reproducibility.

Moreover, if the fiber is broken a new fiber needs to be conditioned and the efficiency of the fiber changes from batch to batch. If USAEME is used those two problems are solved due to for each analysis a new droplet is used and conditioning is not necessary. Besides, for the same reason when the USAEME technique is used there is no need for a cleanup step. However, with SPME is necessary to avoid the carry over effect and the possibility of crystallization may occur if the salt is used during analysis.

For SPME technique an interface (homemade or commercial) is compulsory to couple with a HPLC instrument, whereas the extractant used with USAEME is directly injected.

Table 7.3. *Comparison of the practical characteristics of the microextraction methods developed in this work.*

Characteristics	**SPME**		**USAEME-SFO**
	GC/ECD	**HPLC/DAD**	**HPLC/DAD**
Extraction time (min)	45	90	18
Automation feasibility	High		low
Difficulty level	High		Moderate
Reproducibility affected?	Yes, batch to batch differences		No, every time a new drop is used
Cleanup step	Yes		No
Conditioning	Yes		No
Interface requirement	Specific liner	Yes	No
Extractant material	PDMS/DVB fiber		Undecanol
Cost (€)	398.50 (3 fiber pack)		39.80 (100 g bottle/120.5mL)
Analysis number	150 (~50 per fiber)	180 (~60 per fiber)	~2300 (per bottle)
Cost per extraction* (€)	2.66	2.21	0.02

* Only extraction costs are considered, the chromatography part costs are not included.

Finally, when using SPME technique PDMS/DVB fibers were used. The fiber package containing three units had a price of 398.50 € (last check 2015 January), therefore each fiber costs almost 133 €. Each fiber lasts about 50 uses for GC and 60 extractions if HPLC is used; thus, each extraction cost between 2.21 and 2.66 €. Undecanol was used with USAEME technique. The solvent bottle costs 39.80 € (100 g

bottle and considering the density of the liquid, 120.5 mL). If 50 µL is used for each extraction, with a single bottle can be done more than 2000 analysis. In this case, each extraction will cost around 0.02 €. This price is 100 less than the price with SPME. So USAEME technique is quite cheaper than SPME.

7.2 Comparison of analytical characteristics of the SPME and USAEME methods with other published works

Microextraction techniques have been very useful for different pesticides determination. Next tables (7.4, 7.5 and 7.6) show different developed methods with triazoles used in different matrices. Table 7.4 collects the determination of the triazoles in water matrices, table 7.5 lists several methods in different fruit samples and table 7.6 shows other methods for triazole pesticides determination in more complex liquid matrices. The data collected in these tables correspond only to the triazole analytes studied in this work; thus, other analytes included in the papers are not showed in the tables.

As it can be seen, in the last five years different methods based on liquid microextraction for triazoles determination have been developed. In general, analytes are determined by GC and HPLC, using a wide range of detectors: MS, DAD, UV, FID, ECD, NPD. These detectors have different sensitivities; and consequently, analytical characteristics will depend on this fact. It seems that HPLC have been used more often than GC in water samples.

The detection limit is calculated using different approaches. In this work the calibration line and three as the signal/noise (S/N) ratio have been used. Most of the other researches use also S/N=3, but in many cases is not specified. When HPLC methods are considered, three researches obtained better detection limits than the present work (see table 7.1 and 7.4) [Tang *et al.*, 2010; Wang *et al.*, 2011a; Wang *et al.*, 2012]. These values were ranging from 0.005 to 0.1 µg L^{-1}. Using GC, best results were obtained with DLLME-GC/NPD method [Farajzadeh *et al.*, 2014]. Apart from the mentioned works, other studies obtained similar or even worse values comparing to values showed in table 7.1.

The linear ranges depend on the detector and also on the microextraction technique used. Usually the linear ranges of liquid microextraction techniques are wider, because it depends on the extractant material volume. The volumes of SPME fibers are very limited, but the microlitre volumes used in LPME allows extending the range.

In most of the cases the reproducibilities (expressed as RSD%) were below 10%. In general, the values were higher in SPME than in different liquid microextraction methods. For instance, the RSD values were between 4 and 20% when using DI-SPME-GC/ToF/MS [Souza-Silva *et al.*, 2013]. The RSD values obtained with the microextraction techniques in this work are in the same range as other works.

The recovery studies depend on the matrix type. As it can be seen in Table 7.6, complex matrices results in worse recovery values, such as the studies in cow milk with low values such as 45% [Farajzadeh *et al.*, 2011b]. If the latter work is not taken into account, recoveries are from 70% to 120% [Souza-Silva *et al.*, 2013; Celeiro *et al.*, 2014]. These recoveries are obtained in fruit matrices; but if only water samples are considered, the recovery values are ranging from 74% to 110% [Farajzadeh *et al.*, 2012; Wang *et al.*, 2011a]. The values obtained in the developed methods in this work (see table 7.1) were between 82% and 123.4% in different fruit matrices. These values are comparable with the recoveries from showed references.

Also, the enrichment factor has been compared for the liquid microextraction procedures. The values obtained in this research with the USAEME method were ranging from 226 to 255. The methods based on DLLME (SBSE-DLLME, SEV-DLLME, AALLME), show values slightly higher, but it can be comparables with the present work. Nevertheless, there are surprising values obtained using a DLLME-GC/NPD method, ranging between 1943 and 1988 [Farajzadeh *et al.*, 2014].

As a whole, the data obtained with the methods developed in this thesis are comparable to the results obtained with other liquid microextraction works. Almost all analytical characteristics are in the same order. Detection limits have some exceptions; but generally the values in other works are quite similar with the values obtained in this work. RSD values are similar in all cases, taking into account that there are little differences between SPME and different liquid microextraction techniques that slightly improves the values. As mentioned before recoveries depend on the matrix

complexity. Simpler matrices as water give better results because there are less interferences than in other complex matrices.

In general, the results obtained with SPME and USAEME can be considered similar, but USAEME offers more advantages in front of SPME. Firstly, is easier to handle and secondly, its low cost make the technique more attractive comparing with SPME.

Table 7.4. *Analytical characteristics of the triazole determination with other techniques in water samples.*

Method	Matrix	Analytes	Linearity ($\mu g\ L^{-1}$)	LOD[a] ($\mu g\ L^{-1}$)	RSD[b] (%)	EF[c]	Recovery (%)	Reference
IL-DLLME-HPLC/DAD	Water	TB	122-6830	3.9*	2.5	-	93	[Ravelo-Pérez *et al.*, 2009]
CPE-HPLC/UV	Water	D-TB	0.05-20	0.02-0.03*	4.2-5.3	> 60	93-95	[Tang *et al.*, 2010]
DLLME-SFO-HPLC/DAD	Water	M-TB	0.5-200	0.08-0.1*	4.3-5.7	190-289	86-110	[Wang *et al.*, 2011a]
DLLME-HPLC/UV	Water	TB	2.0-100	1.2	2.8-5.3	82.8	90.6-105.3	[Ye *et al.*, 2012]
GBMNE-HPLC/UV	Water	M-TB	0.05-50	0.005-0.01	4.2-6.6	-	86.0-100.8	[Wang *et al.*, 2012]
DLLME-GC/FID/MS	Water	D-TB	2-5000	0.5-2.0	2-12	306-380	74-99	[Farajzadeh *et al.*, 2012]
DLLME-HPLC/DAD	Water	TB	30-1500	19.8-25.4*	0.9-6.5	-	87.8-103.7	[Luo *et al.*, 2013]
ALLME-GC/FID	Water	D-TB	2-3500	0.4-0.7	3-4	456-504	93-105	[Farajzadeh *et al.*, 2013]

NOTE: The explanation of the method abbreviations is included in the abbreviation list.

[a] LOD: Limit of detection; * Based on S/N= 3.

[b] RSD: Relative standard deviation.

[c] EF: Enrichment factor.

Table 7.5. *Analytical characteristics of the triazole determination with other techniques in fruit samples.*

Method	Matrix	Analytes	Linearity ($\mu g\ L^{-1}$)	LOD[a] ($\mu g\ L^{-1}$)	RSD[b] (%)	EF[c]	Recovery (%)	Reference
SBSE-DLLME-GC/FID	Water Juices	D-TB	10-50000	1.3-1.6*	-	690-710	72-108	[Farajzadeh *et al.*, 2010]
SEV-DLLME-GC/FID	Water Juices	D-TB	0.5-2000	0.14*	3.4	482 (TB)	86-103	[Farajzadeh *et al.*, 2011a]
HF-LPME-GC/MS	Water Grape juice	D	1-5000	0.4	9.0	173.9	83.1-118.9	[Sarafraz-Yazdi *et al.*, 2012]
DI-SPME-GC/ToF/MS	Grape Strawberries	M-TB-FS-D	0.25-1000	0.025-5**	4-20	-	70-120	[Souza-Silva *et al.*, 2013]
AALLME-GC/FID	Water, cucumber, tomato, grape juice	D-TB	2-750	0.56-0.59*	3-6	713-782	100-109	[Farajzadeh and Khoshmaram, 2013]
UAE + PLE-GC/TQ/MS	Grape	TB	2-1000	0.44-22.8*	5.8-9.6	-	91-120	[Celeiro *et al.*, 2014]
USAEMME-GC/FID	Pear, apple and grape juices	M-TB	5-500	1.77-2.26*	3.9-7.73	183-209	91.6-105	[Li *et al.*, 2014]

NOTE: The explanation of the method abbreviations is included in the abbreviation list.

[a] LOD: Limit of detection; * Based on S/N= 3,** LOQ: Limit of quantification.

[b] RSD: Relative standard deviation.

[c] EF: Enrichment factor.

Table 7.6. *Analytical characteristics of the triazole determination with other techniques in complex liquid samples.*

Method	Matrix	Analytes	Linearity ($\mu g\ L^{-1}$)	LOD[a] ($\mu g\ L^{-1}$)	RSD[b] (%)	EF[c]	Recovery (%)	Reference
IL-DLLME-HPLC/DAD	Wine	D	50-2000	9.7*	5.2-9.2	-	74.6-83.5	[Wang *et al.*, 2011b]
DLLME-GC-FID/MS	Cow milk	TB	50-80000	11*	3.6-6.0	156-380	45-96	[Farajzadeh *et al.*, 2011b]
IL-DLLME-HPLC/DAD	Rat blood	M-TB-D	6-500	4-6**	3.0-6.5	178-197	88.9-98.5	[Li *et al.*, 2013]
DLLME-GC/NPD	Honey	D-TB	0.1-45	0.03-0.045*	4-6	1943-1988	97-99	[Farajzadeh *et al.*, 2014]

NOTE: The explanation of the method abbreviations is included in the abbreviation list.

[a] LOD: Limit of detection; * Based on S/N= 3,** LOQ: Limit of quantification.

[b] RSD: Relative standard deviation.

[c] EF: Enrichment factor.

7.3 References

Arthur, C.L. and Pawliszyn, J.; *Solid Phase Microextraction with Thermal Desorption Using Fused Silica Optical Fibers.* **1990.** Analytical Chemistry, 62, 2145-2148.

Bordagaray, A., García-Arrona, R. and Millán, E.; *Optimization of Solid-Phase Microextraction Procedure Coupled to GC-ECD for Triazole Fungicides Determination in Juice Samples.* **2011.** Food Analytical Methods, 4(3), 293-299.

Bordagaray, A., García-Arrona, R. and Millán, E.; *Development and application of a screening method for triazole fungicide determination in liquid and fruit samples using solid-phase microextraction and HPLC-DAD.* **2013.** Analytical Methods, 5(10), 2565-2571.

Bordagaray, A., García-Arrona, R. and Millán, E.; *Determination of Triazole Fungicides in Liquid Samples Using Ultrasound-Assisted Emulsification Microextraction with Solidification of Floating Organic Droplet Followed by Hihg-Performance Liquid Chromatography.* **2014.** Food Analytical Methods, 7(6), 1195-1203.

Celeiro, M., Llompart, M., Lamas, J.P., Lores, M., Garcia-Jares, C. and Dagnac, T.; *Determination of fungicides in white grape bagasse by pressurized liquid extraction and gas chromatography tandem mass spectrometry.* **2014.** Journal of Chromatography A, 1343, 18-25.

Farajzadeh, M.A., Bahram, M., Jafary, F. and Bamorowat, M.; *Combination of Extraction by Silylated Vessel-Dispersive Liquid–Liquid Microextraction as a High-Enrichment Factor Technique: Optimization and Application in Preconcentration of Some Triazole Pesticides from Aqueous Samples Followed by GC-FID Determination.* **2011a.** Chromatographia, 73(3), 393-401.

Farajzadeh, M.A., Djozan, D. and Khorram, P.; *Development of a new dispersive liquid–liquid microextraction method in a narrow-bore tube for preconcentration of triazole pesticides from aqueous samples.* **2012.** Analytica Chimica Acta, 713, 70-78.

Farajzadeh, M.A., Djozan, D., Mogaddam, M.R.A. and Bamorowat, M.; *Extraction and preconcentration technique for triazole pesticides from cow milk using dispersive liquid-liquid microextraction followed by GC-FID and GC-MS determinations.* **2011b.** Journal of Separation Science, 34(11), 1309-1316.

Farajzadeh, M.A., Djozan, D., Nouri, N., Bamorowat, M. and Shalamzari, M.S.; *Coupling stir bar sorptive extraction-dispersive liquid liquid microextraction for preconcentration of triazole pesticides from aqueous samples followed by GC-FID and GC-MS determinations.* **2010.** Journal of Separation Science, 33(12), 1816-1828.

Farajzadeh, M.A. and Khoshmaram, L.; *Air-assisted liquid–liquid microextraction-gas chromatography-flame ionisation detection: A fast and simple method for the assessment of triazole pesticides residues in surface water, cucumber, tomato and grape juices samples.* **2013.** Food Chemistry, 141(3), 1881-1887.

Farajzadeh, M.A., Mogaddam, M.R.A. and Aghdam, A.A.; *Comparison of air-agitated liquid–liquid microextraction technique and conventional dispersive liquid–liquid micro-extraction for determination of triazole pesticides in aqueous samples by gas chromatography with flame ionization detection.* **2013.** Journal of Chromatography A, 1300, 70-78.

Farajzadeh, M.A., Mogaddam, M.R.A. and Ghorbanpour, H.; *Development of a new microextraction method based on elevated temperature dispersive liquid–liquid microextraction for determination of triazole pesticides residues in honey by gas chromatography-nitrogen phosphorus detection.* **2014.** Journal of Chromatography A, 1347, 8-16.

Jeannot, M.A. and Cantwell, F.F.; *Solvent Microextraction into a Single Drop.* **1996.** Analytical Chemistry, 68(13), 2236-2240.

Lambropoulou, D.A. and Albanis, T.A.; *Methods of sample preparation for determination of pesticide residues in food matrices by chromatography–mass spectrometry-based techniques: a review.* **2007.** Analytical and Bioanalytical Chemistry, 389(6), 1663-1683.

Lee, J. and Lee, H.K.; *Fully Automated Dynamic In-Syringe Liquid-Phase Microextraction and On-Column Derivatization of Carbamate Pesticides with Gas Chromatography/Mass Spectrometric Analysis.* **2011.** Analytical Chemistry, 83, 6856-6861.

Li, Y., Yang, X., Zhang, J., Li, M., Zhao, X., Yuan, K., Li, X., Lu, R., Zhou, W. and Gao, H.; *Ultrasound-assisted emulsification magnetic microextraction: a fast and green method for the determination of triazole fungicides in fruit juice.* **2014.** Analytical Methods, 6, 8328-8336.

Li, Y., Zhang, J., Peng, B., Li, S., Gao, H. and Zhou, W.; *Determination of triazole pesticides in rat blood by the combination of ultrasound-enhanced temperature-controlled ionic liquid dispersive liquid-liquid microextraction coupled to high-performance liquid chromatography.* **2013.** Analytical Methods, 5(9), 2241-2248.

Luo, M., Liu, D., Zhou, Z. and Wang, P.; *A New Chiral Residue Analysis Method for Triazole Fungicides in Water Using Dispersive Liquid-Liquid Microextraction (DLLME).* **2013.** Chirality, 25(9), 567-574.

Pinto, M.I., Sontag, G., Bernardino, R.J. and Noronha, J.P.; *Pesticides in water and the performance of the liquid-phase microextraction based techniques. A review.* **2010.** Microchemical Journal, 96(2), 225-237.

Psillakis, E. and Kalogerakis, N.; *Developments in liquid-phase microextraction.* **2003.** Trends in Analytical Chemistry, 22(9), 565-574.

Ravelo-Pérez, L.M., Hernández-Borges, J., Asensio-Ramos, M. and Rodríguez-Delgado, M.A.; *Ionic liquid based dispersive liquid–liquid microextraction for the extraction of pesticides from bananas.* **2009.** Journal of Chromatography A, 1216(43), 7336-7345.

Sarafraz-Yazdi, A. and Amiri, A.; *Liquid-phase microextraction.* **2010.** Trends in Analytical Chemistry, 29(1), 1-14.

Sarafraz-Yazdi, A., Assadi, H. and Ibrahim, W.A.W.; *Determination of Triazole Fungicides Using Hollow Fiber Liquid Phase Microextraction Prior to Gas Chromatography–Mass Spectrometry Analysis.* **2012.** Industrial & Engineering Chemistry Research, 51(7), 3101-3107.

Souza-Silva, E.A., Lopez-Avila, V. and Pawliszyn, J.; *Fast and robust direct immersion solid phase microextraction coupled with gas chromatography–time-of-flight mass spectrometry method employing a matrix compatible fiber for determination of triazole fungicides in fruits.* **2013.** Journal of Chromatography A, 1313, 139-146.

Tang, T., Qian, K., Shi, T., Wang, F., Li, J. and Cao, Y.; *Determination of triazole fungicides in environmental water samples by high performance liquid chromatography with cloud point extraction using polyethylene glycol 600 monooleate.* **2010.** Analytica Chimica Acta, 680(1-2), 26-31.

Wang, C., Wu, Q., Wu, C. and Wang, Z.; *Application of dispersion–solidification liquid–liquid microextraction for the determination of triazole fungicides in environmental water samples by high-performance liquid chromatography.* **2011a.** Journal of Hazardous Materials, 185(1), 71-76.

Wang, S., Ren, L., Xu, Y. and Liu, F.; *Application of ultrasound-assisted ionic liquid dispersive liquid-phase microextraction followed high-performance liquid chromatography for the determination of fungicides in red wine.* **2011b.** Michrochimica Acta, 173, 453-457.

Wang, W., Ma, X., Wu, Q., Wang, C., Zang, X. and Wang, Z.; *The use of graphene-based magnetic nanoparticles as adsorbent for the extraction of triazole fungicides from environmental water.* **2012.** Journal of Separation Science, 35(17), 2266-2272.

Ye, C., Liu, Q., Wang, Z. and Fan, J.; *Dispersive liquid-liquid microextraction combined with high-performance liquid chromatography-ultraviolet detection for the determination of three triazole derivatives in environmental water samples.* **2012.** International Journal of Environmental Analytical Chemistry, 92(10), 1176-1186.

8. Conclusions

8. Conclusions

Taking into account the planned objectives and the results obtained in this thesis, it can be concluded:

1) <u>Development of microextraction procedures for triazole determination</u>

The procedures were based on solid phase microextraction and liquid phase microextraction. These techniques allow preconcentration and isolation of analytes from different matrices. Direct immersion mode was chosen for SPME and the use of ultrasonication was selected among other LPME techniques due its potential to disperse the organic droplet and hence, to increase the mass transfer rate.

In SPME-GC/ECD procedure (CHAPTER 3) satisfactory results were obtained for three of the four analyzed analytes (tetraconazole, myclobutanil and diniconazole). Tebuconazole was not very sensitive with the method. Nevertheless, the procedure was considered simple with barely sample treatment. Also it was supported by green chemistry procedures due to absence of organic solvents.

In order to overcome the problems related with voluminous analytes, the work was continued with SPME-HPLC/DAD procedure (CHAPTER 4). Analytes were not very volatiles and hence, HPLC/DAD determination was considered more appropriate than GC/ECD. Two more analytes were included in this part of the work (flusilazole and fluquinconazole). Regarding analytical characteristics, good results were obtained for all the analytes, although no complete resolution of peaks was achieved.

But, some of the disadvantages of SPME were remarkable, such as the cost and the fiber fragility, especially if direct immersion mode was used. Trying to avoid these lacks, other procedures such as liquid phase microextraction techniques were considered; specifically, microextraction assisted by

ultrasound. USAEME provided fast extractions due to the increase in contact surface that raised the transfer rate.

USAEME-SFO-HPLC/DAD procedure (CHAPTER 5) was developed. The obtained results were good for all the analytes, especially data regarding reproducibilities. Each analysis started with a new extractant material and therefore carry-over effects or problems like fiber aging were not present in the procedure. Besides, the handling with USAEME technique was much easier and less demanding than with SPME.

2) <u>Wide use of GC and HPLC chromatographic techniques</u>

Gas Chromatography (GC) and High Resolution Liquid Chromatography (HPLC) are common techniques used for separation and determination of analytes. In this work with HPLC some different parameters that affect separation, such as, composition of eluent and flow rate, solvent gradients, etc. were studied. Best separation conditions were obtained taking into account the studied factors. The use of SPME can be restricted by the utilization of some organic solvents.

Electron Capture Detection (ECD) with GC and Diode Array Detection (DAD) with HPLC have been used. ECD gave satisfactory results for triazole pesticides, because its halogen atoms. DAD provides three-dimensional data; therefore, more than one wavelength can be chosen for quantification, UV spectra can be obtained for identification and second order advantage can be useful for further chemometrics studies when there are overlapping problems, as it can be seen in the CHAPTER 6.

Those instruments with the mentioned detectors have a reasonable cost affordable for common laboratories. Therefore, their use allows many different applications in routine analysis as first step. Later, more sensible and confirmatory techniques such as GC/MS or LC/MS could be used.

3) Improvements with the use of chemometric methods

- **Experimental design**

There are many experimental variables affecting the microextraction processes. In this context, the selection of the significant variables and the attainment of its optimum values are very important tasks. The use of experimental designs allows obtaining both the significant variables and the best experimental conditions. Fractional factorial designs for screening (in CHAPTERS 3 and 4) and central composite designs for optimization (in CHAPTERS 3, 4 and 5) were used. Moreover, the global response for the selected variables was studied using desirability surface responses.

In the SPME procedures, after applying the screening designs, the qualitative variables and more favorable levels for non significant variables were fixed. The extraction time and temperature variables were considered for optimization. In the case of SPME-HPLC/DAD method also salt addition was included in the design. After application of global desirability response in both methods, the extraction temperature at 60^{o}C and long extraction times (45 min in SPME-GC/ECD and 90 min in SPME-HPLC/DAD) were chosen. When NaCl addition was taken into account, the selected value (180 g L^{-1}) was near to the optimum. This was because excessive salt amount can result in fiber coating damage.

In the USAEME-SFO-HPLC/DAD procedure the extraction time and temperature and NaCl addition variables were considered in the optimization design. After the evaluation of response surface for global desirability, the best experimental conditions were 30^{o}C, 18 min and 250 g L^{-1} of NaCl.

- **Multivariate analysis**

This method approaches may help to solve overlapping problems in chromatography when partial resolution between peaks is obtained. That was the case in SPME-HPLC/DAD procedure where tetraconazole, myclobutanil, flusilazole, fluquinconazole and tebuconazole analytes were not well resolved. PARAFAC2, MCR and PARALIND algorithms have been used and the results

compared (CHAPTER 6). PARAFAC2 and MCR, which are two of the most used techniques for second order data, did not give good results. However, PARALIND takes into consideration rank-deficiency problems and can solve the overlapping peaks problem as it has been shown in the presented case. Models with PARALIND gave good quantitative results, while PARAFAC2 and MCR failed especially with myclobutanil and tebuconazole analytes, which showed very similar spectra.

4) Applicability of the procedures in fruit and liquid samples

The developed methods were applied to apple and grape samples. In SPME-GC/ECD (CHAPTER 3) fruit juices have been used and some triazole pesticide residues have been found. SPME-HPLC/DAD (CHAPTER 4) method was used in fruit and liquid sample analysis. Previously, apple and grape trees were treated by commercial products which contain pesticides and samples were collected within a period of two weeks. Samples were separated into peel, pulp and juice. Also rain samples were taken. The study showed that those products remained as superficial treatment, since the residues were only found in peel and rain samples. USAEME-SFO-HPLC/DAD (CHAPTER 5) was used for some recovery studies performed in commercial juices. USAEME procedure showed its adequacy when analyzing fruit juices, but the method did not work properly with alcoholic beverages.

Summarizing, microextraction techniques showed a high potential for determination of triazole pesticides in fruit and liquid matrices. Some tools, such as, chemometrics, help to obtain the best results when using these techniques. Among different microextraction techniques, regarding difficulties and costs, USAEME provides more practical advantages, although it might not be suitable enough for some complex matrices.

PUBLICATIONS

RESEARCH ARTICLES

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Optimization of solid-phase microextraction procedure coupled to GC-ECD for triazole fungicides determination in juice samples.* **2011**. Food Analytical Methods, 4,293–299.

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Development and application of a screening method for triazole fungicide determination in liquid and fruit samples using solid-phase microextraction and HPLC-DAD.* **2013**. Analytical Methods, 5, 2565-2571.

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Determination of triazole fungicides in liquid samples using ultrasound-assisted emulsification microextraction with solidification of floating organic droplet followed by high-performance liquid chromatography.* **2014**. Food Analytical Methods, 7,1195–1203.

Bordagaray, Ane, Amigo, José Manuel, *Modelling highly co-eluted peaks of analytes with high spectral similarity*. **(Accepted in TrAC, Trends in Analytical Chemistry)**

CONTRIBUTIONS TO CONFERENCES

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Experimental design application for triazole fungicides determination in food liquid samples by solid-phase microextraction and GC/ECD*. VII Colloquium Chemiometricum Mediterraneum, Granada (Spain), **21-24 June 2010.**

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Triazole fungicides determination using Solid- Phase Microextraction (SPME)*. Cross Border Doctorials, Anglet (France), **17-21 October 2011.**

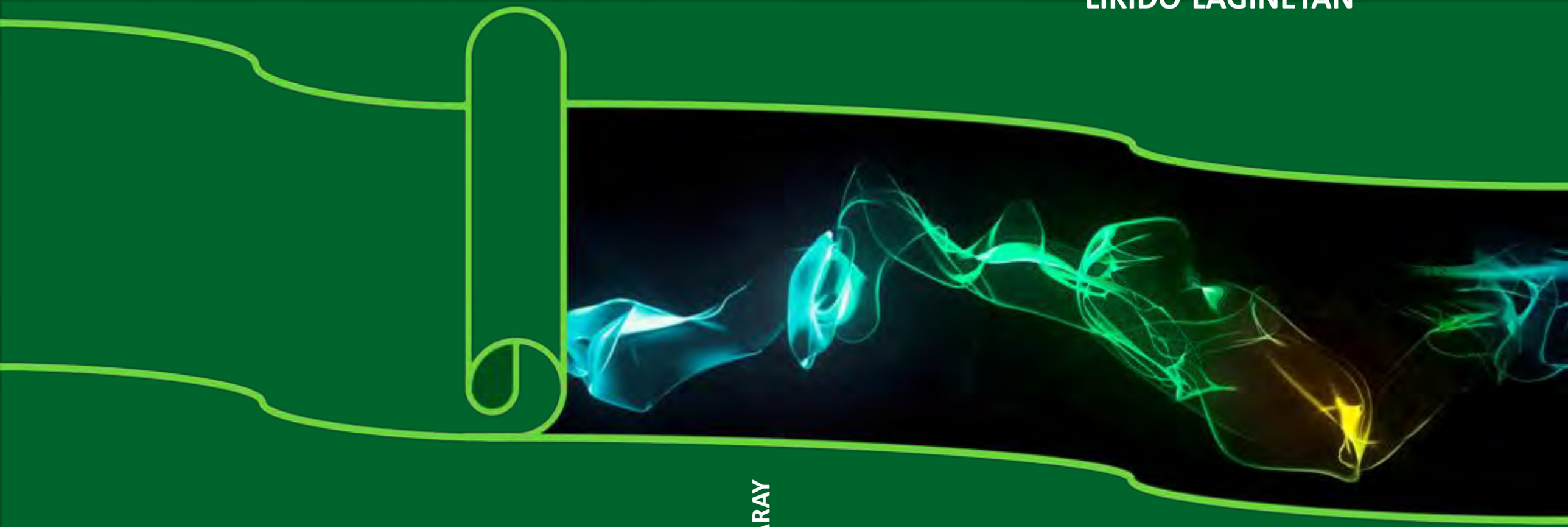
Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda, Ostra, Miren; *Multi-way chemometric methods for the determination of triazole fungicides in environmental samples by SPME-HPLC-DAD*. XIII. Chemometrics in Analytical Chemistry, Budapest (Hungary), **25-29 June 2012.**

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Determination of triazole compounds in liquid samples using ultrasound-assisted liquid phase microextraction with solidification of floating droplet (USALPME-SFO) followed by HPLC-DAD*, XVII. Euroanalysis, Warsaw (Poland), **25-29 August 2013**.

emari ta zabal zazu

Doktorego Tesia

MIKROERAUZKETA TEKNIKA ETA BANAKETA KROMATOGRAFIKOAN OINARRITUTAKO PROZEDUREN GARAPENA TRIAZOL FUNGIZIDEN DETERMINAZIORAKO FRUITU ETA LIKIDO LAGINETAN



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DOKTOREGO TESIA

Mikroerauzketa teknika eta banaketa kromatografikoan oinarritutako prozeduren garapena triazol fungiziden determinaziorako fruitu eta likido laginetan

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Donostia 2015

Laburpena

Laburpena

Onddo, intsektu, larba eta beste organismo ezberdinak sortutako gaixotasunek, uztak kaltetu ditzakete ekoizpen tasa murriztuz. Efektu negatibo hauek nekazaritzan, zenbait produktu eta pestizida erabiliz kontrola daitezke. Besteak beste, konposatu triazolak produktu eraginkorrak dira onddo batzuen aurka egiteko, esaterako *Oidium*-a. Halere, fungizida hauen arrastoak kontsumitzailearen osasunean naiz nekazaritza-produktu deribatuetan eragin dezaketela ikusi da. Horregatik, Europar Batasunak gizartearen segurtasuna bermatzeko, hainbat erregulazio argitaratu ditu, pestizida arrastoen maila maximoak finkatzeko.

Beraz, garrantzitsua da konposatu mota hauek determinatzeko metodoak garatzea. Kasu gehienetan, gas eta likido kromatografia (GC, LC) banaketak erabili dira laginen analisirako, baina horren aurretik beharrezkoa da laginen tratamendu egokia egitea, analitoak isolatu eta prekontzentratzeko. Helburu hori betetzeko, mikroerauzketa teknikak erreminta egokiak dira, izan ere, prekontzentrazio eta erauzketa etapak pausu bakar batean egiteko gai dira, horrekin errore-iturri kopurua murriztuz.

Hori horrela izanda, azken urteetan bi teknika azpimarragarri erabili dira: solido faseko mikroerauzketa (SPME) eta likido faseko mikroerauzketa (LPME) bere aukera ezberdinekin. Bi teknikek disolbatzaile toxiko eta kutsagarrien erabilera gutxitu edo saihestu dezakete eta teknika kromatografikoei akoplatuz, emaitza kuantitatibo onak lor daitezke hainbat matrize eta konposatuentzat.

Lan honetan SPME eta ultrasoinuz lagundutako emultsifikazio mikroerauzketa (USAEME) metodo sinple eta fidagarriak garatu dira, GC eta LC determinazioen laguntzaz. Proposatutako metodoek μg L^{-1} mailan triazol konposatuak determinatzeko gaitasun ona erakutsi dute fruitu lagin mota ezberdinetan.

Gainera, tresna kimiometrikoak erabili dira metodoen arrakasta ziurtatzeko. Hasteko, erauzketetan eragiten duten aldagai ezberdinen aukeraketarako eta optimizaziorako eta baita kromatografian ageri daitezkeen gainezarpen arazoak konpondu ahal izateko. Diseinu faktorial zatikatuak erabili dira aldagai esperimentalen miaketarako eta diseinu konposatu zentralak erabili dira aldagaien balio optimoak

aurkitzeko. Lortutako datu motei egokitutako algoritmoak hautatu dira gainezarpen arazoei aurre egiteko, beraz*, Parallel Factor Analysis 2* (PARAFAC2), *Multivariate Curve Resolution* (MCR) eta *Parallel Factor Analysis with Linear Dependencies* (PARALIND) erabili eta elkarrekin konparatu dira.

Euskal Herriko bereziak diren sagardo eta txakolinaren ekoizpena dela eta, mahats eta sagar laginak hautatu dira. Uzta horiek, sarritan triazol konposatuekin tratatzen baitira onddoek sortutako arazoak konpontzeko. Horregatik, mahats eta sagarren azal eta pulpak aztertu dira, zuku eta beste produktu komertzial batzuez gain, hala nola, muztio eta sagardoa. Lagin mota hauekin metodoaren egokitasuna ikusi da fruitu eta lagin mota hauetan.

AURKIBIDEA

Laburduren zerrenda

Laburdura	Definizioa
AALLME	Airez lagundutako likido likido mikroerauzketa
ANOVA	Bariantzaren analisia
AOAC	Kimiko analitikoen elkarte ofiziala
CCD	Diseinu konposatu zentrala
CPE	Laino puntuko erauzketa
D	Dinikonazol
DI	Murgiltze zuzena
DLLME	Likido likido mikroerauzketa dispertsiboa
DSDME	Zuzenean ezarritako tantaren mikroerauzketa
ECD	Elektroi harrapaketa detektagailua
FS	Flusilazol
FQ	Flukinkonazol
GBMNE	Grafenoan oinarritutako nanopartikula magnetiko bidezko erauzketa
GC	Gas kromatografia
GC/ECD	Gas kromatografia- Elektroi harrapaketa detektagailua
GC/FID	Gas kromatografia- sugar ionizazio detektagailua
GC/FID/MS	Gas kromatografia- sugar ionizazio detektagailua/ masa espektrometria
GC/MS	Gas kromatografia- masa espektrometria
GC/MS/MS	Gas kromatografia- masa espektrometria tandema
GC/NPD	Gas kromatografia- nitrogeno fosforo detektagailua
HF-LPME	Zuntz hutseko likido faseko mikroerauzketa
HPLC	Bereizmen handiko likido kromatografia
HPLC/DAD	Bereizmen handiko likido kromatografia- lerrokatutako diodoen detektagailua
HPLC/MS	Bereizmen handiko likido kromatografia- masa espektrometria
HPLC/MS/MS	Bereizmen handiko likido kromatografia- masa espektrometria tandema
HPLC/UV	Bereizmen handiko likido kromatografia- ultramore detektagailua
HS	Buru-gunea
IL-DLLME	Likido ioniko bidezko likido likido mikroerauzketa dispertsiboa
M	Miklobutanil
MCR	Aldagai anitzeko kurben ebazpena
MRL	Gehienezko hondakin muga
MSPE	Solido faseko erauzketa magnetikoa
PA	Poliakrilatoa
PARAFAC	Faktore paraleloen analisia
PARALIND	Menpekotasun linealdun faktore paraleloen analisia
PCA	Osagai nagusien analisia

Laburdura	Definizioa
PDMS/DVB	Polidimetilsiloxano/ dibinilbentzenoa
PLE	Presurizatutako likido erauzketa
RSM	Erantzun azaleren metodologia
SBSE-DLLME	Barratxo nahasgailuaren sortzio erauzketa- likido likido mikroerauzketa dispertsiboa
SDME	Tanta bakarraren mikroerauzketa
SFO	Tanta organiko flotatzailearen solidifikazioa
SPE	Solido faseko erauzketa
SPME	Fase solidoko mikroerauzketa
SEV-DLLME	Sililatutako erauzketa ontziko likido likido mikroerauzketa dispertsiboa
QuEChERS	Azkarra, erraza, merkea, eraginkorra, iraunkorra eta segurua
TB	Tebukonazol
ToF	Hegaldi-denbora detektagailua
TT	Tetrakonazol
UAE	Ultrasoinuz lagundutako erauzketa
USAEME	Ultrasoinuz lagundutako emultsifikazio mikroerauzketa
USAEMME	Ultrasoinuz lagundutako emultsifikazio mikroerauzketa magnetikoa
UPLC-MS/MS	Ultra bereizmeneko likido kromatografia- masa espektrometria tandema

1. Sarrera

1. Sarrera

Frutak ezinbesteko elikagaiak dira gizakien dietan bitamina eta mineralen iturburu bezala. Gizartearen betebeharrak asetzeko eta gero eta uzta hobeagoak lortzeko ohikoa da laborantzetan pestizida ezberdinen erabilera, izan ere, landareen ekoizpena handiagotu baitezakete kalteak eragiten dituzten eragile ezberdinak kontrolatuz. Ikerketa ezberdinek erakusten dute pestizidak eraginkorrak direla landareen gaixotasunak murrizteko [Scherm *et al.*, 2009] eta baita ere landareen zahartzea eragiten duten prozesu oxidatiboak atzeratzen ere, lurren emaria handiagotuz [Zhang *et al.*, 2010]. Fruituak, kontsumitzailearengana modu ezberdinetan iristen dira, osorik ala produktu prozesatu moduan, hots, ardo edo edari bezala. Beraz, beharrezkoa da uztak seguruak direla ziurtatzea, pestizida arrasto edo bere metabolitoek osasunean arazoak ekar baititzake. Horregatik, pestizida arrastoak fruituetan daudela ziurtatzeko, beharrezkoa da konposatu kaltegarri horien presentzia determinatuko duen metodo bat garatzea.

Eragiteko moduagatik bereizten diren pestizida mota ezberdinak daude: adibidez, intsektizidak, landareak intsektuengandik babestuko dituzte; fungizidak, onddoen gaixotasunak kontrolatuko dituzte, eta bakterizidak, akarizidak eta algazidak beste mikroorganismo espezifikoengandik babestuko dute uzta. Horiez gain, landareen hezkuntza ere bultzatzen duten beste hainbat produktu ere erabil daitezke [Tomlin, 2000].

1.1 Triazol pestizidak

Triazol konposatuak laborantzan onddoetatik babesteko erabiltzen diren pestizidak dira. Fungizida hauen presentziak bi eragin kaltegarri eragin ditzakete, alde batetik osasun arazoak (batez ere sagu eta arratoietan ikertu dira beren eraginak) eta bestetik, fruituetatik eratorritako edarien prozesuetan eragin ditzaketen kalteak.

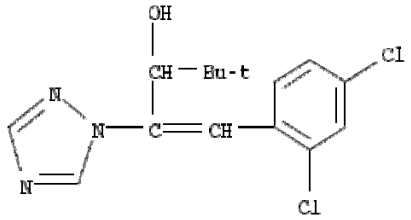
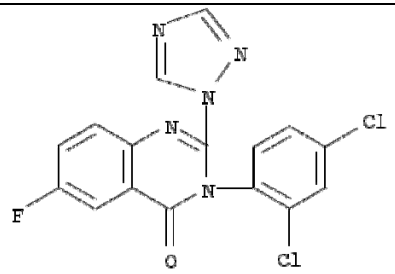
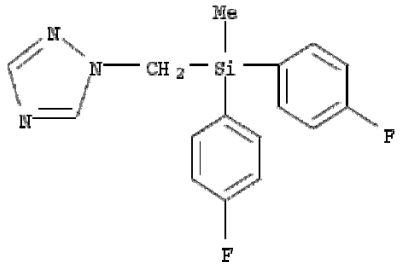
Azol taldea duten konposatu hauek zelula mintzen oinarrizko konposatua den ergosterolaren inhibizioaren bidez eragiten dute, P450 zitokromo entzimaren

aktibitatea mugatuz [Ekman *et al.*, 2006]. Ikerketa asko burutu dira pestizida hauek eragin ditzaketen efektuekin, besteak beste efektu tumoregenikoak [Wolf *et al.*, 2006], endokrino sistemaren gelditzeak [Goetz *et al.*, 2007] eta ugaltze sistemaren aldakuntzak [Taxvig *et al.*, 2007] ikusi dira arratoietan, eta baita ere gibel tumoreak saguetan ere [Allen *et al.*, 2006]. Gainera, konposatu hauek euriaren eraginez beste inguruneetara iristen direnean, hots, uzten ondoko ur inguruneetan kalteak eragin ditzakete *Daphnien* (krustazeo txikiak) heriotza tasa handiagotuz, inguruko landareen hazkuntza mugatuz eta bertako arrainetan estresa sortuz eragin antiklogeniko eta oxidatiboen bidez [Echeverría-Sáenz *et al.*, 2012].

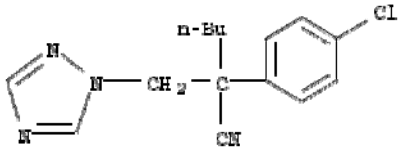
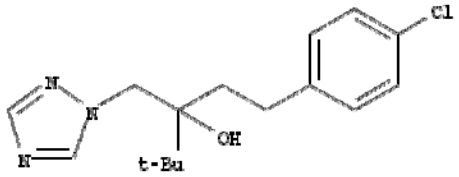
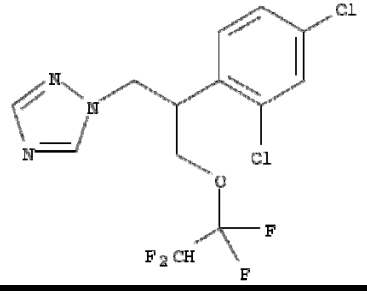
Pestizida hauek oso erabiliak dira edariak egiteko bideratutatko sagasti eta mahastietan. Garagardoa egiteko prozesuetan ikusi da hartziduraren moteltzea eta kasu batzuetan guztizko gelditzea ere eragin dezaketela konposatu hauek [Navarro *et al.*, 2011]; bereziki oktanol/ur partizio koefizientea (K_{OW}) 2 baino handiagoa den kasuetan, konposatuak maltan geratu baitaitezke. Ardoetan eginiko beste ikerketa batzuetan, tebukonazolak lurrinean eragin dezakeela ikusi da [Noguerol-Pato *et al.*, 2011].

Lan honetan ikertu diren analitoak aipatutako K_{OW} muga gainditzen dute 1.1 taula ikus daitekeen moduan. Taula honek tesi honetan determinatutako analitoen propietate fisiko-kimikoak eta egitura molekularra erakusten ditu. Aztertutako triazolak dinikonazola (D), flukinkonazola (FQ), flusilazola (FS), miklobutanila (M), tebukonazola (TB) eta tetrakonazola (TT) izan ziren. Pestizidak oro har, fruituetan mantentzen dira denbora batez, horregatik, pestizidak dituzten produktuek jaso aurretiko denbora mugak ezarrita dituzte, kasu batzuetan hilabeteraino iris daitezkeenak. Gainera, konposatu hauek lurrean absorbatuak gera daitezke, prezipitazioak eramateko gai ez badira. Halere, lurreko geruza ezberdinetan eginiko azterketek pestizidak geruza sakonetara eta lur azpiko uretara ez direla iristen erakutsi dute [Tomlin, 2000].

1.1. Taula. *Ikertutako triazol fungiziden egitura eta propietate fisiko-kimikoak.*

Izena [CAS zenb.]	***Formula / M_w (g mol^{-1})***	***Egitura***	***K_{ow} log P***	***Henry konstantea (Pa m^3 mol^{-1})***	***Disolbagarritasuna uretan (mg L^{-1})***
Dinikonazola (D) [70217-36-6]	*$C_{15}H_{17}Cl_2N_3O$ / 326.2*		*4.3 (25 ^oC)*	*$4.0 \cdot 10^{-2}$*	*4 [95 metanoletan (g kg^{-1})]*
Flukinkonazola (FQ) [72850-64-7]	*$C_{16}H_8Cl_2FN_5O$ / 376.2*		*3.24 (pH 5.6)*	*$2.1 \cdot 10^{-6}$*	*1 [$3 \cdot 10^{-3}$ etanoletan]*
Flusilazola (FS) [85509-19-9]	*$C_{16}H_{15}F_2N_3Si$ / 315.4*		*3.74 (pH 7, 25 ^oC)*	*$2.7 \cdot 10^{-4}$*	*45 (pH 7.8) [$> 2 \cdot 10^{-6}$ disolbatzaile organiko batzuetan]*

1.1. Taula (jarraipena). *Ikertutako triazol fungiziden egitura eta propietate fisiko-kimikoak.*

Izena [CAS zenb.]	***Formula / M_w ($g\ mol^{-1}$)***	***Egitura***	***K_{ow} log P***	***Henry konstantea ($Pa\ m^3\ mol^{-1}$)***	***Disolbagarritasuna uretan ($mg\ L^{-1}$)***
Miklobutanila (M) [88671-89-0]	$C_{15}H_{17}ClN_4$ / 288.8		*2.94 (pH 7-8, 25 ºC)*	$4.3 \cdot 10^{-4}$	*142 [5-10 · 10^{-4} disolbatzaile organiko arruntetan]*
Tebukonazola (TB) [107534-96-3]	$C_{16}H_{22}ClN_3O$ / 307.8		*3.7 (20 ºC)*	$1.0 \cdot 10^{-5}$	*36 (pH 5-9, 20 ºC) [5-10 · 10^{-4} toluenotan]*
Tetrakonazola (TT) [112281-77-3]	$C_{13}H_{11}Cl_2F_4N_3O$ / 372.1		*3.56 (20 ºC)*	$3.6 \cdot 10^{-4}$	*156 (pH 7, 20 ºC) [metanoletan berehalako disolbagarritasuna]*

Pestizida eskuliburutik jasotako datuak [Tomlin, 2000].

Fruitu eta barazkien etengabeko kontsumoagatik eta gizartearen osasuna ziurtatzeko, Europar Batasunak (EU) gehienezko hondakin mugak (MRL) ezartzen ditu hainbat pestizidentzako eta janari mota ezberdinentzako. Lehen erregulazioak 2005ean argitaratu ziren [Regulation EC 396/2005], baina MRL balioak aldatu eta eguneratu egin dira. Erregulazio eguneratuek lan honetako analitoentzako ezarritako balioak 1.2 taulan ageri dira. Fruitu eta analitoen arabera muga hauek 0.01 eta 1 mg kg^{-1} balioen artekoak dira.

1.2. Taula *Europar Batasunak araututakoo MRL balio eguneratuak sagar eta ardo-mahatsetan.*

Analitoa	**Fruitua**	**MRL (mg kg^{-1})**	**Erregulazioa**
Flukinconazola (FQ)	**Sagarra**	0.1	[Regulation EC 149/2008]
	Ardo-mahatsa	0.5	
Miklobutanila (M)	**Sagarra**	0.5	[Regulation EC 149/2008]
	Ardo-mahatsa	1	
Flusilazola (FS)	**Sagarra**	0.02	[Regulation EC 459/2010]
	Ardo-mahatsa	0.2	
Tetrakonazola (TT)	**Sagarra**	0.3	[Regulation EU 34/2013]
	Ardo-mahatsa	0.5	
Dinikonazola (D)	**Sagarra**	0.01	[Regulation EU 1317/2013]
	Ardo-mahatsa	0.01	
Tebukonazola (TB)	**Sagarra**	0.3	[Regulation EU 61/2014]
	Ardo-mahatsa	1	

Horregatik, segurtasun denbora ezartzeko eta EU-k ezarritako mugak betetzeko beharrezkoa da pestizida arrastoak determinatzeko metodoak garatzea

1.2 Erauzketa teknikak

Prozedura analitikoen helburua matrize ezberdinetan aurki daitezkeen informazioa jasotzea da. Prozedura horiek, zenbait pausuren bidez burutzen dira, besteak beste, laginketa, lagin prestaketa, banaketa, kuantifikazioa eta datuen analisia. Laginen prestaketa funtsezkoa da pestizida aztarnak modu eraginkor batean eta zehaztasunez determinatzerako orduan, ez bakarrik aurrekontzentrazio helburuekin, baita matrize ezberdinetatik intereseko konposatuak isolatzeko ere [Zhang *et al.*, 2012b].

Lagin prestaketarako teknika ezberdin asko erabili izan dira. Determinazioak oro har, metodo kromatografikoen bidez egin ohi dira, banaketa eta kuantifikazioa une berean egin daitezkeelako. Baina matrize batzuen konplexutasunak eta ezpurutasunek, analisia oztopa dezakete teknika kromatografikoak bakarrik aplikatuz gero. Gainera, ingurunean aurki daitezkeen aztarna mailak detektatzeko askotan aurrekontzentrazio pausuen beharra dago [Ridgway *et al.*, 2007; Gilbert-López *et al.*, 2009; Zhang *et al.*, 2012b].

Azken urteetan, beste tekniken artean erauzketa tekniken bertsio miniaturizatu ezberdinak garatu dira erauzketa likido-likidoan (LLE) eta solido faseko erauzketan (SPE) oinarrituak. Teknika horiek, analitoek disolbatzaile organikoekin duten disolbagarritasunean eta beraien partizio koefizientean oinarritzen dira eta beraien aurrekontzentrazio ahalmenagatik oso erabiliak izan dira urteetan zehar.

Baliteke LLE izatea erauzketa metodorik ohikoena eta zaharrena [Zhang *et al.*, 2012b], fase emaile eta hartzailearen arteko analito kontzentrazioaren orekan oinarritzen da teknika. Oro har, fase akuoso eta organiko bat izaten dira disolbaezinak diren bi faseak, eta analitoak duen afinitatea bakoitzarekiko ezberdina da. Agitazio modu batez lagunduz, bi faseen arteko kontaktu gainazala handiagotzen da, analitoen transferentzia lagunduz. Erauzketa gertatu ondoren, bi faseak bereizten dira eta dekantazio-inbutu baten bidez banatzen dira. Agitazioaren ondorioz emultsioak sor daitezke kasu batzuetan, baina arazo hori gatza gehituz ala zentrifugazio bidez konpon daiteke [Ridgway *et al.*, 2007]. Teknika eraginkorra bada ere, neketsua eta denbora eta disolbatzaile asko kontsumitzen duen teknika da eta hori ez da gomendagarria kimika berdearen ikuspuntutik.

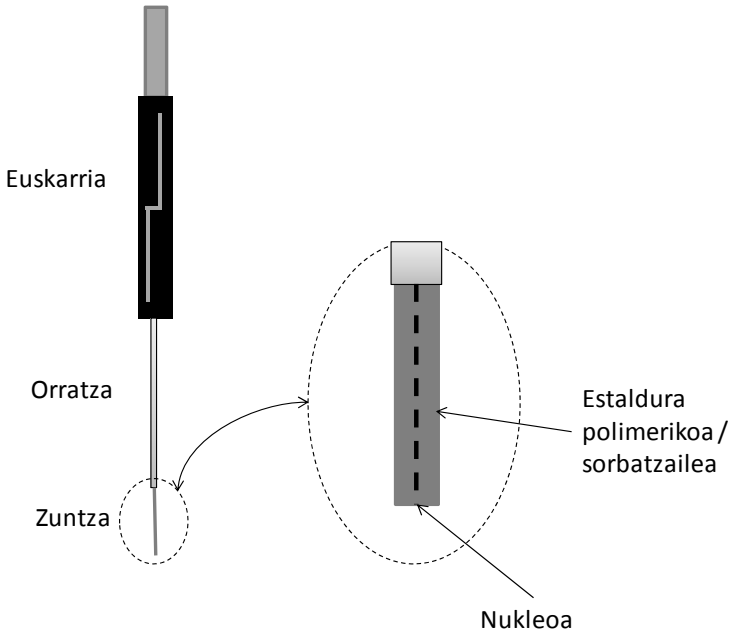
Beste alde batetik, SPE teknika analitoek solido fase batekin eta likido eramaile batekin duten arteko distribuzioan oinarritzen da. Disoluzio bat material erauztailea duen kartutxo batetik zehar pasatzen dira, non analitoak atxikiak geratzen diren. Interferentziak garbiketa disoluzio ezberdinen bidez garbi daitezke, intereseko analitoak kartutxoan bertan geratzen diren bitartean eta azkenik, analitoak eramango dituen beste disoluzio bat pasarazten da solido fasetik zehar atxikitako analitoak askatu eta determinatu daitezen [Zhang *et al.*, 2012b]. Hala eta guztiz ere, SPE-ren eraginkortasuna mugatua izan daiteke kasu batzuetan, analitoak fase solidora nahikoa ez direlako atxikitzen. Horri aurre egiteko SPE sorbatzaile mota ezberdinak garatu dira: alderantzizko-fasea (C8, C18 fase apolarrak), fase-normala (silika, alumina), ioi-trukaketa fasea edo funtzionalizatutako faseak [Buszewski eta Szultka, 2012].

Teknika hauek, ordea, disolbatzaile eta baliabide askoren beharra dute eta horren ondorioz teknika neketsua eta automatizatzeko zaila egiten du. Gainera, kimika berdearen ikuspuntuak disolbatzaile organikoen erabilera minimizatzea bultzatzen du, hondakin toxikoak murrizte aldera, eta LLE eta SPE teknikek ez dute ideia hori laguntzen. Horrez gain, teknika hauek pausu bat baino gehiagokoak direnez, errore iturriak ere handiagotzen dira, etapa ezberdinetan gerta litekeen analito galeraren ondorioz. Arrazoi horiengatik, teknika hauen bertsio miniaturizatuak garatzen hasi ziren. Laurogeita hamargarren hamarkadan hasi ziren fase solidoko mikroerauzketa teknikak garatzen eta geroago agertu ziren likido faseko mikroerauzketa teknikak. Horien ezaugarri nagusia, fase erauztailearen bolumen txikia da, mikrolitro eskalako bolumenekoa.

1.2.1 Fase solidoko mikroerauzketa (SPME)

SPME Pawliszynek aurkeztu zuen 1990ean, aurrekontzentrazio teknika ohiko alternatiba moduan [Arthur eta Pawliszyn, 1990]. Teknika hau, zuntz polimeriko batean oinarritzen da eta laginarekin kontaktuan jarri ondoren analitoak erauzten ditu (ikus 1.1 irudia). Horretarako zuntza gordetzen den orratzak lagina duen ontziaren septa zulatzen du eta ondoren zuntza kanporantz bultzatzen da laginarekin kontaktuan jartzeko. Analitoak zuntzean absorbatu edo adsorbatu ondoren, zuntza berriz orratzean jaso eta desortzioa gertatuko den instrumentuetara eramaten dira. Bertan

disolbatzaile edo tenperatura altuen laguntzaz desorbatzen dira, ondoren teknika kromatografiko ezberdinen bidez banatu eta kuantifikatzeko. Teknika honek erauzketa eta aurrekontzentrazio une berean egiten dituenez, denbora eta errore iturriak murrizten ditu.



1.1. Irudia. *Zuntz eta euskarri komertzial baten eskema.*

Zuntzak hiru modutan jarri daitezke laginarekin kontaktuan [Pawliszyn, 1997]: laginean murgilduz (DI), buru-guneko fasean (HS) edota mintzez babestua. Analitoen lurrunkortasuna eta matrizearen arabera aukeratuko da modurik egokiena. Murgiltze kasuan, zuntza lagin likidoan bertan sartzen da eta buru-gune kasuan, zuntza likidoaren gaineko gunean kokatzen da. Azken hau bereziki lurrunkorrak diren analitoentzat gomendatzen da, laginarekin kontaktu zuzena saihesten denez, zuntzaren bizitza areagotzen baita. Mintzez babestutako modua matrize konplexuentzat gomendatzen da, mintzak interferentziez babesten baitu zuntza.

1.2.1.1 SPME oinarria

Mikroerauzketa prozesua amaitutzat ematen da zuntza eta laginaren arteko oreka iristen denean. Prozesuan bi fasek bakarrik parte hartzen dutenean (murgiltze kasuan bezala) masaren kontserbazio legea 1.1 ekuazioaren bidez adieraz daiteke [Pawliszyn, 1997].

$$C_0 \cdot V_s = C_s^{eq} \cdot V_s + C_f^{eq} \cdot V_f \quad (1.1)$$

C hizkiak kontzentrazioa adierazten du, azpi-indizean *0* dagoenean hasierako kontzentrazioa dela esan nahi du, *s* eta *f* daudenean, aldiz, lagineko eta zuntzeko kontzentrazioak adierazten ditu, hurrenez hurren. *Eq* goi-indizeak orekako kontzentrazioak direla adierazten du. *V* hizkiak lagina eta zuntzaren bolumenak adierazten ditu.

Analitoaren distribuzio koefizientea (K_{fs}) horrela defini daiteke:

$$K_{fs} = \frac{C_f^{eq}}{C_s^{eq}} \quad (1.2)$$

1.1 eta 1.2 ekuazioak berrantolatuz:

$$C_f^{eq} = C_0 \cdot \frac{K_{fs}V_s}{K_{fs}V_f + V_s} \quad (1.3)$$

Ekuazioko mol kantitatea kontsideratuz, ekuazioa honela geratuko da:

$$n = C_f^{eq} \cdot V_f = C_0 \cdot \frac{K_{fs}V_s}{K_{fs}V_f + V_s} \cdot V_f \quad (1.4)$$

Zuntzaren bolumena oso txikia dela kontuan hartuz, $K_{fs}V_f$ terminoa arbuiatu daiteke V_s-rekin konparatuz gero. Horrela, ekuazioa sinplifika daiteke:

$$\boldsymbol{n = C_0 \cdot K_{fs} \cdot V_f} \quad (1.5)$$

1.5 ekuazioa ikusita, erauzitako mol kantitatea hasierako kontzentrazioarekiko zuzenik proportzionala dela ikus daiteke, zuntz bolumena eta partizio koefizienteak konstanteak baitira. Lagin bolumenak, beraz, erauzketan zehar eraginik ez duela ikusten da.

Erauzketa buru-gunean emanez gero, gas faseari dagokion beste termino bat gehitu behar da hasierako masa balantzearen ekuazioan. Halere, emaitzak konparagarriak dira 1.5 ekuazioan erakutsitakoarekin eta erauzitako analito kantitatea hasierako kontzentrazioarekiko proportzionala izango da 1.6 ekuazioan erakusten den bezala.

$$C_0 \cdot V_s = C_s^{eq} \cdot V_s + C_f^{eq} \cdot V_f + C_h^{eq} \cdot V_h \quad (1.6)$$

Kasu honetan, bi distribuzio konstante daude ekuazioetan inplikaturik HS-SPME kasuan hiru fase ezberdin daudelako (lagina, buru-gunea eta zuntza). *h* azpi-indizeak buru-gunea adierazten du.

$$K_{hs} = \frac{C_h^{eq}}{C_s^{eq}} \quad (1.7)$$

$$K_{fh} = \frac{C_f^{eq}}{C_h^{eq}} \quad (1.8)$$

$$K_{fs} = K_{fh} K_{hs} \quad (1.9)$$

Lehen egin bezala, amaierako ekuazioaren itxura honakoa da:

$$\boldsymbol{n = \frac{K_{fs} V_f V_s}{K_{fs} V_f + K_{hs} V_h + V_s} \cdot C_0} \quad (1.10)$$

Beraz, edozein modua hautatuta ere (murgiltzea edo buru-gunea) erauzitako kantitatea hasierako kontzentrazioarekiko proportzionala da. Hemendik ondoriozta daiteke, zuntzaren kokapena erauzketarekiko independentea dela, baldin eta bolumenak konstante mantentzen badira (lagin bolumena, buru-gune bolumena eta zuntzaren bolumena).

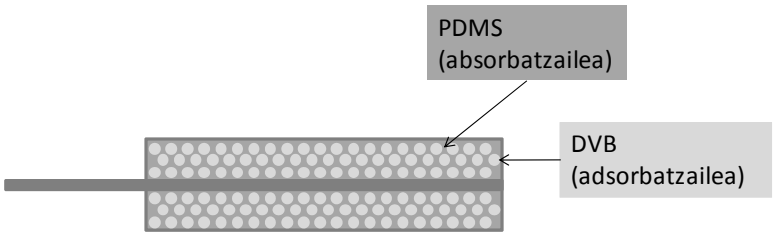
1.2.1.2 SPME prozesuan eragiten duten aldagaiak

SPME prozesua eragiten duten zenbait aldagai daude. Horien araberakoa izan daiteke erauzketa bat arrakastatsua ala ez. Nahiz eta esan bezala, erauzketa hasierako

kontzentrazioarekiko proportzionala den, aldagai batzuk aztertu behar dira [Peñalver *et al.*, 1999]. Aldagai hauek optimizatu eta finkatzea beharrezkoa da, determinazio zehatz eta errepikakorrak lortzeko. Aldagai horietako batzuk ondoren azalduko dira:

- **Estaldura mota**

Zuntz mota ezberdinak aurki daitezke komertzialki eskuragarri [Pawliszyn, 2009] estaldura, lodiera, polaritate eta erauzketa mekanismo ezberdinekoak. Poliakrilatoa (PA), polidimetilsiloxanoa (PDMS), eta dibinilbentzenoa (DVB) eta beraien arteko konbinazioak dira gehien erabiliak (ikus 1.2 irudia)



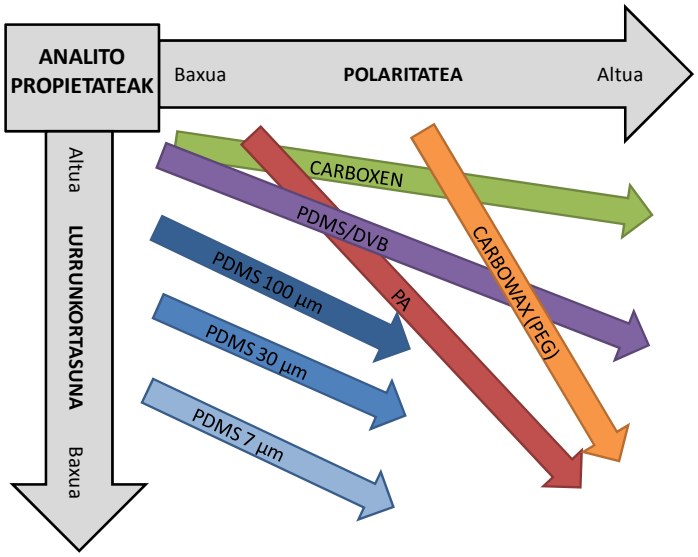
1.2. Irudia. *PDMS/DVB estaldura baten irudi eskematikoa.*

Absortzioa edo/eta adsortzioa eman daiteke, estaldura polimerikoaren arabera. Hots, PDMS materiala absortzioan oinarrituta dagoen bitartean DVB estaldurak analitoa adsorbatzen du. Erauzitako kantitatean zerikusi handia du zuntzaren lodierak, zuntz lodiagoak, betiere analito kantitate gehiago erauziko baitu, baina denbora gehiago ere beharko du. Estaldura hauek "likido-erako" polimeroak direla esaten da, fluidoen propietateak baitituzte. Izan ere, analitoak estalduran zehar migratzen baitute eta analitoen atxikipena, zuntzaren lodieran eta polaritatean oinarritzen baita.

Bestalde, material adsorbatzailearen eraginkortasuna, gainazal azalera, poro kantitatea eta poroen tamainaren araberakoa izango da. Kasu honetan ematen diren interakzioak pi-pi (π-π) lotura, hidrogeno-zubia edota van der Walls motako interakzio intramolekularrak dira.

Estaldura aukeratzerakoan analitoen izaera hartu behar da kontuan. 1.3 irudian ikus daitekeen bezala polaritatea eta lurrunkortasuna ezinbesteko faktoreak dira. PDMS materiala ez-polarra da, baina DVB-rekin konbinatzean estaldura bipolar bat

osatzen dute, analito ezberdin asko erauzketetarako osagarriak diren propietateak bilduz.



1.3. Irudia. *Komertzialki eskuragarri dauden zuntz estaldura ezberdinen erabilera esparrua.*

Sigma-Aldrichen (Madril, Espainia) bidez eskuragarri dauden hainbat zuntz daude 1.3 taulan ikus daitekeen moduan. Taula horretan erabilera gomendioak ageri dira.

PDMS/DVB oso erabilia izan da hainbat analito eta matrizeetan: esaterako fungizida halogenatuen determinazioan [Millán *et al.*, 2003] eta ardotan okratoxin A-ren determinazioan [Aresta *et al.*, 2006]; marrubietan [Wang *et al.*, 2000] eta tomateetan [Ravelo-Pérez *et al.*, 2008] hainbat pestizida determinatzeko; luzoker eta angurrian piretroideak determinatzeko [Parrilla-Vázquez *et al.*, 2008]; sagarretan karbedazima eta tiabendazolak determinatzeko [Hu *et al.*, 2008]; behi-esnean pestizida arrastoak aurkitzeko [Fernandez-Alvarez *et al.*, 2008]; eta haur-janarietan fungizida estrobilurinoak determinatzeko [Viñas *et al.*, 2009]. Ingurugiro laginetan ere oso erabilia izan da PDMS/DVB estaldura: fenitrotiona eta bere metabolitoak ingurugiro uretan [Sánchez-Ortega *et al.*, 2005]; nekazaritza lurretan pestizida organoklorodunak aurkitzeko [Vega Moreno *et al.*, 2006] edota pestizida arrastoak ur laginetan [Beceiro-González *et al.*, 2007; Mmualefe *et al.*, 2009].

1.3. Taula. *Zuntz komertzialen erabilera gida.*

Zuntz estaldura	Lodiera (µm)	pH tartea	Gehienezko tenperatura (°C)	Gomendatutako tenperatura tartea (°C)
PDMS	100	2-10	280	200-280
	30	2-11	280	200-280
	7	2-11	340	220-320
PDMS/DVB	65	2-11	270	200-270
PA	85	2-11	320	220-300
Carboxen/PDMS	75	2-11	320	250-310
PEG	60	2-9	250	200-250
DVB/CAR/PDMS	50/30	2-11	270	230-270

- **Erauzketa denbora**

Erauzketa denbora aldez aurretik finkatu beharra dago. Nahiz eta erauzketa guztiz osoa ez izan, oreka bat azkar lor daiteke denbora laburretan, analito eta estalduraren izaeraren arabera. Denborak kontrolatua egon behar du, analisia errepikakorra eta kuantifikazio egoki bat eman dadin.

- **Erauzketa tenperatura**

Tenperatura optimizatzea beharrezkoa da, kontrako bi eragin ikus baitaitezke prozesuan [Abdulra'uf *et al.*, 2012]. Oro har, tenperaturak eragin positiboa du erauzketa prozesuetan, analitoen difusio koefizientearen lagungarria baita. Bereziki HS-SPMErentzat lagungarria da, fase likidotik gaserako masa-transferentzia hori laguntzen baitu, lurrun dentsitatea areagotuz [Aulakh *et al.*, 2005]. Baina, gehiegizko tenperaturak distribuzio konstantean negatiboki ere eragin dezake, erauzketa tamalgarriagoak emanez.

- **Gatz gehikuntza**

Gatzaren gehikuntza gomendagarria izan daiteke, disoluzioaren indar ioniko handitzen duelako, horrela konposatu organikoak fase akuosoan gutxiago disolbatuz eta partizio koefizientea handituz [Aulakh *et al.*, 2005]. Halere, gehiegizko gatzak efektu negatiboak izan ditzake, izan ere, matrizea gatzez ase daiteke eta guztiz

disolbatu ez diren gatzak zuntz estalduraren leku aktiboetan koka daitezke. Horregatik, garbiketa etapa bat gehitzea gomendagarria da analisi bakoitzaren ondoren, gatzaren kristalizazioak zuntzaren bizitza labur baitezake. Gatz ezberdinen artean sodio kloruroa eta sodio sulfatoa oso erabiliak dira.

- **pH-ren eragina**

pH-a kontuan hartu beharreko aldagaia litzateke, bereziki konposatu azido edo basikoak baditugu. Analitoak forma ez disoziatuan egon beharko dute, izan ere forma disoziatuan erauzketaren eraginkorra jaitsi daiteke. Kasu horietan, pH-a doituz, molekulek erauzteko egokia den forma hartuko dute.

Halere, bereziki DI-SPME moduan, pH-a kontrolatu behar da, zuntzak pH oso azido edo basikoetan kalte baitaitezke. Buru-gunean erabiliz gero, tartea zabalagoa izan daiteke. Halere, fabrikatzaileek beti gomendatzen dute estaldura bakoitzarentzat pH tarte erabilgarria (ikus 1.3 taula).

- **Agitazioa**

Agitazioak masa transferentzia lagundu eta difusio geruza murrizten du [Beltran *et al.*, 2000], oreka egoera azkarrago helduz. Hala eta guztiz ere, gehiegizko agitazioak burbuilak sor ditzake eta arraintxo magnetikoak eman ditzaken kolpeak zuntza kaltetu dezake. Horregatik modu kontrolatu batean eman behar da agitazioa, eta beharrezko agitazio mota ezberdinak hartu beharko lirateke kontuan kasu bakoitzerako: hala nola, agitazio magnetikoa, vortex teknika, zuntzaren agitazioa, fluxua edo sonikazioa [Alpendurada, 2000].

- **Lagin eta buru-gune bolumena**

Batez ere buru-gunea erabiltzen denean, garrantzitsua da lagin/buru-gune bolumena optimizatzea. Nahiz eta lagin bolumena zuntza laginean murgilduta dagoenean ez izan erabakigarria ekuazioetan ikusi den bezala, beharrezkoa da bolumen hauek konstante mantentzea analisi ezberdinetan zehar.

- **Desortzioa**

Desortzio mota segidan etorriko den determinazio metodoaren araberakoa izango da. Hasieran, gas kromatografia (GC) erabili zen helburu horrekin, baina azken

urteetan bereizmen handiko likido kromatografia (HPLC) [Lord, 2007] eta elektroforesi kapilarra (CE) [Kumar eta Malik, 2009] erabili dira.

Desortzioa GC batean ematen bada, injektorea SPME-rentzat egokia den ator batez hornitua egongo da. Izan ere, bolumen handiagoko atorrak, gailur kromatografikoen zabaltzea eragin baitezake. Injektorean desortzioa tenperatura altuen bitartez ematen da, horregatik kontuan izan behar dira 1.3. taulan adierazitako estaldura bakoitzarentzat baimendutako tenperatura maximoak.

HPLC kasuan berriz, desortzioa sei portutako balbula bati lotua dagoen ganbara batean ematen da. Zuntza, bertan sartu eta desortzioa disolbatzaile eta/edo fase mugikorraren bidez ematen da. Kasu honetan, bi desortzio mota eman daitezke: estatikoa edo dinamikoa. Modu dinamikoan, HPLC-ko fase mugikorraren etengabeko fluxua pasatzen da desortzio ganbaratik, zuntzetik analitoak eramanez. Modu estatikoan, aldiz, desortzio ganbara disolbatzailez edo fase mugikorrez bete eta zuntza bertan edukitzen da denbora tarte batean, fluxurik gabe. Modu estatikoa gomendagarriagoa da analitoak zuntz polimerikoari oso atxikiak badaude [Kataoka *et al.*, 2000; Aulakh *et al.*, 2005].

Disolbatzaileak aukeratzerako orduan, muga batzuk izan behar dira kontuan, izan ere, disolbatzaile batzuk zuntzaren estaldura puztu dezakete eta horrek, zuntza hondatu, berriro orratzean jaso nahi denean. Ondorioz, metanola bezalako disolbatzaile protikoak eragoztea komeni da.

Aldagai guzti hauek optimizatzea beharrezkoa da, kasu gehienetan prozesua orekara ez delako iristen. Kuantifikazioa posible da orekara iritsi gabe, baldin eta baldintzak konstante mantentzen badira [Ai, 1997] eta kuantifikazioa fidagarria eta errepikakorra izan dadin aldagaiak optimizatu eta finkatu egin behar dira.

Hurrengo 1.4 taulan SPME-ren abantaila eta desabantaila batzuk ageri dira [Alpendurada, 2000; Kataoka *et al.*, 2000; Pawliszyn eta Pedersen-Bjergaard, 2006; Spietelun *et al.*, 2013]. Azpimarragarria da, disolbatzailerik gabeko erauzketa teknika dela eta estaldura aukera ezberdinek, hainbat matrize eta analitoentzako erabilgarria egiten duela. Bestalde, ordea, zuntzen hauskortasuna da desabantaila nagusia. Kontu handiz erabiltzea ezinbestekoa da zuntzen bizitza luzea izatea nahi bada.

1.4. Taula. *SPME teknikaren abantailak eta desabantailak.*

SPME	
Abantailak	***Desabantailak***
• *Disolbatzailerik gabeko erauzketa.* • *Azkarra (LLE eta SPE moduko teknikekin konparatuz)* • *Zuntz estaldura aukera zabala.* • *Erabilerraza.* • *Automatizatzeko aukera.*	• *Zuntz hauskorrak.* • *Zuntz garestiak.* • *Zuntzen arteko ezberdintasunak.* • *Zuntzen egokitzapena beharrezkoa.* • *Pisu molekular altuko konposatuak zuntzean adsorbaturik gera daitezke modu itzulezin batean.* • *Era daitezkeen burbuilak masa transferentzian eragiten dute.* • *Analisi ondorengo garbiketa etapen beharra, analito arrastoak eta gatzaren kristalizazioa eragozteko, batez ere murgiltze modua erabiltzen bada.*

1.2.2 Likido faseko mikroerauzketa (LPME)

SPME-ren mugak gainditzeko erauztaile organiko tanta ñimiño batean oinarritutako metodoa garatzen hasi zen 1996 urtean [Jeannot eta Cantwell, 1996]. LLE miniaturizatzeko ideia baten moduan hasi zen uretan disolbaezina zen tanta organikoaren erabilera. Urte gutxien buruan, metodoaren zenbait adar edo aldaera agertu dira. Baina oinarria, bera da, tanta organiko bat laginarekin kontaktuan jarri (zuzenean fase akuosoan, edo baita buru-gunean ere) eta erauzketa eman ondoren era batera edo bestera tanta jaso eta ondorengo determinazio kromatografikoa ematen da.

Likido faseko mikroerauzketa (LPME) ere fase emailea eta hartzailearen arteko orekan oinarrituta dago. Tanta organikoan erauzitako kantitatea hasieran ezarritako kontzentrazioarekiko zuzenki proportzionala da 1.11 ekuazioan adierazten den moduan [Jeannot eta Cantwell, 1996]:

$$C_d^{eq} = K_{ds} C_s^{eq} = \frac{K_{ds}\, C_0}{1 + K_{ds}\, V_d/V_s} \qquad (1.11)$$

C_0 eta C_s^{eq} hasierako eta orekako kontzentrazioak dira fasea akuoso edo laginean, C_d^{eq}, aldiz, orekan tanta organikoak duen analitoaren kontzentrazioa izango da; V_d eta V_s tanta organikoa eta laginaren bolumenak izango dira, hurrenez hurren eta azkenik K_{ds} distribuzio koefizientea izango da, ondoren adierazten den bezala definitua:

$$K_{ds} = \frac{C_d^{eq}}{C_s^{eq}} \tag{1.12}$$

Lurrunkorrak diren analitoentzat buru-gunea ere kontuan hartu behar da 1.11 ekuazioan eta kasu horretan, honela geratuko litzateke ekuazioa [Jeannot *et al.*, 2010]:

$$\boldsymbol{C_d^{eq}} = \frac{\boldsymbol{K_{ds}\, C_0}}{\boldsymbol{1+(K_{hs}\, V_h/V_s)+(K_{ds}\, V_d/V_s)}} \tag{1.13}$$

Berriz ere, *h* terminoa buru-guneari dagokion azpi-indizea izango da.

Edozein kasutan ere, tantaren orekako kontzentrazioa (C_d^{eq}) beti izango da laginaren hasierako kontzentrazioaren proportzionala (C_0) eta partizio koefizientearen araberakoa izateaz gain, tantaren eta laginaren bolumenaren araberakoa izango da.

Aberastasun faktorea (EF) honela defini daiteke: analitoaren orekako kontzentrazioa fase organikoan (C_d^{eq}) eta hasierako fase akuosoan zuen kontzentrazioaren (C_0) arteko erlazioa [Mohamadi eta Mostafavi, 2010; Pakade eta Tewary, 2010]. EF altuek erauzketa eraginkorra izan dela adierazten dute, eta metodo balidazioko parametro batzuk hobeagotu daitezkeela, esaterako, detekzio-muga.

$$EF = \frac{C_d^{eq}}{C_0} \tag{1.14}$$

Likido mikroerauzketa teknika mota ezberdinen sailkapen ugari aurki daitezke bibliografian [Lambropoulou eta Albanis, 2007; Pena-Pereira *et al.*, 2010; Pinto *et al.*, 2010; Sarafraz-Yazdi eta Amiri, 2010; Mahugo-Santana *et al.*, 2011; Han eta Row, 2012; Dehghani Mohammad Abadi *et al.*, 2012; Spietelun *et al.*, 2014; Andrašcíková *et al.*, 2015]. Nahiz eta izen ezberdin ugari eman teknikaren adar ezberdinei, oro har honela sailka daitezke:

- **Tanta bakarraren mikroerauzketa (SDME)**

Disolbatzaile organiko baten kantitate oso txiki bat (1-3 µL) laginarekin kontaktuan jartzen da (DI edo HS moduan) mikroxiringa baten puntan esekita. Erauzketa denboraren ondoren, tanta mikroxiringarekin berarekin berriz jaso eta determinaziora eramaten da. Metodo hau izan zen abiapuntua, tefloi hodiak erabiltzetik mikroxiringak erabiltzen hasi zirenean (ikus 1.4.a irudia).

- **Zuzenean ezarritako tantaren mikroerauzketa (DSDME)**

Kasu honetan, tanta organikoa zuzenean likidoaren gainean ezartzen da, kanpo euskarrien laguntzarik gabe. Tanta, dentsitatearen arabera likidoaren gainean edo edukiontziaren hondoan kokatuko da. Edozein kasutan ere, erauzketa denboraren ondoren, tanta hori jaso behar da (ikus 1.4.b irudia)

- **Likido-likido mikroerauzketa dispertsiboa (DLLME)**

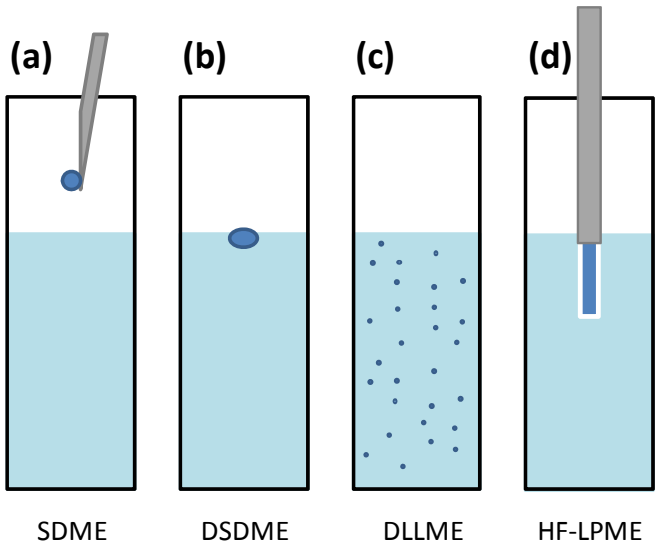
Izenak dioen moduan, tanta organikoa fase akuosoan zehar dispertsatzen da, laino erako fase bat sortuz. Dispertsio hau, beste disolbatzaile organikoen laguntzaz eman daiteke (disolbatzaile dispertsatzailea) edo agitazio edo ultrasoinua erabiliz. Erauzketa denboraren ondoren zentrifugazioa beharrezkoa da laino fasea berriz, tanta organiko bakar bat bihurtu dadin (ikus 1.4.c irudia).

- **Zuntz hutseko likido-likido mikroerauzketa (HF-LPME)**

Zuntz huts itxura duen hodi porotsu bat erabiltzen da fase organikoaren euskarri modura. Beraz, zuntz-hutsa laginean sartzen da erauzketa denboran zehar eta ondoren jaso egiten da (ikus 1.4.d irudia).

Erauzketa euskarriren bat erabiliz eginez gero, tanta ere era berean jaso ohi da. Baina tantak ez badu inolako euskarririk, tanta jasotzeko beste metodo batzuk erabili behar dira. Gehien erabilia tanta organikoa flotatzailearen solidifikazioa (SFO) da, tanta likidoaren gaineko aldean dagoenean, tenperatura baxuen laguntzaz izoztu eta behin solidifikatuta dagoenean, espatula batekin jaso daiteke. Tanta organikoa bildu edo jaso ondoren giro tenperaturan urtu daiteke eta gero kromatografia teknika batean injektatu. Helburu honetarako, tanta organikoak zenbait ezaugarri izan behar ditu, alde batetik, urtze tenperatura baxuak, giro tenperaturan izoztutako tanta urtu dadin (10 -

30 ^{o}C artean), bestalde, erauztailearen dentsitatea urarenak baino baxuagoa izan behar du, fase akuosoaren gainean kokatu dadin [Ghambarian *et al.*, 2013].



1.4. Irudia. *Likido mikroerauzketa metodo batzuen eskema: (a) Tanta bakarraren mikroerauzketa; (b) zuzenean ezarritako tantaren mikroerauzketa; (c) likido-likido mikroerauzketa dispertsiboa eta (d) zuntz hutseko likido faseko mikroerauzketa.*

SPME kasuan bezala, likido faseko mikroerauzketa baldintzatuko duten hainbat aldagai daude [Regueiro *et al.*, 2008; Ganjali *et al.*, 2010]. Ondoren azalduko dira batzuk:

- **Erauztailea**

Erauztaile moduan erabiltzen den disolbatzaile aukera zabala dago, baina zenbait baldintza betetzea ezinbestekoa da [Ganjali *et al.*, 2010; Han eta Row, 2012]:

1. Intereseko analitoekin afinitate ona izatea.
2. Urarekin disolbaezina izatea.
3. Erauzketa denboran zehar egonkorra izatea.
4. Portaera kromatografiko egokia izatea (GC edo HPLC analisirako egokia) edo beste determinazio teknikekin bateragarria izatea.

5. SFO kasuan, urtze tenperaturak giro tenperatura baino baxuagoa izatea, baina ura baino izozte puntu altuagoa izatea (oro har, ur bainu batean izozten baita tanta).

- **Erauztaile bolumena**

Erauztaile bolumena oso garrantzitsua da, bolumen txikiek aberastasun faktorea (EF) hobeagotzen baitute, baina tanta txikiegiak maneiatzeko konplexuagoak izan daitezke. Beraz, tantaren tamainarekin oreka bat aurkitu behar da, tanta bideragarria izan dadin, nahikoa handia izan behar da, baina era berean diluzioa saihesteko handiegia ezin da izan. Bestalde, kontuan izan behar da masa transferentzia gainazal azalera handiagoekin hobeagoa izango dela [Krylov *et al.*, 2011], baina esan bezala, bolumen handiek EF balioa murriztuko dute.

- **Erauzketa denbora**

Prozeduraren errepikakortasuna hobeagotzeko, oreka egoerara iristeko behar bezain denbora behar da, nahiz eta erauzketa guztiz osoa ez izan. Masa transferentziaren abiaduraren araberakoa izango da denbora, baina era berean, baita tanta bolumenarena ere. Erauzketa ez-oreka baldintzetan ematea ere posible da, DLLME kasuan ezik, bere kontaktu gainazala oso handia denez, orekara denbora laburrean iritsi ohi da [Ganjali *et al.*, 2010; Krylov *et al.*, 2011].

- **Erauzketa tenperatura**

Tenperaturak masa transferentzia laguntzen du, beraz, beharreko denbora murrizten da. Halere, tenperaturak tanta organikoaren disolbagaritasuna handiagotu dezake fase akuosoan, eta horrek prozesua ezegonkortzeaz gain, analitoen partizio koefizientearen murriztea eragin dezake [Krylov *et al.*, 2011]. Erauzketa buru-gunean ematen bada, egoera are konplexuago bat eman daiteke, izan ere, erauztailea lurruntzen bada erauzketaren eraginkortasuna eta errepikakortasunaren murriztea eragin dezake.

- **Gatz gehikuntza**

Gatz gehikuntzak efektu kontrajarriak izan ditzake LPME kasuan. Alde batetik, gatzak erauzketa kaltetu dezake analitoak fase batetik bestera igarotzea oztopa dezakeelako biskositatearen igoeragatik. Baina bestalde, analitoen masa transferentzia lagundu dezake, izan ere urak gatz ioien inguruan sortzen dituen hidratazio esferek

analitoek fase akuosoan duten disolbagarritasuna murriztu baitezakete [Ganjali *et al.*, 2010; Krylov *et al.*, 2011].

- **Agitazioa**

Agitazioa, oro har, masa transferentziaren lagungarri da, baina agitazioa indartsuegia bada, tanta ezegonkortu dezake [Ganjali *et al.*, 2010; Krylov *et al.*, 2011]. Agitazio metodo ezberdinetatik erabiliena iman txiki baten bidezko agitazio magnetikoa da.

- **Disolbatzaile dispertsatzailea**

DLLME kasuan, dispertsioa eragingo duen disolbatzaile bat erabili ohi da. Laino itxura duen emultsio bat sortzen da, milaka mikrotanta eratuz, eta horren ondorioz azalera gainazala handitzen denez, masa transferentzia azkartzen da. Bere toxikotasun eta kostu baxuagatik, metanola, azetona, etanola eta azetonitriloa oso erabiliak dira [Sarafraz-Yazdi eta Amiri, 2010]. Halere, bere eraginkortasuna mugatua egon daiteke, bolumen handiek analitoek erauztailearekiko duten partizio koefizientea murriztu dezaketelako [Saraji eta Boroujeni, 2014].

Disolbatzaile bat ez bada ere, laino itxura eman diezaioke ultrasonikazioak [Regueiro *et al.*, 2008]. Ultrasoinuak (US) tanta organikoa dispertsa dezake beste disolbatzaileren beharrik gabe.

Desortzio etapa ez da beharrezkoa zuzenean SPME kasuan bezala, izan ere tanta jaso ondoren GC edo HPLC instrumentuetara injektatu daiteke xiringa baten laguntzaz. Halere, HPLC-rekin beste disolbatzaile batekin diluitu beharra gerta liteke, erauztailea fase mugikorrarekin bateratu ahal izateko.

1.2.2.1 Ultrasoinuz lagundutako emultsifikazio mikroerauzketa tanta organiko flotatzailearen solidifikazioarekin (USAEME-SFO)

Denbora murrizteko gaitasunagatik DLLME geroz eta gehiago erabiltzen da. Metodo hau tanta organikoaren dispertsioan oinarritzen da, milaka mikrotanta sortuz, laino itxura duen disoluzio bat osatzen da. Ondorioz, kontaktu gainazala asko handitzen da eta horrek masa transferentzia laguntzen duenez, erauzketa denborak laburragoak dira.

Aurretik aipatutako baldintzez gain, kasu honetan erauztaileak beste baldintza bat bete behar du: emultsio egoki bat formatzea, baina ondoren zentrifugazioaren laguntzaz berriz tanta osatu ahal izatea. Laino itxurako disoluzioa lortzeko disolbatzaile dispertsatzaile bat erabil daiteke (metanola, azetona, etanola eta azetonitriloa esaterako) [Saraji eta Boroujeni, 2014]. Baina ez da dispertsioa eratzeko modu bakarra. Ultrasoinu (US) bainu batek ere laino itxura duen emultsioa sor dezake (ikus 1.5 irudia) eta erauzketa prozesua lagundu [Regueiro *et al.*, 2008].

1.5. Irudia. *Ultrasoinua aplikatu ondoren sortzen den laino itxurako disoluzioa.*

Ultrasoinuaren energia askotan erabili izan da lagin prestaketa prozedura askotan [Luque de Castro eta Priego-Capote, 2007]. Ultrasonikazioa eraginkorra dela

ikusi da sintesietan, emultsifikazio produktuetan eta material garbiketetan; ospitaleetako kirurgia prozeduretan ere erabili izan da teknika ez-inbasibo moduan.

Ultrasoinu uhinak materialetan zehar garraiatzen dira konpresio/espantsio zikloak sortuz molekulen artean. Zabalkuntza zikloak burbuilak edo kabitazioak sortzen ditu likidotan eta bertako gasen konpresio azkarrak disoluzioak duen tenperatura eta presioaren igoera eragiten du. Sortzen diren burbuila edo hutsune horiek likido bolumen totalarekin konparatuz gero oso txikiak dira, eta beraz ez dira ikusi ere egingo. Ondorioz sortzen den beroa azkar barreiatzen da. Fenomeno honi kasu batzuetan “irakite-hotza” ere deitzen zaio.

Ultrasoinuaren erabilerak likido-likido mikroerauzketa prozesuetan alde batetik masa transferentzia laguntzen du, eta bestetik, sortzen den emultsifikazioaren sorrerak tanta organikoaren eta fase akuosoaren arteko kontaktu gainazala handiagotzen du. Desabantailetako bat tantaren birformakuntzarako behar duen denbora litzateke, izan ere, erauzketaren ondoren tanta berriz jaso ahal izateko moduan egon behar da. Halere, tanta bolumena handia ez denez, zentrifugazioaren bidez prozesua azkar egin daiteke.

Dispertsiorako ultrasoinua erabiltzen den lau kasu aurki daitezke bibliografian; lehenengo kasuak US bakarrik erabiltzen du, beste inolako disolbatzaileren beharrik gabe [Ozcan *et al.*, 2010; Wei *et al.*, 2011; Cortada *et al.*, 2011]. Beste adar batek dispertsatzaile bolumen txiki bat erabiltzen du (0.2-0.5 mL) [Yan *et al.*, 2011; Zhang *et al.*, 2012a]. Hirugarren kasu batean surfaktante batzuk erabiltzen dira emultsifikazioa laguntzeko [Cheng *et al.*, 2011a; Cheng *et al.*, 2011b; Xia *et al.*, 2012; You *et al.*, 2013] eta azken adar batek nanopartikula magnetikoak erabiltzen ditu tantaren birformakuntza laguntzeko zentrifugazioaren ordez [Li *et al.*, 2014].

Hurrengo 1.5 taulan ikus daitezke USAEME teknikaren zenbait abantaila eta desabantaila [Mohamadi eta Mostafavi, 2010; Mahugo-Santana *et al.*, 2011; Han eta Row, 2012; Saraji eta Boroujeni, 2014; Spietelun *et al.*, 2014]. Nahiz eta matrize konplexuetan erabilterraza ez bada ere, beste teknika asko baino azkarragoa, merkeagoa eta sinpleagoa da teknika hau.

1.5 Taula. *USAEME metodoaren abantailak eta desabantailak.*

USAEME	
Abantailak	***Desabantailak***
• *Hondar-efekturik ez.* • *Azkarra.* • *Merkea.* • *Disolbatzaile organikoen erabilera minimoa.* • *EF altuak.* • *Erabiltzeko sinplea.* • *Helburu bakoitzerako egokitu daitezkeen adar ezberdinen aukera.* • *Determinazio teknikekin akoplamendu erraza, bestelako interfaseren beharrik eza.*	• *Matrize konplexuekin erabiltzeko zaila.*

1.3 Teknika kromatografikoak

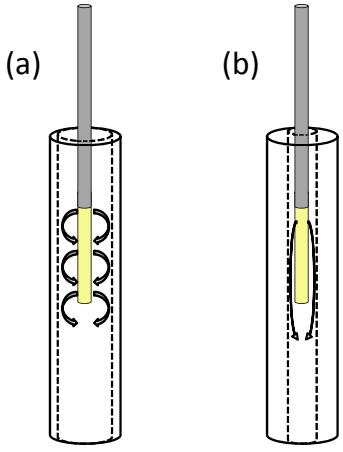
Nahiz eta 60 hamarkadaren hasieran gas kromatografiaren inguruko erreferentzia bat aurki daitekeen [Stross eta Ambrose, 1960], IUPAC elkarteak kromatografia honela definitu zuen 1993. urtean: *"banaketa metodo fisiko bat, non konposatuak fase geldikor bat eta norabide definitu batean mugitzen den fase mugikor baten artean banatzen diren"* [Ettre, 1993]. Beraz, kromatografia teknikek konposatu ezberdinak bereizi eta ondoren eluitzailearen konposizioaren aldaketa nabariko duen tresna baten bidez neurtuko dituzte. Neurketa honek, sustantzia bakoitzaren kontzentrazioarekiko zuzenki proportzionala den seinale bat emango du. Horregatik nahasteen kuantifikazio helburuentzat oso erabilia da kromatografia.

Aurrekontzentrazioaren ondoren, analitoak bereizi eta determinatu behar direnez, tesi honetan erabilitako teknikak elektroi-harrapaketaren detektagailu duen gas kromatografia (GC-ECD) eta lerrokatutako diodoen detektagailuz hornitua dagoen bereizmen handiko kromatografia likidoa (HPLC-DAD) erabili dira. Erabilera konkretu honetarako berezitasun batzuk azalduko dira laburki ondorengo atalean.

1.3.1 Gas kromatografia elektroi harrapaketa detektagailuarekin (GC/ECD)

Gas kromatografia (GC) oso erabilia izan da banaketa eta determinazio helburuekin. Zutabe kapilar, gas eluitzaile baten interakzioetan eta tenperatura gradiente batean oinarritzen da teknika.

SPME teknika garatu zenean, determinazioak GC bidez egin ziren. Zuntza gordetzen den orratza GC-ko injektorean sar daiteke inongo interfase bereziren beharrik gabe. Atorra da kontuan hartu beharreko bereizgarri bakarra. Izan ere, atorraren bolumenak zerikusi handia izan dezake batez ere lurrunkorrak diren konposatuekin, horregatik, ator estu bat (ikus 1.6.b. irudia) erabiltzea gomendatzen da, gailur kromatografikoen zabalera ekiditeko [Kataoka *et al.*, 2000; Lord eta Pawliszyn, 2000]. Barne-diametro zabala duten atorretan (1.6.a. irudia), analitoak behin eta berriz desorbatu eta absorbatzen dira SPME zuntzean eta horren eraginez gailur kromatografikoen zabalera eta formaren deformazioa gerta daiteke.



1.6. Irudia. *Atorraren barne-diametroaren lodieraren efektua: (a) barne-diametro zabala duen atorra eta (b) SPME-rentzat bereziki diseinatutako barne-diametro txikiko atorra.*

Injektoreko tenperaturak lurrunkortasun txikiena duen analitoaren irakite tenperatura baino altuagoa izan behar du, baina beti ere zuntz estaldurarenaren

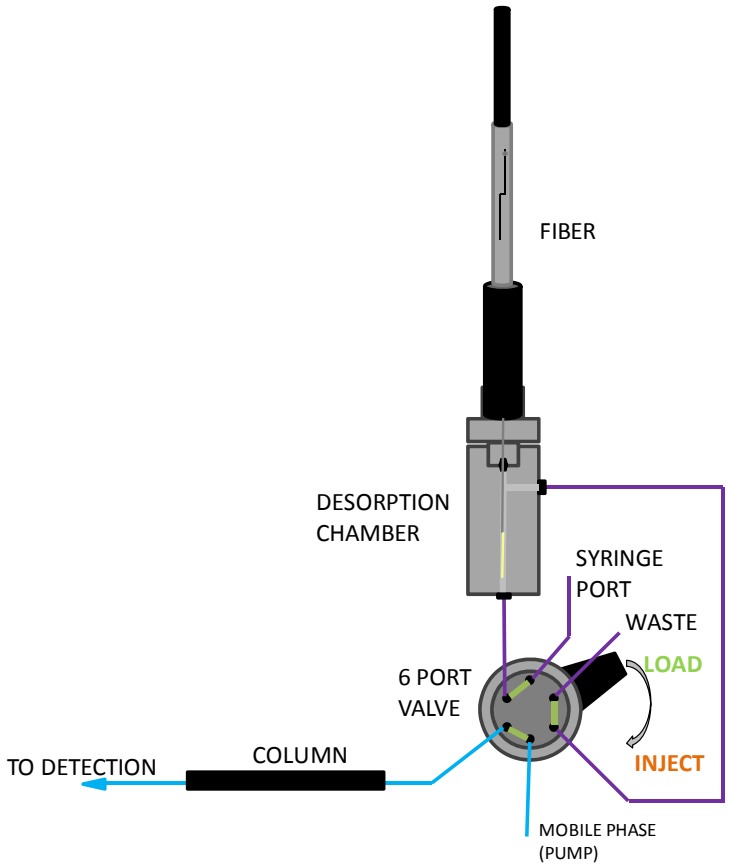
erabilera gidan gomendatzen den tenperatura maximoa aintzakotzat hartuz. [Abdulra'uf *et al.*, 2012].

Erabilitako detektagailua Elektroi Harrapaketa Detektagailua (ECD) izan zen. Detektagailu honek, elektroiak askatzen dituen konposatu bat du (^{63}Ni) eta askatutako elektroiak positiboki kargatuta dauden anodorantz azeleratuak izan ondoren seinale elektriko bat sortzen dute. Elektroi horiek xurgatzen dituen konposaturen bat tartetik pasatuz gero seinale elektrikoa aldatuko da eta aldakuntza hori analitoaren kontzentrazioarekiko proportzionala izango da. Halogenoak, beraien elektronegatibotasun propietatearengatik, elektroi hartzaile onak dira eta detektagailu mota honetarako egokiak dira. Triazol konposatuak halogeno atomoren bat dutenez, detektagailu mota honentzat detektagarriak dira.

Halere, injektoreko tenperatura altuek zuntzen estaldura polimerikoak degradatzea eragin dezakete eta gainera termikoki ezegonkorrak diren konposatuak ezin dira GC-n erabili. Ondorioz, beste banaketa eta determinazio teknikak erabiltzea beharrezkoa izan daiteke kasu batzuetan.

1.3.2 Bereizmen handiko kromatografia likidoa lerrokatutako diodoen detektagailuarekin (HPLC/DAD)

Bereizmen handiko kromatografia likidoa (HPLC) fase geldikorra den zutabe batean eta disolbatzaile ezberdinez osatutako fase mugikorrean oinarritzen da. Polaritate ezberdineko hainbat disolbatzaile eta beraien arteko gradienteak erabiltzen dira bereizketa burutzeko. SPME HPLC-rekin erabili ahal izateko zailtasunik handiena zuntza eta injektorea bateratzea izan zen. Laurogeita hamargarren hamarkadan Pawliszyn eta bere taldeak lehen interfaseak proposatu zituzten [Chen eta Pawliszyn, 1995]. Geroztik, komertzialki eskuragarri dauden zenbait interfase merkaturatu ditu Sigma-Aldrich-ek (Madril, Espainia). 1.7 irudiak SPME-HPLC Rheodyne® interfase balbula bat erakusten du.



1.7. Irudia. *SPME-HPLC interfasearen 6 portutako Rheodyne® balbularen irudi eskematikoa.*

Interfasearen berezitasuna desortzio ganbaran dago. Beste kasuetan kiribil bat dagoen bitartean, kasu honetan zuntza sartuko den ganbara bat du interfaseak.

Determinazioa lerrokatutako diodo detektagailu baten bidez egin zen. Detektagailu honen abantaila nagusia detekzioa uhin-luzera tarte zabal batean egiten duela da eta ez uhin-luzera bakar batean. Gainera, eluzio-denbora bakoitzeko ultramore (UV) espektro oso bat jasotzea ahalbidetzen du, bigarren mailako abantaila emanez. Beraz, aldagai anitzeko analisiaz gain, hainbat uhin luzeretan ikus daitezke kromatograma ezberdinak. UV espektroa hatz-marka bezala ere erabil daitekeenez, liburutegi nahikoa izanez gero, konposatuak ere identifikatzea posible litzateke.

1.4 Kimiometria

Kimiometria: "*matematikak, estatistika eta logika erabiltzen dituen kimikaren diziplina (I) prozedura esperimental optimoak diseinatu edota aukeratzeko; (II) datu kimikoak aztertuz ahalik eta informazio gehien lortzeko; eta (III) sistema kimikoen jakinduria zabaltzeko"* [Stalikas *et al.*, 2009].

1.4.1. Diseinu esperimentala

Mikroerauzketen eraginkortasuna finkatu beharreko aldagai ezberdin askoren araberakoa da. Edozein konposatu kuantifikatzeko kalibrazio eta balidazio prozesuekin hasi aurretik, garrantzitsua da aldagai ezberdinak optimizatzea. Izan ere, aldagaien balio egokiak aukeratuta erantzun hobeago bat lor daiteke, eta ondorioz detekzio-muga, errepikakortasuna eta tarte lineala bezalako parametroak hobe daitezke.

Prozesua aldagai askoren menpe dagoenean, aldagai edo faktore bakoitza banan-bana optimizatzea oso nekagarria eta denbora asko eskatzen duen lana izan daiteke. Gainera, kasu horretan egon daitezkeen aldagaien arteko interakzioak ezin dira aurreikusi [Dejaegher eta Vander Heyden, 2009], sistema osoarentzat onuragarriak diren balio optimo globalak ezin dira aurkitu eta amaieran lortzen diren balio optimoak hasiera puntuaren araberakoak izan daitezke [Dejaegher eta Vander Heyden, 2011]. Aldagai anitzeko optimizazio batek, aldiz, maila ezberdinetako interakzioak detekta ditzake. Diseinu esperimentala (DoE), analisi talde bat da non aldi berean hainbat aldagai ezberdin, zenbait mailetan aztertzen diren, esperimentu kopuru zehatz baten bidez.

DoE metodologiak aldagai bakoitzaren efektuak eta beraien arteko interakzioak nabarmendu ditzake. Horrez gain, esperimentu kopuru mugatu batek laborategi baten baliabideak eta horren dakartzan gastuak murriztuko ditu.

Stalikas-en arabera [Stalikas *et al.*, 2009], prozedura analitiko baten optimizaziorako kontuan hartu beharreko puntuak dira ondorengoak:

I. Arazoaren aldagai eta erantzun egokien definizioa miaketa azterketen bidez: aztertzaileak prozesua baldintzatuko dituen aldagaiak aukeratu behar ditu. Mikroerauzketen kasuan: lagin bolumena, erauzketa denbora eta tenperaturak, agitazioa etab. Bestalde, prozesua balioetsiko duen irizpidea hautatu beharko da, hau da, optimizatu nahi den erantzuna: azalera kromatografikoak, erauzketa etekina, aberastasun-faktorea, etab.

II. Diseinu esperimentalaren aukeraketa: diseinu mota ezberdinen artean, kasu bakoitzerako diseinurik egokiena hautatu beharko da. Aldagai kopurua handia ez bada diseinu faktorial osoa erabil daiteke, baina kopurua handia izanez gero, esperimentu askoren beharra egongo litzateke eta kasu horretan diseinu murriztuagoak egokiagoak lirateke. Besteak beste, diseinu faktorial zatikatua, Plackett-Burman, Doehlert, Box-Behnken eta diseinu konposatu zentralak nahikoa bereizmen eskaintzen dute esperimentu kopuru mugatu batekin.

III. Aldagai mailen hautaketa eta kodifikazioa: aldagai balio tarteak aukeratu behar dira, aldaerak ikusi ahal izateko eta errorearekiko duten aldea ikusteko. Aldagai balioen tartea txikiegia bada, ikus daitekeen aldaera ausazko errorearekin nahas daiteke, eta aldaeraren interpretazio okerra eman daiteke. Ondoren, aukeratutako balioak [-1], [+1], [0], [-α] eta [+α] kodeekin izendatuko dira.

IV. Modelo matematikoen doitzea: joera esperimentala modelo matematiko bati doitu behar zaio: lineala, karratua, interakzioduna, etab. Interakziorik gabeko modelo lineal sinple batek honako itxura izango luke adibidez:

$$y = b_0 + \sum b_i x_i + \varepsilon \qquad (1.15)$$

V. Modelo egokitasunaren egiaztapena: doitutako modeloa egokia den ziurtatu beharko da eta sistemaren egiazko informazioa ematen duen baieztatu. Hori horrela dela jakiteko bariantzaren analisia (ANOVA), doikuntza, hondar balioen analisia eta karratu minimoaren estimatzailea (R^2) behatuko dira.

VI. Modeloaren azterketa eta efektuen estimazioak: *t*-Student motako testak erabili ohi dira erregresio koefizienteak errorearengandik bereizten diren ala ez zehazteko. *p* balioak eta *F* testak beste informazio

estatistiko bat emango digute, kasu honetan adierazgarritasunari buruz. Azkenik irudikapen grafikoak bisualki adierazgarriak direneko informazioa emango digute: Pareto grafikoak, azalera erantzunak, desiragarritasun funtzioa, etab.

VII. Balio optimoaren estimazioa: helburua desiragarria den erantzuna maximizatzea edo minimizatzea da. Hori irudikapen grafikoen bidez (erantzun azalerak, desiragarritasun funtzioak, etab.) edo funtzio matematikoak ebatziz (funtzio matematikoaren deribatuak eginez) lor daiteke.

VIII. Modelo sendotasunaren egiaztapena: metodoak eman dituen emaitzak zehatzak eta errepikakorrak direla ziurtatzea da egiaztatu behar dena, hau da, aldaketa txikiekin sistema egonkor mantentzen dela baieztatzea.

Laburtuz, DoE erabiltzerakoan bi etapa nagusi bereiz daitezke: miaketa eta optimizazioa. Miaketaren helburua adierazgarriak diren aldagaiak hautatzea da, hau da, sisteman gehien eragiten duten aldagaiak aurkitzea eta eraginik ez duten aldagaiak finkatzea. Behin esanguratsuak diren aldagaiak hautatuta, beraien balio optimoak bilatuko dira optimizazio prozeduren bidez. Balio optimo horietan, sistemak desiragarritasun maximoa duen erantzuna emango du, hots, erantzun kromatografiko maximoa (azalera edo altuera) edo erreakzio kimiko baten etekin hoberena.

Diseinu mota ezberdinak ikusten hasi aurretik zenbait terminoren definizioak ematea komenigarria da [Bezerra *et al.*, 2008]:

- *Faktoreak edo aldagai independenteak:* finkatu nahi diren aldagaiak izango dira, beraien artean independenteak. Ohiko aldagaiak izan daitezke tenperatura, pH-a, esperimentuen iraupena, etab. Faktoreak kuantitatiboak (jarraiak) edota kualitatiboak (kategorikoak edo aldagai diskretuak) izan daitezke.
- *Aldagaien mailak:* optimizatuko den aldagai balioen tartea. Aldagai kuantitatiboetan 1 balio ematen zaio baliorik altuenari, -1 txikienari eta 0 balioa erdiko puntuari. Aldagai kualitatiboei, +1 eta -1 ausazko balioak emango zaizkie.
- *Erantzunak ala mendeko aldagaiak:* ekipamendu batek ematen duen erantzuna: azalera kromatografikoa, absorbantzia, esperimentu baten etekina, etab. beste era batera esan da, maximora (edo minimora) eraman nahi dugun neurria.

Diseinu faktorial osoa, zatikatua eta konposatu zentrala azalduko dira laburki ondorengo lerroetan.

1.4.1.1. Miaketa. Diseinu faktorialak

Miaketa diseinuen helburua esanguratsuak diren aldagaiak aurkitzea da. Metodo bat garatzerakoan sisteman eragiten duten hainbat aldagai daudela ikus daiteke, baina horietatik batzuk bakarrik izan daitezke benetan adierazgarriak direnak. Hau da, aldagaien balioa aldatuz gero, erantzunean aldaketa eragingo dutenak. Ondoren diseinu mota hauetako batzuk azalduko dira:

- **Diseinu faktorial osoa**

Diseinu osoa da, posible diren konbinazio denak kontuan hartuko dituena. Esperimentu kopurua $N=l^f$ izango da, non l maila kopurua (2 izango da -1 eta +1 maila kodifikatutan aztertzen bada) eta f aldagai kopurua izango dira. Aldagai bakoitzaren bi mailen konbinazio posible guztiak aztertzen direnez, esperimentu kopurua oso handia izan daiteke aldagai asko baldin baditugu (ikus 1.6 taula) [Brereton, 2003].

1.6. Taula. *Aldagai bakoitza bi mailetan aztertuz gero beharrezkoak diren esperimentu kopuruak diseinu faktorial oso batean.*

l=2	**Faktore edo aldagai independente kopurura (f)**									
	2	3	4	5	6	7	8	9	10	11
$N=2^f$	4	8	16	32	64	128	256	512	1024	2048

1.6 taulan ikus daitekeen moduan, diseinuari aldagai kopuru handia ezarriz gero, esperimentu kopurua izugarri handitzen da, eta hori kasu batzuetan ezinezkoa gerta daiteke laborategian burutzea.

Oro har, aldagai bakoitza bi maila ezberdinetan aztertzen da, bien artean nahikoa ezberdintasun dagoen ala ez ikusteko. Lehen aipatu bezala, aldagaien balioak kodifikatu egin ohi dira +1 balioa emanez balio altuenari eta -1 balioa baxuenari. Aldagai kualitatiboak izanez gero +1 eta -1 balioak ausaz emango zaizkie. Aldagai kuantitatiboetan ordea zeinu positiboak eta negatiboak aldagaiaren balioaren zentzua emango digu: kode positiboak balio altuak hobeagoak direla esaten du eta negatiboak, aldiz, balio txikienak direla erantzun hoberenak. 1.7 taulan 3 aldagai dituen sistema baten diseinu faktorial oso bat ageri da. Ikus daitekeen bezala konbinazio posible guztiak ageri dira. Esperimentu kopurua $N=l^f$ denez, 8 esperimentu aski dira diseinu honetarako ($2^3 = 8$).

1.7. Taula. *3 faktore eta 2 mailez (altua [+1] eta baxua [-1]). osatutako diseinu faktorial osoa.*

Esp. kop.	**A faktorea**	**B faktorea**	**C faktorea**
1	+1	+1	+1
2	+1	+1	-1
3	+1	-1	+1
4	+1	-1	-1
5	-1	+1	+1
6	-1	+1	-1
7	-1	-1	+1
8	-1	-1	-1

Diseinu mota honek efektu nagusiak eta aldagaien arteko interakzio guztiak estimatu ditzake. Modeloa matematikoan interakzio linealak bakarrik aintzakotzat hartuz, ondoko ekuazioa beteko luke:

$$y = b_0 + b_a x_a + b_b x_b + b_c x_c + b_{ab} x_a x_b + b_{ac} x_a x_c + b_{bc} x_b x_c + b_{abc} x_a x_b x_c \quad (1.16)$$

Kasu honetan 8 koefiziente ezezagun daude ebazteko (b_0, b_a, b_b, b_c, b_{ab}, b_{ac}, b_{bc} eta b_{abc}). Behin ekuazioa ebatzita, koefiziente horiek esanguratsuak diren ala ez *t*-Student testaren bidez jakin daiteke. Koefizienteren bat ez bada esanguratsua, bera ordezkatzen duen aldagaia (edo aldagaien arteko interakzioa) garrantzitsua ez dela esan nahi du. Kasu horretan, aldagaia finkatuz aurrera jarrai daiteke. Halere,

koefizientearen zeinua kontuan hartzea komeni da, adierazgarria izan ez arren, zein baliok emango duen erantzun hoberena esaten digu.

- **Diseinu faktorial zatikatua**

Aldagai independente asko izanez gero, diseinu faktorial oso batek esperimentu kopuru altua eskatzen du, batzuetan egiteko ezinezkoa. Horregatik, informazio garrantzitsua galdu gabe, esperimentu kopurua murriztea beharrezkoa izan daiteke.

Diseinu faktorial oso bat koefiziente denak kalkulatzeko gai da, efektu nagusiak eta maila ezberdinetako interazioak. Baina interakzio horietako asko baztergarriak izan daitezke. Ondorioz, eta informazioa galdu litekeela onartuz, esperimentu kopurua murriztu daiteke. Kasu horretarako bereizmen kontzeptua definitu zen [Box *et al.*, 1978]:

- III bereizmena: ez ditu efektu nagusiak elkarrekin nahasten baina efektu nagusiak bi aldagaien interakzioekin nahas ditzake.
- IV bereizmena: ez ditu efektu nagusiak beste bi aldagaien interakzioekin nahasiko, baina bi aldagaien interakzio ezberdinak nahas ditzake.
- V bereizmena: ez ditu efektu nagusiak, ezta bi aldagaien interakzio ezberdinak nahasiko, baina bi aldagaien arteko interakzioak hiru aldagaien interakzioekin nahas ditzake.

Diseinu ezberdinen artean faktorial zatikatua, Plackett-Burman eta Taguchi bereiz daitezke, besteak beste [Brereton, 2003]. Matrize ezberdinak eratuko ditu diseinu bakoitzak, betiere esperimentu kopuru mugatu batez osatuak.

Diseinu faktorial zatikatuan esperimentu kopurua $N=l^{f-k}$-k definitua dator, non k termino berriak esperimentu kopurua biz zenbat aldiz zatitua izan den adierazten duen. Beraz k=1 izanez gero, esperimentu kopurua bi aldiz zatituko litzateke, etab. Adibidez, 5 aldagaiko diseinu faktorial oso bat izanez gero, esperimentu kopurua 32 izango zen, baina k=1 erabiliz eta diseinu zatikatua hautatuz, analisi kopurua erdira jaitsiko zen (16 esperimentu); aldiz, k=2 bada, esperimentu kopurua 8 izango zen.

Beraz, diseinu faktorial zatikatuak esperimentu kopurua murriztuko du, bereizmena zertxobait galduz. 1.8 taulan zenbait diseinuren adibideak ikus daitezke, 5

eta 6 faktore erabiliz, eta k ezberdinak erabiliz lor daitezkeen bereizmen ezberdinak ikus daitezke.

1.8. Taula. *Miaketa diseinu ezberdinak bereizmen ezberdinak eta aldagai kopuru ezberdinak kontsideratuz.*

Esperimentu kopurua	5 faktore										6 faktore					
	2^{5-1} diseinua (V bereizmena)					2^{5-2} diseinua (III bereizmena)					2^{6-2} diseinua (IV bereizmena)					
	Faktorea					Faktorea					Faktorea					
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	F
1	-1	-1	-1	-1	+1	-1	-1	-1	+1	+1	-1	-1	-1	-1	-1	-1
2	+1	-1	-1	-1	-1	+1	-1	-1	-1	-1	+1	-1	-1	-1	+1	-1
3	-1	+1	-1	-1	-1	-1	+1	-1	-1	+1	-1	+1	-1	-1	+1	+1
4	+1	+1	-1	-1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	-1	+1
5	-1	-1	+1	-1	-1	-1	-1	+1	+1	-1	-1	-1	+1	-1	+1	+1
6	+1	-1	+1	-1	+1	+1	-1	+1	-1	+1	+1	-1	+1	-1	-1	+1
7	-1	+1	+1	-1	+1	-1	+1	+1	-1	-1	-1	+1	+1	-1	-1	-1
8	+1	+1	+1	-1	-1	+1	+1	+1	+1	+1	+1	+1	+1	-1	+1	-1
9	-1	-1	-1	+1	-1						-1	-1	-1	+1	-1	+1
10	+1	-1	-1	+1	+1						+1	-1	-1	+1	+1	+1
11	-1	+1	-1	+1	+1						-1	+1	-1	+1	+1	-1
12	+1	+1	-1	+1	-1						+1	+1	-1	+1	-1	-1
13	-1	-1	+1	+1	+1						-1	-1	+1	+1	+1	-1
14	+1	-1	+1	+1	-1						+1	-1	+1	+1	-1	-1
15	-1	+1	+1	+1	-1						-1	+1	+1	+1	-1	+1
16	+1	+1	+1	+1	+1						+1	+1	+1	+1	+1	+1

Emaitzak estatistikoki interpretatu ohi dira bariantzaren analisiaren bidez (ANOVA). Aldagai bakoitzaren eragina errorearekin alderatzen da eta *F* testa burutzen da. $F_{kritiko}$ balioa bilatzen da askatasun graduen arabera eta kalkulatutako F balioarekin konparatzen da. Kalkulatutako balioa kritikoa baino txikiagoa bada, aldagai hori adierazgarria ez dela esan nahi du. Bestalde, *p* balioak aldaera bat ausaz gertatu den ala ez esaten du, beraz muga batean ezarritako balioa gainditzen bada ANOVA taulan (oro har, *p*=0.05 balioa), aldaera ausazkoa dela esan nahiko du.

ANOVA taulez gain, Pareto grafikoen bidez irudika daitezke efektu adierazgarriak. Grafikoko barrak p=0.05-k ezarritako muga gainditzen badu, aldagaia

garrantzitsua dela esan nahi du. Ikusten den zeinuak, balio gogokoena adierazten du. Beraz, zeinu positiboak, [+1] moduan kodifikatutako balioa hobeagoa dela esan nahiko du, eta alderantziz, zeinu negatiboak [-1] hobea dela. Miaketako emaitzak aztertu ondoren adierazgarriak suertatu diren aldagaiak optimizazio etapara eramango dira, eta ez-adierazgarriak diren aldagaiak finkatuko dira.

1.4.1.2 Optimizazioa. Diseinu konposatu zentrala

Optimizazioaren helburua, erantzun hoberena emango duen balioa lortzea da. Oro har, bi edo hiru faktore optimizatu ohi dira, aldagai kopurua handiagoa izanez gero diseinu matrizeak esperimentu kopuru handia beharko bailuke eta. Horregatik, miaketa etapan aldagai kopurua murriztea komeni da. Optimizazioan aldagai kuantitatiboak bakarrik azter daitezke, kualitatiborik ez. Izan ere, erantzuna faktoreen funtzio moduan modelatzen baita [Dejaegher eta Vander Heyden, 2011].

Beraz, bigarren mailako modelo polinomial bat eraikitzen da datuak deskribatzeko (ikus 1.17 ekuazioa)

$$y = b_0 + \sum_{i=1}^{f} b_i x_i + \sum_{1 \le i < j}^{f} b_{ij} x_i x_j + \sum_{i=1}^{f} b_{ii} x_i^2 \qquad (1.17)$$

1.17 ekuazioak modelo matematiko orokor bat erakusten du f aldagaientzat. Bertan efektu nagusiak (b_i), interakzio linealak (b_{ij}) eta efektu karratuak (b_{ii}) adierazten dira.

Diseinu konposatu zentral batentzat, diseinu faktorialari puntuak gehitzen zaizkio: izar puntuak eta puntu zentraleko errepikapenak. Puntu zentraleko errepikapenak bi helburu dituzte; alde batetik, errorea estimatzea eta bestetik, aurresandako erantzunaren bariantza egonkortzea [Ferreira *et al.*, 2007]. Kasu honetako esperimentu kopurua honela kalkula daiteke:

$$N = N_f + N_s + N_c \qquad (1.18)$$

N_f puntu faktorialen adierazgarria da eta $N_f = 2^f$ eginez kalkula daiteke; N_s izar puntuen adierazgarria da eta $N_s = 2f$ eginez kalkulatzen da. N_c gehitutako puntu zentral kopurua izango da. Hots, 3 aldagaiko sistema bat badugu (f=3), esperimentu

kopuru totala hemeretzi izango da bost puntu zentral gehituz gero [N= 2^3 + (2 x 3) + 5 = 8 + 6 + 5 = 19].

Izar puntuak puntu zentraletik distantzia jakin batera kokatzen dira, hurrengo bi baldintzetako bat betez [Brereton, 2003]:

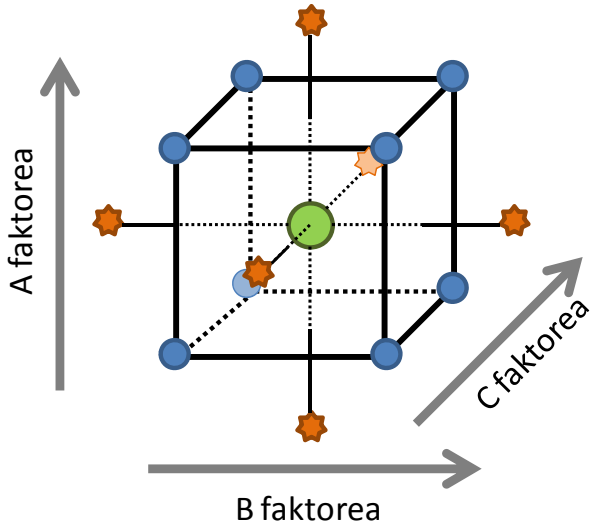
a) Errotabilitatea: aurresandako balioen konfiantza diseinuaren zentrotik dagoen distantziaren araberakoa da. Beraz, puntu zentraletik zenbat eta urrunago egon balio horren konfiantza txikiagoa izango da. Izar puntuak 1.19 ekuazioan adierazten den bezala kalkulatu eta α distantzian kokatzen dira.

$$\alpha = \sqrt[4]{N_f} \qquad (1.19)$$

b) Ortogonalitatea: 1.17 ekuazioko termino guztiak (linealak, karratuak eta interakzioak) beraien artean ortogonalak direla esan nahi du, beraz, ez dago bi terminoen arteko korrelaziorik (korrelazio koefizientea zero da). Errotabilitatea baino baldintza konplexuagoa da, ez baita erraza termino karratuen artean 0 korrelazioa aurkitzea. Kasu honetan α distantzia ondorengo ekuazioaren bidez kalkulatzen da:

$$\alpha = \sqrt{\frac{\sqrt{N \cdot N_f} - N_f}{2}} \qquad (1.20)$$

1.8 irudiak 3 aldagaiko sistema baten esperimentu puntuak erakusten ditu. Beraz, diseinua puntu faktorialez (1.8. irudia, puntu urdinak), izar puntuez (1.8. irudia, puntu laranjak) eta puntu zentralez (1.8. irudia, puntu berdea) osaturik dago.



1.8. Irudia. *Hiru aldagaientzako diseinu konposatu zentrala.*

Behin bigarren mailako modelo polinomiala eraiki denean emaitzak grafikoki ala estatistikoki eman daitezke. Oro har, modeloak bi eta hiru dimentsioko irudikapenen bidez ikus daitezke, optimoak nabarmenduz. Baina aldagai asko daudenean grafiko ezberdinen interpretazio bateratua egin behar da.

Horregatik, desiragarritasun funtzioa erabiltzen da, efektu orokorrak aurkitzeko eta aldi berean aldagaiak optimizatzeko. Funtzio honen oinarria erantzunen transformazioan datza. Beraz modeloak aurresandako erantzuna ($\hat{y}$) desiragarritasun-funtzio bilakatzen da (d) eta hau aldi berean maximizatuko da (edo minimizatu, kasuaren arabera) [Costa *et al.*, 2011]. Derringer eta Suich-ek [Derringer eta Suich, 1980] definitu zuten ondorengo desiragarritasun funtzioa (ikus 1.21 ekuazioa). Funtzio horretan sistemaren erantzun bakoitza transformatzen da eta 0 eta 1 arteko desiragarritasun balio bati egokitzen zaio (0 < d < 1), non 1 baliorik desiragarriena den (lortu nahi den erantzunik hoberena).

$$d = \begin{Bmatrix} 0 & , & \hat{y} \leq U \\ \left(\frac{\hat{y}-U}{T-U}\right)^s & , & U < \hat{y} < T \\ 1 & , & T \leq \hat{y} \end{Bmatrix} \qquad (1.21)$$

Ekuazio honetan, T helburu-balioa izango da (1), U onargarria den balio minimoa (0) eta s erabiltzaileak aukeratutako parametro bat (s>0). Ondorioz, d=1 izango da $\hat{y}$=T bada. Behin erantzunak desiragarritasun balioetara aldatu ondoren, desiragarritasun globalak erakusten dituzten azalerak irudikatzen dira eta bertan balio optimoak erraz aurki daitezke.

Behin prozesua baldintzatzen duten aldagaien balioak grafikoki edo matematikoki optimizatu ondoren, metodoaren balidazioarekin jarrai daiteke.

1.4.2. Aldagai anitzeko analisia

Kimiometriak datuak hobeto ulertzeko eta informazio gehiago lortzeko balio duten erremintak eskaintzen ditu. Aldagai anitzeko analisiak metodo ezberdinen bidez, informazioa ematen digu aldagai asko tartean daudenan. Oro har, metodo gehienak aldagai berriak sortzen dituzte (aldagai latenteak edo osagaiak) eta hauek, era berean, gure datuen inguruko informazio baliagarria emango dute. Metodo hauek matematikoki ebazten dira, algoritmo ezberdinak erabiliz. Horietako batzuk ondoren azalduko dira laburki.

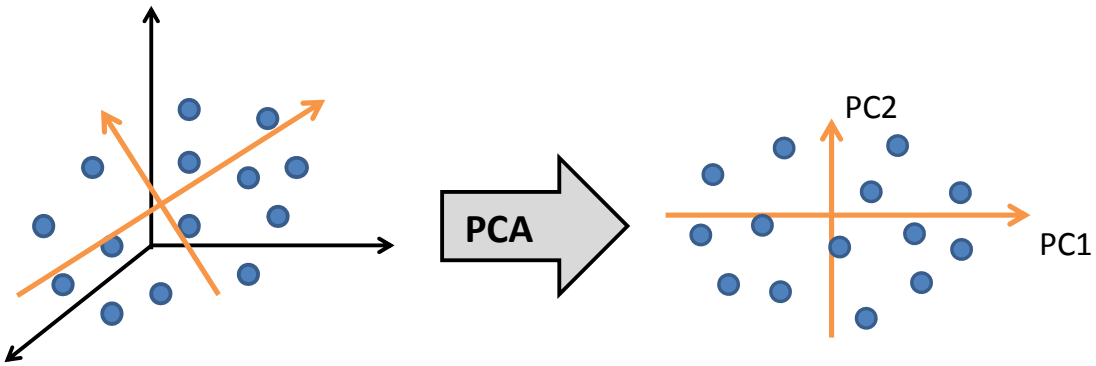
1.4.2.1. Nomenklatura

Hasteko, datozen orrietan erabiliko diren terminoak azalduko dira, modeloak deskribatzeko bereizgarriak izan daitezke eta. Formuletan hizki bakunak ageri direnean (adb. b) eskalarrak edo zenbaki soilak direla adieraziko dute; hauek letra lodiz badaude, ordea, bektoreak ordezkatuko dituzte (adb. **a**). Matrizeak letra larriz eta lodiz adieraziko dira, bi dimentsiokoak (*2-way*) badira (adb. **X**) eta azpimarratuak ere bai hiru dimentsiokoak (*3-way*) izanez gero (adb. **<u>X</u>**). "Modu" terminoa erabili ohi da, matrizearen dimentsio bakoitza adierazterakoan. Ordena handiagoko matrizeak egon badaude (*multi-way*), baina tesi honetan ez dira ageri. I, J eta K hizkiek moduen dimentsioa adierazteko erabiliko diren letrak izango dira, beraz (I x J x K) matrize bat ikusiz gero, matrize horrek hiru modu dituela adieraziko du: lehenengo moduaren

aldagai kopurua I izango da, bigarrenak J aldagai izango ditu eta hirugarren moduak K aldagai. T goi-indizeak matrizearen alderantzizkoa adierazten du (adb. $\mathbf{X}^T$). Atal honetan ageri ez diren beste sinbolo batzuk testuan bertan azalduko dira.

1.4.2.2. Principal Component Analysis (PCA)

PCA (*Principal Component Analysis*) elkarrekin erlazionatuta dauden datuen dimentsionalitatea murrizten duen deskonposaketa metodo bat da, betiere ahalik eta datuek ordezkatzen duten aldaerari buruzko informazio maximoa mantenduz [Kumar *et al.*, 2014]. Jatorriko aldagaiak osagai nagusiak (*Principal Component*, PC) izeneko aldagai berri batzuetara eraldatzen dira eta hauek datuen joera nagusiak deskribatuko dituzte (ikus 1.9 irudia). Lehen PC-ak datuen aldakortasun maximoa deskribatuko du, bigarren PC-a aurrenekoarekiko ortogonala izango da eta bigarren aldakortasun maximoa aurkituko du; hirugarrena, aurrekoekin ere ortogonala izango da eta ondorengo aldaera maximoa aurkituko du, etab. PC bakoitzak datuen bariantza zehatz bat deskribatuko du eta metatutako azaldutako bariantza bezala adieraziko da. Azaldutako bariantza horrek gure datuak deskribatzeko behar diren PC kopuruari buruzko informazioa emango digu, halere erabakia ikerlariaren menpe dago, eta horretarako beste parametro batzuk ere hartu beharko dira kontuan.



1.9. Irudia. *Datu multzo baten PCA eraldaketa 2 osagai nagusi (PC) erabiliz.*

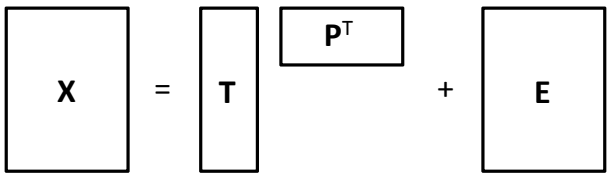
Oro har, PCA-k jatorrizko aldagaiak, PC kopuru murritzago batzuetan bihurtzen ditu. 1.9 irudiko adibidean ikus daiteke, hasierako datuak hiru dimentsiotako sistema batean kokatuta daudela, bada PCA eginez, datu horien aldakortasuna deskribatzeko 2 osagai nahikoa direla ikus daiteke. Aldagai berriak (PC1 eta PC2) elkarrekiko ortogonalak dira, eta datuak deskribatuko dituzten "ardatz" berriak izango dira. Hala eta guztiz ere, PCA-ren interpretazioa nekeza izan daiteke, ez baitu benetako informazio kimikoaren profilarekin bat egiten beste kasu batzuetan bezala.

PCA-k modelo bilineal bat jarraitzen du, hau da 1.22 ekuazioaren forma izango du. **X** matrizea I lagin eta J aldagaiez osatuta egongo da eta matrizeko elementu bakoitza (x_{ij}) honela deskribatuko da, R osagai kopuruarekin:

$$x_{ij} = \sum_{r=1}^{R} t_{ir} p_{jr} + e_{ij} \qquad (1.22)$$

PCA-k jatorrizko matrize datua (**X** (IxJ)) *score* matrize (**T** (IxR)) eta *loading* matrize (**P** (JxR)) batean bilakatuko ditu. *Loading*-ak PC berrien eta jatorrizko ardatzen arteko erlazioa adieraziko dute; *score*-ak, aldiz, laginek aldagai berri horietan (*loading*-etan) duten proiektatutako balioa adierazten dute [Bro eta Smilde, 2014]. **E** (IxJ) hondar-matrizea izango da, hasierako matrizearen dimentsio berdinekoa eta modeloak azaldu ezin duen zatia azalduko du, beste era batera esanda, errorea.

$$\boldsymbol{X} = \boldsymbol{T}\boldsymbol{P}^{T} + \boldsymbol{E} \qquad (1.23)$$



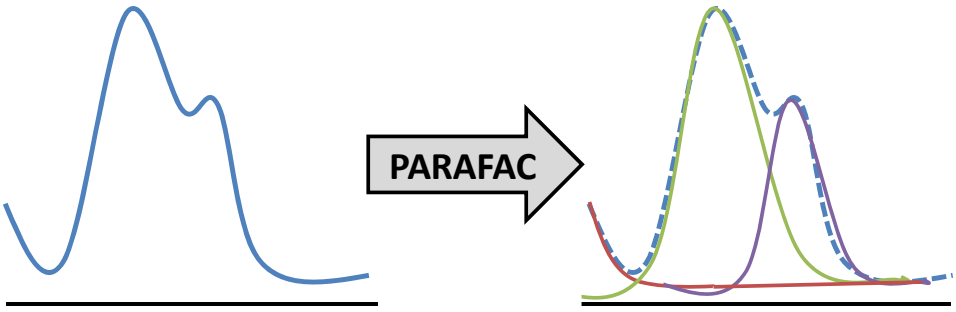
***1.10. Irudia**. PCA modelo matematikoaren irudikapen grafikoa. Lerroak bektoreak adierazten dituzte, aldiz, karratuek 2-dimentsioko matrizeak.*

PCA-k 1.23 ekuazioa jarraitzen du, eta ekuazio hau grafikoki 1.10 irudian ikus daiteke. PCA helburu kualitatiboekin erabil daiteke, informazio interesgarria eman baitezake, baina ezin da kalibrazio helburuekin erabili. Bibliografia zabala aurki daiteke PCA-ri buruz [Munck *et al.*, 1998; Bro, 2003; Smilde *et al.*, 2004; Bro eta Smilde, 2014].

Beste metodo asko PCA-n oinarrituta daude, esaterako, PCR, PLS, PARAFAC edota MCR.

1.4.2.3. Parallel Factor Analysis (PARAFAC)

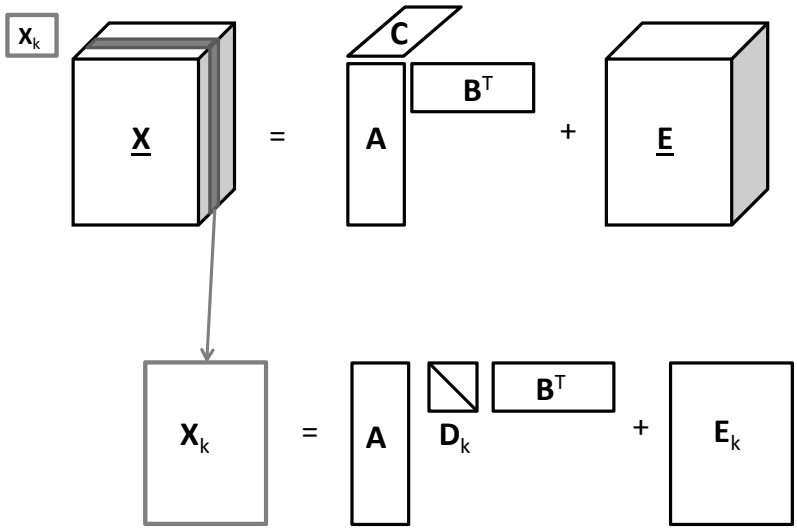
PARAFAC (Parallel Factor Analysis) PCA-ren jarraipena bezala kontsidera daiteke maila altuagoko datuentzat [Bro, 1997], baina emaitza bisualki interpretatzeko errazagoak ematen ditu. Helburua bera da, aldagai kopurua murriztea, aldagai berri batzuen bitartez. PCA-ren interpretazioa zaila eta abstraktua izan daiteke kasu batzuetan, aldiz, PARAFAC interpretatzeko errazagoa da, osagaiek informazio kimikoa ordezkatzen dutelako. 1.11 irudian ikus daiteke adibide bat: bertan kromatograma zati bat ikus daiteke gailur gainezarriekin; PARAFAC-ek jatorri kimikoak dituen osagaiak aurkituko ditu, eta kasu honetan hiru kontribuzio ezberdin aurki ditzake. Alde batetik, gailur baten buztana den zati bat (gorria), eta bestetik gainezartzen diren bi gailurrak (berdea eta morea). Izan ere, kromatografiaren kasuan, PARAFAC osagai bakoitzak analito bakoitzaren eluzio eta espektro-profilak deskriba ditzake [Amigo *et al.*, 2010].



1.11. Irudia. *Hiru osagaiekin egindako PARAFAC deskonposaketa.*

Grafikoki modu berean interpretatzen ez bada ere, PCA-k eta PARAFAC-ek antzekotasun handia dute modelo matematikoak begiratuz gero. PCA modelo bilineal bat bada, PARAFAC hiru dimentsiotara zabaltzen den modelo trilineala izango da (ikus 1.12 irudia) [Smilde *et al.*, 2004]:

$$x_{ijk} = \sum_{r=1}^{R} a_{ir} b_{jr} c_{jr} + e_{ijk} \quad (1.24)$$



1.12. Irudia. *R osagaidun PARAFAC modeloaren irudikapena. Lerroek bektoreak ordezkatzen dituzte, karratuek eta kuboek bi eta hiru dimentsioko matrizeak, hurrenez hurren.*

X matrizeko x_{ijr} elementuak horrela deskribatuko dira eta deskonposaketan ikus daitekeen k geruza honela deskribatzen da [Smilde *et al.*, 2004]:

$$\boldsymbol{X}_k = \boldsymbol{A}\boldsymbol{D}_K\boldsymbol{B}^T + \boldsymbol{E}_k \quad (1.25)$$

$\mathbf{X}_k$ geruza bakoitza A eta B *loading* berdinarekin deskribatzen dira (eluzio eta espektro *loading*-ak), baina geruza bakoitzak izango duen pisu edo kontzentrazio erlatiboa ($\mathbf{D}_k$) ezberdina izango da. $\mathbf{D}_k$ matrize hau k lerro dituen matrize diagonala izango da non **C** (KxR) matrizearen informazioa jasoko duen eta (c_{k1}, c_{k2}…, c_{kR}) elementuez osatuta egongo da. **C** osagai bakoitzaren kontzentrazioak ordezkatuko dituen *loading*-a izango da.

PARAFAC helburu kualitatiboekin zein kuantitatiboekin erabil daiteke, izan ere, kontzentrazio erlatiboei buruzko informazioa ematen baitu, eta hauek benetako kontzentrazioekin erlaziona daitezke. PARAFAC-en abantaila nagusia emaitza bakar bat lortzen duela, hein-urritasun arazorik ez badago behintzat [Sidiropoulos eta Bro, 2000; Omidikia *et al.*, 2013].

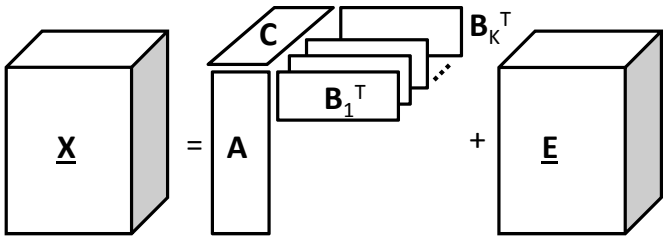
1.4.2.4. Parallel Factor Analysis 2 (PARAFAC2)

Aldagai anitzeko metodo gehienek, analisi ezberdinen arteko profilak guztiz berdinak direla onartzen dute, baina kasu batzuetan, analisien arteko ezberdintasunak egon daitezke. Hots, kromatografian eluzio-denboren desplazamenduak ikustea ohikoa da [Bro *et al.*, 1999]. Kontrolaezinak diren baldintza esperimentalak alda daitezke, hala nola, presioa, fluxua edota erabileraren ondorioz gerta daitezkeen zutabe kromatografikoaren aldaketa txikiak. Horrek eluzio-denboran aldaketak eragin ditzake. Oro har, aldagai anitzeko metodoak aldaketa hauek aurresateko ez dira gai, baina PARAFAC2 aldaketa txiki hauek barneratzeko gaitasunarekin sortu zen [Kiers *et al.*, 1999].

PARAFAC2-ak PARAFAC modura datu matrizea 3 kontribuzio ezberdinetan banatzen du: kontzentrazio erlatiboak (pisua), eluzio-profila eta espektro-profila. Baina kasu honetan, PARAFAC-ek ez bezala, PARAFAC2-ak lagin kopurua bezain beste eluzio-profil ematen ditu, hau da, lagin bakoitzak du bere eluzio-profil konkretua. Horrez gain, PARAFAC2-ak ez du inongo mugatzailerik jartzen profil horretan, laginek forma eta luzera bera dutela onartzen baitu [Amigo *et al.*, 2008].

Matematikoki PARAFAC2 PARAFAC-en oso antzekoa da. Esan bezala ezberdintasuna **B** matrizean dago, kasu honetan bakar bat ez, K aldagai (lagin kopuru) adina **B** matrize izango ditugu (ikus 1.13 irudia).

$$\boldsymbol{X}_k = \boldsymbol{A}\boldsymbol{D}_k\boldsymbol{B}_k^T + \boldsymbol{E}_k \qquad (1.26)$$



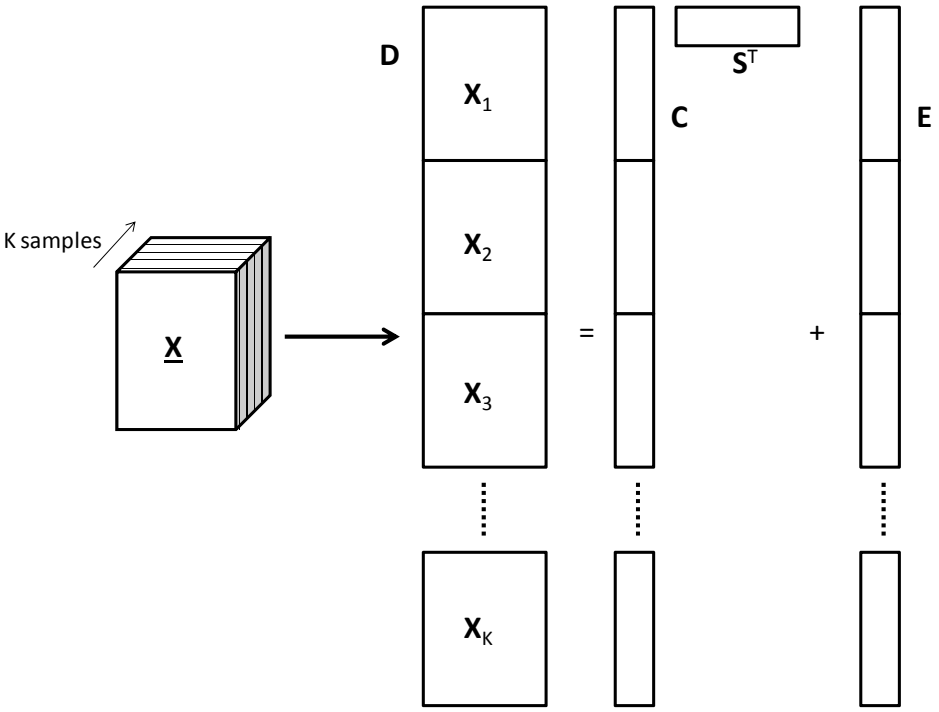
1.13. Irudia. *PARAFAC2 modeloaren irudikapen grafikoa. Lerroek bektoreak ordezkatzen dituzte, karratuek eta kuboek bi eta hiru dimentsioko matrizeak, hurrenez hurren.*

Beraz, PARAFAC2-ak emango digun emaitza honakoa izango da: kontzentrazio erlatibo edo pisuaren profila (**A**), espektro profil bat (**C**) eta lagin bezain beste eluzio-profil (**B**).

Eluzio-profil independenteak lortuko ditugunez, PARAFAC2-aren abantaila nagusia PARAFAC-en aurrean, denbora eskatzen duten aurretratamenduen (lerrokatzea) beharrik eza izango litzateke.

1.4.2.5. Multivariate Curve Resolution (MCR)

MCR (*Multivariate Curve Resolution*) modelo bilineal bat da, baina datu trilinealak maneiatzeko ere gai da, matrizearen berrantolaketa baten bidez. Helburu honetarako **X** (IxJxK) matrizea zutabe-norabidean antolatzen da matrize berri bat sortuz (**D** (IKxJ)) 1.14 irudian ikus daitekeen bezala. MCR-k matrizeak bi osagaietan banatuko ditu: kontzentrazio (**C** (IKxR)) eta espektro profilak (**S** (JxR)), R osagai kopurua izango delarik. Iterazio metodo bat da MCR, beraz hasierako estimazio baten optimizazioaren bidez iritsiko da konbergentzia puntu batera ALS (*Alternating Least Squares*) erabiliz [Tauler, 1995]. MCR datu trilinealekin nahiz ez-trilinealekin erabil daiteke, PARAFAC metodoak, ordea, datu trilinealekin bakarrik erabil daitezke. Ondorioz, MCR-ak teknika ezberdinekin neurtutako datuak elkarrekin aztertzea ahalbidetu dezake.



***1.14**. **Irudia**. MCR modeloaren irudikapena. Lehenengo matrizearen berrantolaketa ikus daiteke eta ondoren kontzentrazio (**C**) eta espektro-profilaren (**S**) deskonposaketa.*

MCR modelo matematikoaren itxura PCA-renaren oso antzekoa da, nahiz eta esanahi grafikoa guztiz ezberdina izan. Izan ere, MCR-ko osagaiak ez dira elkarrekiko ortogonalak eta informazio kimiko esanguratsua dute, interpretatzeko errazagoa izanik [Jaumot eta Tauler, 2010].

$$\boldsymbol{D} = \boldsymbol{C}\boldsymbol{S}^{T} + \boldsymbol{E} \qquad (1.27)$$

MCR-k, ordea, ez du emaitza edo soluzio bakar bat ematen, anbiguotasun errotazionala ager daitekeelako. Benetako soluzio bakar bat lortu ahal izateko modeloa mugatzaileen bidez kontrolatu behar da [Abdollahi eta Tauler, 2011]. Mugatzaile hauek forma matematiko batera eramaten dira prozesu iteratiboa baldintzatuz [de Juan eta Tauler, 2003]. Oro har, gehien erabiltzen diren mugatzaileak ez-negatibotasuna, unimodalitatea edo masa-balantzea (propietate kimikoak ordezkatzen dituztenak) eta selektibotasun-tartea, egitura trilineala, etab. (esanahi matematikoa dutenak) izan daitezke [de Juan eta Tauler, 2006].

1.4.2.6. Parallel Factor Analysis with Linear Dependencies (PARALIND)

PARALIND (*Parallel Factor Analysis with Linear Dependencies*), PARAFAC-en oinarritutako metodo bat da, hein-urritasuna duten datuentzako diseinatua. Aldakortasun iturriek (seinale kromatografiko bat esaterako), efektu independenteak izan ditzakete bi moduetan, baina elkarren arteko mendekotasun bat egon daiteke hirugarren moduan. Hein-urritasuna deritze eta soluzio ez bakarretara eta nahasgarrietara eraman gaitzake PARAFAC modeloa erabiliz gero [Bro *et al.*, 2009].

PARALIND-ek dependentzia matrize bat gehitzen du hein-urritasuna modeloan sartzeko. Beraz, PARAFAC modeloa jarrai dezake [Bahram eta Bro, 2007]:

$$\boldsymbol{X}_k = \overline{\boldsymbol{A}}\boldsymbol{D}_K\boldsymbol{B}^T + \boldsymbol{E}_k \quad (1.28)$$

Baldin eta:

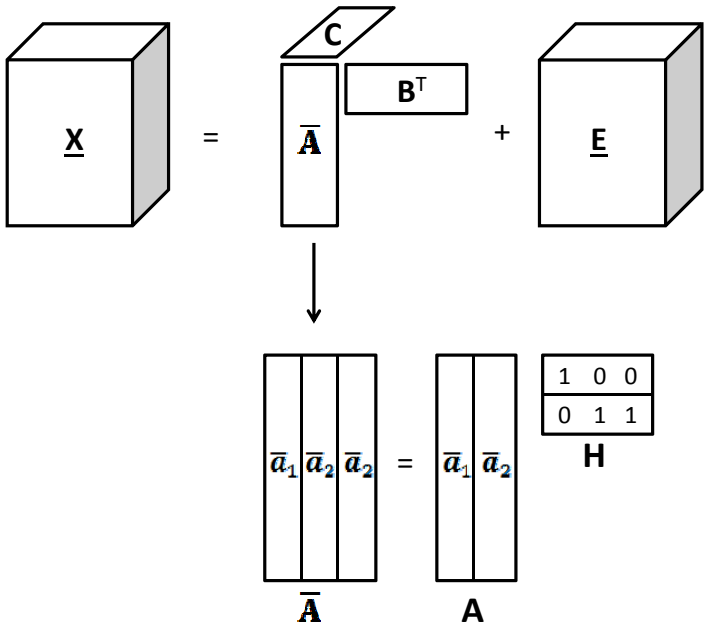
$$\overline{\boldsymbol{A}} = \boldsymbol{AH} \quad (1.29)$$

Non, **A** (IxR) matrizea eta **H** (RxS) matrizeak diren. Ondorioz, $\overline{\boldsymbol{A}}$ (IxS) matrizea izango da PARAFAC modeloan hein-urritasuna sartuko duen matrizea. R hein-urritasuneko moduaren osagai kopurua izango da eta, aldiz, S beste bi moduek izango duten osagai kopurua. Demagun N + M → P erreakzio bat dugula, eta N eta M-ren hasierako kontzentrazioak berdinak direla. Beraien estekiometria 1:1 denez, beraien agortzea modu berdinean emango da, eta beraz 2 kontzentrazio profil ezberdin izango ditugu (N eta M-k profil bera dutelako), nahiz eta 3 konposatu egon. Kasu horretan, dependentzia matrizeak honelako itxura izango du [Bro *et al.*, 2009]:

$$\boldsymbol{H} = \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 1 \end{bmatrix} \quad (1.30)$$

Dependentzia matrize horrek modeloak bi profil berberak direla jakin dezake. Beraz, $\boldsymbol{A} = [\overline{\boldsymbol{a}}_1\overline{\boldsymbol{a}}_2]$ izanik, $\overline{\boldsymbol{A}} = \boldsymbol{AH} = [\overline{\boldsymbol{a}}_1\overline{\boldsymbol{a}}_2\overline{\boldsymbol{a}}_2]$ izango baita. Hau kimikoki honela interpreta daiteke: lehenengo moduak bi fenomeno deskribatuko ditu, baina beste bi moduetan 3 fenomenoekin deskribatuko dira datu hauek. Beste era batera esanda, $\overline{\boldsymbol{A}}$-k 3 fenomeno ordezkatzen ditu, beste bi moduak bezala, baina $\overline{\boldsymbol{A}}$ hori **A** eta **H**-ren

araberakoa izango da eta guri interpretatu ahal izateko **A** interesatzen zaigu (ikus 1.15 irudia).



1.15. Irudia. *PARALIND modelo matematikoaren irudikapena, non* $\bar{A}$ *matrizea hein-urritasuna duen* ***A*** *matrizean eta bere* ***H*** *dependentzia matrizean deskonposatzen den.*

Beraz, PARALIND algoritmoak A profila emango digu (kasu honetan kontzentrazio profilari dagokiona), bi fenomeno deskribatuko dituena (beraz, 2 osagai ditu), eta beste aldetik **B** eta **C** profilak (eluzio eta espektro profilak) emango dizkigu bakoitza 3 osagaiekin, hondar-matrizeaz (**E**) gain.

1.5 Erreferentziak

Abdollahi, H. eta Tauler, R.; *Uniqueness and rotation ambiguities in Multivariate Curve Resolution methods.* **2011.** Chemometrics and Intelligent Laboratory Systems, 108(2), 100-111.

Abdulra'uf, L.B., Chai, M.K. eta Tan, G.H.; *Applications of Solid-Phase Microextraction for the Analysis of Pesticide Residues in Fruits and Vegetables: A Review.* **2012.** Journal of AOAC International, 95(5), 1272-1290.

Ai, J., *Headspace Solid Phase Microextraction. Dynamics and Quantitative Analysis before Reaching a Partition Equilibrium.* **1997.** Analytical Chemistry, 16, 3260-3266.

Allen, J.W., Wolf, D.C., George, M.H., Hester, S.D., Sun, G., Thai, S., Delker, D.A., Moore, T., Jones, C., Nelson, G., Roop, B.C., Leavitt, S., Winkfield, E., Ward, W.O. eta Nesnow, S.; *Toxicity Profiles in Mice Treated with Hepatotumorigenic and Non-Hepatotumorigenic Triazole Conazole Fungicides: Propiconazole, Triadimefon, and Myclobutanil.* **2006.** Toxicologic Pathology, 34(7), 853-862.

Alpendurada, M.F., *Solid-phase microextraction: a promising technique for sample preparation in environmental analysis.* **2000.** Journal of Chromatography A, 889(1–2), 3-14.

Amigo, J.M., Skov, T. eta Bro, R.; *ChroMATHography: Solving Chromatographic Issues with Mathematical Models and Intuitive Graphics.* **2010.** Chemical Reviews, (110), 4582-4605.

Amigo, J.M., Skov, T., Bro, R., Coello, J. eta Maspoch, S.; *Solving GC-MS problems with PARAFAC2.* **2008.** Trends in Analytical Chemistry, 27(8), 714-725.

Andrašcíková, M., Matisová, E. eta Hrouzková, S.; *Liquid Phase Microextraction Techniques as a Sample Preparation Step for Analysis of Pesticide Residues in Food.* **2015.** Separation & Purification Reviews, 44, 1-18.

Aresta, A., Vatinno, R., Palmisano, F. eta Zambonin, C.G.; *Determination of Ochratoxin A in wine at sub ng/mL levels by solid-phase microextraction coupled to liquid chromatography with fluorescence detection.* **2006.** Journal of Chromatography A, 1115(1-2), 196-201.

Arthur, C.L. eta Pawliszyn, J.; *Solid Phase Microextraction with Thermal Desorption Using Fused Silica Optical Fibers.* **1990.** Analytical Chemistry, 62, 2145-2148.

Aulakh, J.S., Malik, A.K., Kaur, V. eta Schmitt-Kopplin, P.; *A Review on Solid Phase Micro Extraction—High Performance Liquid Chromatography (SPME-HPLC) Analysis of Pesticides.* **2005.** Critical Reviews in Analytical Chemistry, 35(1), 71-85.

Bahram, M. eta Bro, R.; *A novel strategy for solving matrix effect in three-way data using parallel profiles with linear dependencies.* **2007.** Analytica Chimica Acta, 584(2), 397-402.

Beceiro-González, E., Concha-Graña, E., Guimaraes, A., Gonçalves, C., Muniategui-Lorenzo, S. eta Alpendurada, M.F.; *Optimisation and validation of a solid-phase microextraction method for simultaneous determination of different types of pesticides in water by gas chromatography–mass spectrometry.* **2007.** Journal of Chromatography A, 1141(2), 165-173.

Beltran, J., López, F.J. eta Hernández, F.; *Solid-phase microextraction in pesticide residue analysis.* **2000.** Journal of Chromatography A, 885(1-2), 389-404.

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S. eta Escaleira, L.A.; *Response surface methodology (RSM) as a tool for optimization in analytical chemistry.* **2008.** Talanta, 76(5), 965-977.

Box, G.E.P., Hunter, W.G. eta Hunter, J.S.; *Statistics for experimenters. An introduction to design, data analysis, and model building.* **1978.** John Wiley & Sons. New York (USA).

Brereton, R.G.; *Chemometrics. Data Analyisi for the Laboratory and Chemical Plant.* **2003.** 1 edn. John Wiley & Sons Ltd. Chichester (UK).

Bro, R., *PARAFAC. Tutorial and applications.* **1997.** Chemometrics and Intelligent Laboratory Systems, 38(2), 149-171.

Bro, R., *Multivariate calibration: What is in chemometrics for the analytical chemist?* **2003.** Analytica Chimica Acta, 500(1–2), 185-194.

Bro, R., Andersson, C. eta Kiers, H.A.L.; *PARAFAC2: Part II. Modeling chromatographic data with retention time shifts.* **1999.** Journal of Chemometrics, 13(3-4), 295-309.

Bro, R., Harshman, R.A., Sidiropoulos, N.D. eta Lundy, M.E.; *Modeling multi-way data with linearly dependent loadings.* **2009.** Journal of Chemometrics, 23(7-8), 324-340.

Bro, R. eta Smilde, A.K.; *Principal component analysis.* **2014.** Analytical Methods, 6, 2812-2831.

Buszewski, B. eta Szultka, M.; *Past, present, and future of solid phase extraction: A review.* **2012.** Critical Reviews in Analytical Chemistry, 42, 198-213.

Chen, J. eta Pawliszyn, J.; *Solid Phase Microextraction Coupled to High- Performance Liquid Chromatography.* **1995.** Analytical Chemistry, 67(15), 2530-2533.

Cheng, J., Matsadiq, G., Liu, L., Zhou, Y. eta Chen, G.; *Development of a novel ultrasound-assisted surfactant-enhanced emulsification microextraction method and its application to the analysis of eleven polycyclic aromatic hydrocarbons at trace levels in water.* **2011a.** Journal of Chromatography A, 1218(18), 2476-2482.

Cheng, J., Xia, Y., Zhou, Y., Guo, F. eta Chen, G.; *Application of an ultrasound-assisted surfactant-enhanced emulsification microextraction method for the analysis of diethofencarb and pyrimethanil fungicides in water and fruit juice samples.* **2011b.** Analytica Chimica Acta, 701(1), 86-91.

Cortada, C., Vidal, L. eta Canals, A.; *Determination of geosmin and 2-methylisoborneol in water and wine samples by ultrasound-assisted dispersive liquid–liquid microextraction coupled to gas chromatography–mass spectrometry.* **2011.** Journal of Chromatography A, 1218(1), 17-22.

Costa, N.R., Lourenço, J. eta Pereira, Z.L.; *Desirability function approach: A review and performance evaluation in adverse conditions.* **2011.** Chemometrics and Intelligent Laboratory Systems, 107(2), 234-244.

de Juan, A. eta Tauler, R.; *Chemometrics applied to unravel multicomponent processes and mixtures: Revisiting latest trends in multivariate resolution.* **2003.** Analytica Chimica Acta, 500(1–2), 195-210.

de Juan, A. eta Tauler, R.; *Multivariate curve resolution (MCR) from 2000: progress in concepts and applications.* **2006.** Critical Reviews in Analytical Chemistry, 36(3-4), 163-176.

Dehghani Mohammad Abadi, M., Ashraf, N., Chamsaz, M. eta Shemirani, F.; *An overview of liquid phase microextraction approaches combined with UV–Vis spectrophotometry.* **2012.** Talanta, 99, 1-12.

Dejaegher, B. eta Vander Heyden, Y.; *The use of experimental design in separation science.* **2009.** Acta Chromatographica, 21(2), 161-201.

Dejaegher, B. eta Vander Heyden, Y.; *Experimental designs and their recent advances in set-up, data interpretation, and analytical applications.* **2011.** Journal of pharmaceutical and biomedical analysis, 56(2), 141-158.

Derringer, G. eta Suich, R.; *Simultaneous optimization of several response variables.* **1980.** Journal of Quality Technology, 12(4), 214-219.

Echeverría-Sáenz, S., Mena, F., Pinnock, M., Ruepert, C., Solano, K., de la Cruz, E., Campos, B., Sánchez-Avila, J., Lacorte, S. eta Barata, C.; *Environmental hazards of pesticides from pineapple crop production in the Río Jiménez watershed*

(Caribbean Coast, Costa Rica). **2012.** Science of The Total Environment, 440, 106-114.

Ekman, D.R., Keun, H.C., Eads, C.D., Furnish, C.M., Murrell, R.N., Rockett, J.C. eta Dix, D.J.; *Metabolomic evaluation of rat liver and testis to characterize the toxicity of triazole fungicides.* **2006.** Metabolomics, 2(2), 63-73.

Ettre, L.S., *Nomenclature for chromatography (IUPAC recommendations 1993).* **1993.** Pure and Applied Chemistry, 65(4), 819-872.

Fernandez-Alvarez, M., Llompart, M., Lamas, J.P., Lores, M., García-Jares, C., Cela, R. eta Dagnac, T.; *Development of a solid-phase microextraction gas chromatography with microelectron-capture detection method for a multiresidue analysis of pesticides in bovine milk.* **2008.** Analytica Chimica Acta, 617(1-2), 37-50.

Ferreira, S.L.C., Bruns, R.E., da Silva, E.G.P., dos Santos, W.N.L., Quintella, C.M., David, J.M., de Andrade, J.B., Breitkreitz, M.C., Jardim, I.C.S.F. eta Neto, B.B.; *Statistical designs and response surface techniques for the optimization of chromatographic systems.* **2007.** Journal of Chromatography A, 1158(1-2), 2-14.

Ganjali, M.R., Sobhi, H.R., Farahani, H., Norouzi, P., Dinarvand, R. eta Kashtiaray, A.; *Solid drop based liquid-phase microextraction.* **2010.** Journal of Chromatography A, 1217(16), 2337-2341.

Ghambarian, M., Yamini, Y. eta Esrafili, A.; *Liquid-phase microextraction based on solidified drops of organic solvents.* **2013.** Microchimica Acta, 180, 519-535.

Gilbert-López, B., García-Reyes, J.F. eta Molina-Díaz, A.; *Sample treatment and determination of pesticide residues in fatty vegetable matrices: A review.* **2009.** Talanta, 79(2), 109-128.

Goetz, A.K., Ren, H., Schmid, J.E., Blystone, C.R., Thillainadarajah, I., Best, D.S., Nichols, H.P., Strader, L.F., Wolf, D.C., Narotsky, M.G., Rockett, J.C. eta Dix, D.J.; *Disruption of Testosterone Homeostasis as a Mode of Action for the Reproductive Toxicity of Triazole Fungicides in the Male Rat.* **2007.** Toxicological Sciences, 95(1), 227-239.

Han, D. eta Row, K.; *Trends in liquid-phase microextraction, and its application to environmental and biological samples.* **2012.** Microchimica Acta, 176(1), 1-22.

Hu, Y., Yang, X., Wang, C. eta Zhao J.; *A sensitive determination method for carbendazim and thiabendazole in apples by solid-phase microextraction−high performance liquid chromatography with fluorescence detection.* **2008.** Food Additives & Contaminants: Part A, 25(3), 314-319.

Jaumot, J. eta Tauler, R.; *MCR-BANDS: A user friendly MATLAB program for the evaluation of rotation ambiguities in Multivariate Curve Resolution.* **2010.** Chemometrics and Intelligent Laboratory Systems, 103(2), 96-107.

Jeannot, M.A. eta Cantwell, F.F.; *Solvent Microextraction into a Single Drop.* **1996.** Analytical Chemistry, 68(13), 2236-2240.

Jeannot, M.A., Przyjazny, A. eta Kokosa, J.M.; *Single drop microextraction—Development, applications and future trends.* **2010.** Journal of Chromatography A, 1217(16), 2326-2336.

Kataoka, H., Lord, H.L. eta Pawliszyn, J.; *Applications of solid-phase microextraction in food analysis.* **2000.** Journal of Chromatography A, 880(1-2), 35-62.

Kiers, H.A.L., ten Berge, J.M.F. eta Bro, R.; *PARAFAC2: Part I. A direct fitting algorithm for the PARAFAC2 model.* **1999.** Journal of Chemometrics, 13(3-4), 275-294.

Krylov, V., Krylov, A., Mosyagin, P. eta Matkivskaya, Y.; *Liquid-phase microextraction preconcentration of impurities.* **2011.** Journal of Analytical Chemistry, 66(4), 331-350.

Kumar, A. eta Malik, A.K.; *A Review on the Hyphenation of Solid Phase Microextraction with Capillary Electrophoresis and Mass Spectrometry.* **2009.** Critical Reviews in Analytical Chemistry, 39, 81-88.

Kumar, N., Bansal, A., Sarma, G.S. eta Rawal, R.K.; *Chemometrics tools used in analytical chemistry: An overview.* **2014.** Talanta, 123, 186-199.

Lambropoulou, D.A. eta Albanis, T.A.; *Liquid-phase micro-extraction techniques in pesticide residue analysis.* **2007.** Journal of Biochemical and Biophysical Methods, 70(2), 195-228.

Li, Y., Yang, X., Zhang, J., Li, M., Zhao, X., Yuan, K., Li, X., Lu, R., Zhou, W. eta Gao, H.; *Ultrasound-assisted emulsification magnetic microextraction: a fast and green method for the determination of triazole fungicides in fruit juice.* **2014.** Analytical Methods, 6, 8328-8336.

Lord, H.L., *Strategies for interfacing solid-phase microextraction with liquid chromatography.* **2007.** Journal of Chromatography A, 1152(1-2), 2-13.

Lord, H. eta Pawliszyn, J.; *Evolution of solid-phase microextraction technology.* **2000.** Journal of Chromatography A, 885(1–2), 153-193.

Luque de Castro, M.D. eta Priego-Capote, F.; *Ultrasound-assisted preparation of liquid samples.* **2007.** Talanta, 72(2), 321-334.

Mahugo-Santana, C., Sosa-Ferrera, Z., Torres Padrón, M. eta Santana Rodríguez, J.J.; *Application of new approaches to liquid-phase microextraction for the*

determination of emerging pollutants. **2011.** Trends in Analytical Chemistry, 30(5), 731-748.

Millán, S., Sampedro, M.C., Unceta, N., Goicolea, M.A., Rodríguez, E. eta Barrio, R.J.; *Coupling solid-phase microextraction and high-performance liquid chromatography for direct and sensitive determination of halogenated fungicides in wine.* **2003.** Journal of Chromatography A, 995(1-2), 135-142.

Mmualefe, L.C., Torto, N., Huntsman-Mapila, P. eta Mbongwe, B.; *Headspace solid phase microextraction in the determination of pesticides in water samples from the Okavango Delta with gas chromatography-electron capture detection and time-of-flight mass spectrometry.* **2009.** Microchemical Journal, 91(2), 239-244.

Mohamadi, M. eta Mostafavi, A.; *A novel solidified floating organic drop microextraction based on ultrasound-dispersion for separation and preconcentration of palladium in aqueous samples.* **2010.** Talanta, 81(1–2), 309-313.

Munck, L., Nørgaard, L., Engelsen, S.B., Bro, R. eta Andersson, C.A.; *Chemometrics in food science—a demonstration of the feasibility of a highly exploratory, inductive evaluation strategy of fundamental scientific significance.* **1998.** Chemometrics and Intelligent Laboratory Systems, 44(1–2), 31-60.

Navarro, S., Vela, N., Pérez, G. eta Navarro, G.; *Effect of sterol biosynthesis-inhibiting (SBI) fungicides on the fermentation rate and quality of young ale beer.* **2011.** Food Chemistry, 126(2), 623-629.

Noguerol-Pato, R., González-Rodríguez, R.M., González-Barreiro, C., Cancho-Grande, B. eta Simal Gándara, J.; *Influence of tebuconazole residues on the aroma composition of Mencía red wines.* **2011.** Food Chemistry, 124(4), 1525-1532.

Omidikia, N., Abdollahi, H. eta Kompany-Zareh, M.; *Visualization and establishment of partial uniqueness and uniqueness rules in parallel factor analysis.* **2013.** Journal of Chemometrics, 27(10), 330-340.

Ozcan, S., Tor, A. eta Aydin, M.E.; *Determination of polycyclic aromatic hydrocarbons in waters by ultrasound-assisted emulsification-microextraction and gas chromatography–mass spectrometry.* **2010.** Analytica Chimica Acta, 665(2), 193-199.

Pakade, Y.B. eta Tewary, D.K.; *Development and applications of single-drop microextraction for pesticide residue analysis: A review.* **2010.** Journal of Separation Science, 33(23-24), 3683-3691.

Parrilla-Vázquez, P., Mughari, A.R. eta Martínez-Galera, M.; *Solid-phase microextraction (SPME) for the determination of pyrethroids in cucumber and watermelon using liquid chromatography combined with post-column*

photochemically induced fluorimetry derivatization and fluorescence detection. **2008.** Analytica Chimica Acta, 607(1), 74-82.

Pawliszyn, J.; *Handbook of Solid Phase Microextraction.* **2009.** 1 edn. Chemical Industry Press. Beijing (China).

Pawliszyn, J.; *Solid Phase Microextraction. Theory and Practice.* **1997.** 1 edn. Wiley-VCH. New York (USA).

Pawliszyn, J. eta Pedersen-Bjergaard, S.; *Analytical Microextraction: Current Status and Future Trends.* **2006.** Journal of Chromatographic Science, 44(6), 291-307.

Pena-Pereira, F., Lavilla, I. eta Bendicho, C.; *Liquid-phase microextraction techniques within the framework of green chemistry.* **2010.** Trends in Analytical Chemistry, 29(7), 617-628.

Peñalver, A., Pocurull, E., Borrull, F. eta Marcé, R.M.; *Trends in solid-phase microextraction for determining organic pollutants in environmental samples.* **1999.** Trends in Analytical Chemistry, 18(8), 557-568.

Pinto, M.I., Sontag, G., Bernardino, R.J. eta Noronha, J.P.; *Pesticides in water and the performance of the liquid-phase microextraction based techniques. A review.* **2010.** Microchemical Journal, 96(2), 225-237.

Ravelo-Pérez, L.M., Hernández-Borges, J., Borges-Miquel, T.M. eta Rodríguez-Delgado, M.A.; *Pesticide analysis in tomatoes by solid-phase microextraction and micellar electrokinetic chromatography.* **2008.** Journal of Chromatography A, 1185(1), 151-154.

Regueiro, J., Llompart, M., Garcia-Jares, C., Garcia-Monteagudo, J.C. eta Cela, R.; *Ultrasound-assisted emulsification–microextraction of emergent contaminants and pesticides in environmental waters.* **2008.** Journal of Chromatography A, 1190(1–2), 27-38.

Regulation EC 396/2005 of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. Official Journal of the European Union. **(16.3.2005)** L 70/1-16. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EC 149/2008 of 29 January 2008 amending Regulation (EC) No 396/2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto. Official Journal of the European Union. **(1.3.2008)** L 58/1-398. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EC 459/2010 of 27 May 2010 amending Annexes II, III and IV to Regulation (EC) No 396/2005 of hte European Parliament and of the council as regards maximum residue levels for certain pesticides in or on certain products. Official

Journal of the European Union. **(28.5.2010)** L 129/3-49. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 34/2013 of 16 January 2013 amending Annexes II, III and IV to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residuel levels for 2-phenylphenol, ametoctradin, *Aureobasidium pullulans* strains DSM 14940 and DSM 14941, cyproconazole, difenoconazole, dithiocarbamates, folpet, propamocarb, spinosad, spirodiclofen, tebufenpyrad and tetraconazole in or on certain products. Official Journal of the European Union. **(26.1.2013)** L 25/1-48. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 1317/2013 of 16 December 2013 amending Annexes II, III and V to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for 2,4-D, beflubutamid, cyclanilide, diniconazole, florasulam, metolachlor and S-metolachlor, and milbemectin in or on certain products. Official Journal of the European Union. **(17.12.2013)** L 339/1-43. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 61/2014 of 24 January 2014 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for cyromazine, fenopropidin, formetanate, oxamyl and tebuconazole in or on certain products. Official Journal of the European Union. **(24.1.2014)** L 22/1-32. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Ridgway, K., Lalljie, S.P.D. eta Smith, R.M.; *Sample preparation techniques for the determination of trace residues and contaminants in foods.* **2007.** Journal of Chromatography A, 1153(1–2), 36-53.

Sánchez-Ortega, A., Sampedro, M.C., Unceta, N., Goicolea, M.A. eta Barrio, R.J.; *Solid-phase microextraction coupled with high performance liquid chromatography using on-line diode-array and electrochemical detection for the determination of fenitrothion and its main metabolites in environmental water samples.* **2005.** Journal of Chromatography A, 1094(1-2), 70-76.

Sarafraz-Yazdi, A. eta Amiri, A.; *Liquid-phase microextraction.* **2010.** Trends in Analytical Chemistry, 29(1), 1-14.

Saraji, M. eta Boroujeni, M.K.; *Recent developments in dispersive liquid–liquid microextraction.* **2014.** Analytical and Bioanalytical Chemistry, 406(8), 2027-2066.

Scherm, H., Christiano, R.S.C., Esker, P.D., Del Ponte, E.M. eta Godoy, C.V.; *Quantitative review of fungicide efficacy trials for managing soybean rust in Brazil.* **2009.** Crop Protection, 28(9), 774-782.

Sidiropoulos, N.D. eta Bro, R.; *On the uniqueness of multilinear decomposition of N-way arrays.* **2000.** Journal of Chemometrics, 14, 229-239.

Smilde, A., Bro, R. eta Geladi, P.; *Multi-way analysis. Applications in the chemical sciences.* **2004.** John Wiley & Sons Ltd. West Sussex (England).

Spietelun, A., Marcinkowski, Ł, de la Guardia, M. eta Namieśnik, J.; *Recent developments and future trends in solid phase microextraction techniques towards green analytical chemistry.* **2013.** Journal of Chromatography A, 1321, 1-13.

Spietelun, A., Marcinkowski, Ł, de la Guardia, M. eta Namieśnik, J.; *Green aspects, developments and perspectives of liquid phase microextraction techniques.* **2014.** Talanta, 119, 34-45.

Stalikas, C., Fiamegos, Y., Sakkas, V. eta Albanis, T.; *Developments on chemometric approaches to optimize and evaluate microextraction.* **2009.** Journal of Chromatography A, 1216(2), 175-189.

Stross, F.H. eta Ambrose, D.; *Preliminary reccomendations on nomenclature and presentation of data in gas chromatography.* **1960.** Pure and Applied Chemistry, 1(1), 177-186.

Tauler, R., *Multivariate curve resolution applied to second order data.* **1995.** Chemometrics and Intelligent Laboratory Systems, 30(1), 133-146.

Taxvig, C., Hass, U., Axelstad, M., Dalgaard, M., Boberg, J., Andeasen, H.R. eta Vinggaard, A.M.; *Endocrine-Disrupting Activities In Vivo of the Fungicides Tebuconazole and Epoxiconazole.* **2007.** Toxicological Sciences, 100(2), 464-473.

Tomlin, C.D.S.; *The Pesticide Manual.* **2000.** 12 edn. The British Crop Protection Council. Farnham (UK).

Vega Moreno, D., Sosa Ferrera, Z. eta Santana Rodríguez, J.J.; *Sample Extraction Method Combining Micellar Extraction−SPME and HPLC for the Determination of Organochlorine Pesticides in Agricultural Soils.* **2006.** Journal of Agricultural and Food Chemistry, 54(20), 7747-7752.

Viñas, P., Campillo, N., Martínez-Castillo, N. eta Hernández-Córdoba, M.; *Method development and validation for strobilurin fungicides in baby foods by solid-phase microextraction gas chromatography–mass spectrometry.* **2009.** Journal of Chromatography A, 1216(1), 140-146.

Wang, Z., Hennion, B., Urruty, L. eta Montury, M.; *Solid-phase microextraction coupled with high performance liquid chromatography: a complementary technique to solid-phase microextraction-gas chromatography for the analysis of pesticide residues in strawberries.* **2000.** Food Additives and Contaminants, 17(11), 915-923.

Wei, S., Leong, M., Li, Y. eta Huang, S.; *Development of liquid phase microextraction based on manual shaking and ultrasound-assisted emulsification method for analysis of organochlorine pesticides in aqueous samples.* **2011.** Journal of Chromatography A, 1218(51), 9142-9148.

Wolf, D.C., Allen, J.W., George, M.H., Hester, S.D., Sun, G., Moore, T., Thai, S., Delker, D.A., Winkfield, E., Leavitt, S., Nelson, G., Roop, B.C., Jones, C., Thibodeaux, J. eta Nesnow, S.; *Toxicity profiles in rats treated with tumorigenic and nontumorigenic triazole conazole fungicides: propiconazole, tridimefon and myclobutanil.* **2006.** Toxicologic Pathology, 34(7), 895-902.

Xia, Y., Zhi, X., Wang, X., Chen, M. eta Chen, J.; *Ultrasound-enhanced surfactant-assisted dispersice liquid-liquid microextraction and high-performance liquid chromatography for determination of ketoconazole and econazole nitrate in human blood.* **2012.** Analytical and Bioanalytical Chemistry, 402, 1241-1247.

Yan, H., Wang, H., Qin, X., Liu, B. eta Du, J.; *Ultrasound-assisted dispersive liquid–liquid microextraction for determination of fluoroquinolones in pharmaceutical wastewater.* **2011.** Journal of Pharmaceutical and Biomedical Analysis, 54(1), 53-57.

You, X., Wang, S., Liu, F. eta Shi, K.; *Ultrasound-assisted surfactant-enhanced emulsification microextraction based on the solidification of a floating organic droplet used for the simultaneous determination of six fungicide residues in juices and red wine.* **2013.** Journal of Chromatography A, 1300, 64-69.

Zhang, J., Gao, H., Peng, B., Li, Y., Li, S. eta Zhou, Z.; *Simultaneous determination of four synthesized metabolites of mequindox in urine samples using ultrasound-assisted dispersive liquid–liquid microextraction combined with high-performance liquid chromatography.* **2012a.** Talanta, 88, 330-337.

Zhang, L., Liu, S., Cui, X., Pan, C., Zhang, A. eta Chen, F.; *A review of sample preparation methods for pesticide residue analysis.* **2012b.** Central European Journal of Chemistry, 10(3), 900-925.

Zhang, Y., Zhang, X., Chen, C., Zhou, M. eta Wang, H.; *Effects of fungicides JS399-19, azoxystrobin, tebuconazloe, and carbendazim on the physiological and biochemical indices and grain yield of winter wheat.* **2010.** Pesticide Biochemistry and Physiology, 98(2), 151-157.

2. Helburuak

2. Helburuak

Tesi honen helburu nagusia modu erraz eta fidagarri batean triazolen determinaziorako prozedurak garatzea izan zen lagin ezberdinetan. Aztertutako triazolak dinikonazola, flukinkonazola, flusilazola, miklobutanila, tebukonazola eta tetrakonazola izan ziren. Helburu nagusia, ondorengo xede espezifikoen bidez lortu zen:

1. Mikroerauzketetan oinarritutako prozeduren garapena. Mikroerauzketa teknikak proposatu ziren bere gain zuten kimika berdearen ezaugarriengatik. Beraz, fase solidoko mikroerauzketak (SPME) eta ultrasoinuz lagundutako emultsifikazio mikroerauzketak (USAEME) prozedurak hautatu ziren, disolbatzaile organikoen absentzia edota erabilera murritzagatik.

2. GC/ECD eta HPLC/DAD teknika kromatografikoen erabilera zabaltzea. Ekipamendu hauek aukeratu ziren mikroerauzketa teknikei erraz akoplatzeko zuten gaitasunagatik eta baita bere kostua ohiko laborategiengandik eskuragarria zelako.

3. Erreminta kimiometrikoen bidezko prozeduren hobekuntza. Lehenik, diseinu esperimentalarekin mikroerauzketan eragiten zuten aldagaien aukeraketa eta optimizazioa burutzeko. Bigarren, aldagai anitzeko analisiaren bidez, lortutako datuen ondorengo tratamendua egiteko.

4. Lortutako prozeduren aplikazioa fruitu eta likido laginetan. Euskal Herrian pestizida mota hauek duten erabileragatik, mahats eta sagar laginak hautatu ziren metodoaren egokitasuna behatzeko.

3. Triazol fungizidak fruitu zukuetan determinatzeko fase solidoko mikroerauzketa eta GC/ECD bidezko metodoaren garapena

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3. Triazol fungizidak fruitu zukuetan determinatzeko fase solidoko mikroerauzketa eta GC/ECD bidezko metodoaren garapena

3.1 Laburpena

Fase solidoko mikroerauzketa (SPME) elektroi harrapaketa bidezko detektagailudun gas kromatografian oinarritutako prozedura bat garatu da triazol arrastoak determinatzeko. Bi pausutan burututako diseinu esperimental bat egin zen. Lehenik 2^{6-2} diseinu faktorial zatikatu bat egin zen aldagai esperimentalen miaketarako (zuntz-estaldura mota, erauzketa tenperatura, erauzketa denbora, agitazio abiadura, desortzio tenperatura eta desortzio denbora). Ondoren, bi aldagaiekin diseinu konposatu zentral bat egin zen baldintza esperimentalak optimizatzeko. Aukeratutako baldintza esperimentalak ondorengoak izan ziren: zuntza, PDMS/DVB; erauzketa tenperatura, 60^{o}C; erauzketa denbora, 45 min; desortzio denbora, 3 min; desortzio tenperatura 260^{o}C, eta agitazio abiadura, 500 rpm. Baldintza esperimental hauekin tetrakonazol, miklobutanil eta dinikonazolarentzat lortutako detekzio-mugak µg L^{-1} ordenakoa izan zen mahats eta sagar zukuetan. Berreskuratze ehunekoak %93.6 eta %112.1 bitartekoak izan ziren. Desbideratze estandar erlatiboak (% RSD), ordea, %1.2 eta %11.6 artekoak (sagarra) eta %6.7-tik %18.0-rainokoak (mahatsa) izan ziren. Metodoa Nafarroan, Errioxan eta Euskal Herrian jasotako bost mahats laginei eta hamahiru sagar lagini aplikatu zitzaien. Kuantifikazioa estandarren gehikuntza metodoaren bidez egin zen, hiru gehikuntza bi aldiz aztertuz. Miklobutanila aurkitu zen hiru sagar laginetan (110 – 112 µg L^{-1}) eta dinikonazola mahats lagin batean (9.4 µg L^{-1}).

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Optimization of Solid-Phase Microextraction Procedure Coupled to GC-ECD for Triazole Fungicides Determination in Juice Samples

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Abstract A solid-phase microextraction (SPME) procedure followed by gas chromatography electron capture detection (GC/ECD) for the determination of triazole residues was developed. An experimental design with two steps was done. Firstly, a 2^{6-2} fractional factorial design for screening several experimental variables (fiber-coating type, extraction temperature, extraction time, stirring rate, desorption temperature, and desorption time) was done. After, a two-factor central composite design for optimizing, the experimental conditions were carried out. The chosen experimental conditions were: fiber, PDMS/DVB; extraction time, 45 min; extraction temperature, 60 °C; desorption time, 3 min; desorption temperature, 260 °C, and stirring speed, 500 rpm. Using those conditions the limits of detection obtained for tetraconazole, myclobutanil, and diniconazole were in the order of few μg L^{-1} in grape and apple liquid extracts. Recoveries were from 93.6% to 112.1%. Relative standard deviation ranged from 1.2% to 11.6% (apple) and 6.7 to 18.0% (grape). The method was applied to five grape samples and 13 apple samples collected in Navarra, Rioja, and Basque Country. Quantification was performed by the standard addition method. Three standard additions by duplicate covering adequate range concentration were used. Myclobutanil was found in three apple samples (110–122 μg L^{-1}) and diniconazole in one grape sample (9.4 μg L^{-1}).

Keywords Experimental design · Azole fungicides · Apple · Wine · Solid- phase microextraction · GC/ECD

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Introduction

Triazole fungicides are one of the most common groups of pesticides applied to vineyards and apple orchards. Besides its antifungal activity, those compounds are also discussed as a group that disturbs endocrine activity in humans. Azole fungicides used in agriculture are moderately lipophilic and fairly persistent with typical half lives of weeks to months. They may reach the aquatic environment mainly by surface runoff and spray drift. Those chemicals can reach plant tissues leaving residues that can be detected in fruits and processed products. The determination of residue levels is necessary for food safety monitoring and regulatory purposes. In order to protect the health of consumers maximum residue levels (MRLs) in raw products have been established. European Union has fixed MRLs ranging from 0.1 to 2 mg kg^{-1} in apples and grapes for diniconazole, myclobutanil, tebuconazole, and tetraconazole (Regulation EC 396/2005). However, there is no legislation for processed products such as fruit juices.

After some preliminary treatment steps, the determination of pesticides in food matrices mostly use gas chromatography (GC) or liquid chromatography (LC) with mass spectrometry (MS) detectors. In recent years, considerable efforts have been made trying to develop new sample preparation techniques that save time, labor, and solvent consumption. In this respect, several procedures based on solid-phase extraction (SPE), quick, easy, cheap, effective, rugged and safe (QuEChERS) methodology, stir bar sorptive extraction and solid-phase microextraction (SPME) have been used in multiple pesticide residue determination (Lambropoulou and Albanis 2007; Picó et al. 2007; Cunha et al. 2009; Jiang et al. 2009). Referring to azole fungicides methods based on solid-phase microdispersion followed by LC with ultraviolet diode array detection in fruit pulps, LC/MS/MS and dispersive liquid–liquid micro-

extraction, and GC/MS or GC/ECD in wine samples have been developed (Bicchi et al. 2001; Trösken et al. 2005; Montes et al. 2009). SPME is a useful technique that does not require solvents and can be carried out directly from the liquid phase (direct immersion (DI)) or from the headspace (HS) over the samples (Pawliszyn 1997). SPME has been used as a sample preparation method followed by GC/MS for the analysis of different pesticides in water (Beceiro-González et al. 2007), for triazole residues in wine and strawberries (Zambonin et al. 2002), and for pesticide residues in fruit juice (Cortés-Aguado et al. 2008). Also, SPME coupled to LC/MS has been used for fungicide determination in fruits (Blanco et al. 2003) and for determination of carbamate and phenyl urea pesticide residues in fruit juices (Sagratini et al. 2007).

There are several variables affecting the SPME procedure, among them fiber type, extraction temperature, extraction time, stirring rate, and desorption conditions. Experimental designs, that take into account simultaneously several variables and its interaction effects, seem to be an appropriate way to find the convenient experimental conditions with a reduced number of experiments. Response surface designs are used during method optimization to determine optimal conditions for the factors that have the most influence on the response of interest (Van den hauwe et al. 2002). Also, factorial designs have considered to assess the influence of several factors and to obtain the best conditions. This approach has been presented in the developing a SPME followed by the GC/ECD for multiresidue analysis of pesticides in milk (Fernández-Álvarez et al. 2008). Although choice of an experimental design ultimately depends on the objectives of the study and the number of factors to be investigated, a thorough approach takes into account two phases, the screening and the optimization. Screening techniques, such as factorial designs, allow to select which factors are significant and at what levels. During the optimization response surface designs, including central composite design (CCD), are often applied to determine the optimal experimental conditions (Stalikas et al. 2009). The latter approach in two steps has been used for organochlorine pesticides and polychlorinated biphenyls determination in human serum using SPME and GC/ECD (López et al. 2007).

The goal of the work was to develop an appropriate green method for the determination of triazoles in juices samples using SPME followed by GC/ECD. In the method developing the advantageous characteristics of GC and its inherently green character compared with LC, and the SPME technique that avoid the use of clean-up steps and the hazardous solvents were considered. The experimental design was planned in two steps, the first one using a screening design to find which experimental conditions were significant. Secondly, with an optimization design trying to obtain the best values of significant variables for the determination of triazoles. The application of the optimized procedure was checked by analysis of different apple and grape juice samples.

Experimental

Reagents, Materials, and Equipment

Diniconazole (99.8%, Pestanal) and tebuconazole (99.6%, Pestanal) were supplied by Sigma-Aldrich (Madrid, Spain), myclobutanil (99.4%) was acquired at LGC Standards (Barcelona, Spain), and tetraconazole (97.5%) from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The methanol used for the stock solutions was purchased from Teknokroma (Barcelona, Spain). Table 1 shows the chemical structures and characteristics of these triazoles.

In the sample treatment, a Beckman Coulter centrifuge (Fullerton, USA) with a 25.5 rotor (with maximum and minimum radium of 108 mm and 38.5 mm, respectively) and 0.45-μm sterilized filters (Albet- Hahnemuehle, Barcelona, Spain) were used.

SPME holders and fibers [85 μm polyacrilate (PA) coating and 65 μm polydimethylsiloxane/divinylbencene (PDMS/DVB) coating], sample vials (40 mL amber glass) and PTFE silicone septa were obtained from Supelco (Bellefonte, PA, USA). For the extraction a vessel with thermostatic jacket joined to a Lauda RE 104 thermo bath (Lauda, GmbH & Co. KG, Lauda-Königshofen, Germany) was used. Inside this vessel, a 40 mL amber vial with a 20-mm PTFE-coated stir bar was placed.

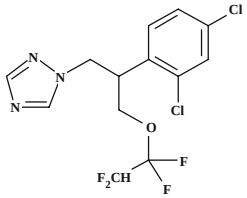
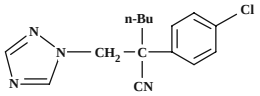
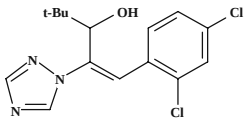
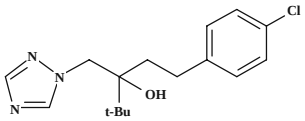
The analyses were performed in a HP 6890N (Agilent, Wilmington, DW, USA) gas chromatographer with an electron capture detector. The liner inter diameter was 0.75 mm and was acquired in Supelco. The column used was an HP-5 (30 m×0.250 mm×0.25 μm film thickness) capillary column (Agilent). The analytes were desorbed in the injector at 260 °C, where the valve was opened 2 min after injection. The carrier gas was helium with a 2.0 mL min^{-1} flow. The oven temperature program was: 60 °C (2 min), then a ramp of 20 °C min^{-1} to 280 °C (4 min). The electron capture detector temperature was fixed at 280 °C.

The experimental designs were performed and the results were evaluated by use of Statistica software (StatSoft, Tulsa, USA).

Grape and Apple Samples

Five grapes and 13 apples as samples were collected in Basque Country, Navarra, and La Rioja (Northern Spain).

Table 1 Names, chemical structures, and characteristics of the studied triazoles

Name	Structure	M_w	Formula	K_{OW} log P	Henry constant (Pa m^3 mol^{-1})
Tetraconazole		372.1	$C_{13}H_{11}Cl_2F_4N_3O$	3.56 (20ºC)	$3.6 \cdot 10^{-4}$
Myclobutanil		288.8	$C_{15}H_{17}ClN_4$	2.94 (pH 7-8, 25ºC)	$4.3 \cdot 10^{-4}$
Diniconazole		326.2	$C_{15}H_{17}Cl_2N_3O$	4.3 (25ºC)	$4.0 \cdot 10^{-2}$
Tebuconazole		307.8	$C_{16}H_{22}ClN_3O$	3.7 (20ºC)	$1.0 \cdot 10^{-5}$

Data from Tomlin (2000)

The grape samples were located in five vineyards. The varieties of harvested grapes were different; two were "tempranillo" and the other three, "Hondarribi zuri". The first variety was used to make red wine and the last one, white wine. The thirteen apple samples were picked in three different farmhouses. The apple varieties were used to making cider. One grape and one apple samples obtained from a pesticide-free farm were used as control samples.

The samples were blended and centrifuged at 21,000 rpm (53,300×*g*) during 8 min. After, the liquid was filtered with a 0.45-μm pore size filter. The obtained juices were maintained at −20 °C until the analysis.

SPME Extraction Procedure

After sample homogenization, 1 mL of the apple or grape juice sample was diluted with 24 mL of double-distillated water in a 40-mL amber vial that was capped with PTFE-coated silicone septum. Sample extraction was performed in DI mode, exposing the 65-μm PDMS/DVB fiber in the stirred samples (500 rpm). The extraction conditions were 60 °C for the temperature and 45 min for the time. After sampling, the fiber was withdrawn into the holder, placed in the GC injector, and thermally desorbed at 260 °C during 3 min.

The quantification of triazoles components was done by standard addition using mixed standards. The standard concentration was different depending of analyte added to the sample. The concentrations in vials varied between 0.78 and 10.4 μg L^{-1} for tetraconazole, 8.7 and 106 μg L^{-1} for myclobutanil, 0.38 and 5.10 μg L^{-1} for diniconazole, and 190–2,560 μg L^{-1} for tebuconazole. Blanks were run periodically during the study to verify the absence of contaminants.

Results and Discussion

In order to develop an adequate method using SPME for triazole determination in fruit liquid sample, it is necessary to consider and optimize several parameters that affect the extraction procedure. An experimental design with two steps (screening and optimization) was used, searching for the best experimental conditions. The concentrations for each analytes used in all the runs were: diniconazole, 1 μg L^{-1}; myclobutanil, 100 μg L^{-1}; tetraconazole, 1 μg L^{-1}; tebuconazole 1 mg L^{-1}. The four triazoles showed retention times between 11 and 14 min (11.5 min, tetraconazole; 12.4 min, myclobutanil; 12.7 min, diniconazole; 13.3 min, tebuconazole).

On the basis of the literature and the experience of the laboratory, different variables were selected to define the experimental field in the SPME procedure (Beltran et al. 2000; Zambonin et al. 2002; Lasa et al. 2006; López et al. 2007; Cortés-Aguado et al. 2008; Zuazagoitia et al. 2009).

Screening Design

A 2^{6-2} fractional factorial design (resolution IV) was made trying to find the significant variables. Taking into account the analytes' characteristics (Table 1), the direct immersion mode for extraction was chosen. Table 2 shows the six considered variables or factors and its levels. Those were: fiber type (85 μm PA and 65 μm PDMS/DVB), extraction time (15 and 30 min), extraction temperature (20 and 50 °C), desorption time (3 and 9 min), desorption temperature (250 and 270 °C), and agitation rate (300 and 500 rpm). The screening was completed with 18 assays, two of them in the central point.

The obtained data was evaluated by analysis of variance (ANOVA) and the main effects were visualized using a Pareto chart. In ANOVA, the significance of the factors was evaluated by F test. In the Pareto chart, the bar lengths are proportional to the absolute values of the main effects. An effect that exceeds a vertical reference line (usually 95%) may be considered significant with regard to the response. The positive and negative signs in Pareto chart indicate that the response is enhanced or reduced, respectively, on passing a given factor from the lowest to the highest level. After this analysis, three factors resulted significant fiber type, time, and temperature of extraction. Table 2 shows the significant results. The other factors without significance were fixed desorption temperature at 260 °C, desorption time in 3 min, and stirring rate at 500 rpm. The categorical variable was also chosen, and the PDMS/DVB fiber was used in the rest of the study.

Optimization Design

The second step of the experimental design was to optimize the significant variables. A CCD was made to optimize the time and temperature of extraction. In this work, 2^2 design was increased by [(2×2)+1] star points. The star points were located at $+\alpha$ and $-\alpha$ from the center of experimental domain. The axial distance α was selected as 1.4142 to establish the rotatability condition. The runs at the center of the experimental field were performed six times more. In total, the matrix of the CCD design consisted of 15 experimental runs that were randomly carried out. A second-degree polynomial model was used including the main effects for the two factors, their interaction and their quadratic components. The R^2 obtained for each of triazoles was considered as indication of adequacy of the model. The regression coefficients obtained are used to compute predicted values of the dependent variable for the different combinations of the levels of the independent variables.

The most common way to summarize the results of a CCD experiment is in the form of a response surface. This can be done in 3D plots selecting two factors (i.e., temperature and time). In this study, four 3D plots belonging to each of the triazole compounds can be obtained. Instead of these independent response surfaces, we chose to get the global desirability surface. First, the desirability function for each dependent variable was fixed by assigning desirability values of 0.0 (for undesirable, lowest result in this work), 0.5 (medium), and 1.0 (for very desirable, highest result in this work). After doing the specifications graphs, the global desirability surface response developed by the model in 3D plot was obtained (Fig. 1). This graph is useful for interpreting graphically the effect on overall response desirability of the independent variables time and temperature. As can be seen, in the experimental domain the best global response was reached

Table 2 Considered variables, levels, and significance for 2^{6-2} fractional factorial screening and CCD optimization designs

Screening

Variable	Low level	High level	Result
Fiber type	PA	PDMS/DVB	Significant, PDMS/DVB
Extraction temp. (°C)	20	50	Significant, for optimization
Extraction time (min)	15	30	Significant, for optimization
Desorption temp. (°C)	250	270	Not significant, 260 °C
Desorption time (min)	3	9	Not significant, 3 min
Agitation (rpm)	300	500	Not significant, 500 rpm

Optimization

Variable	Level			Result
	Low	Medium	High	Optimized values
Extraction temp. (°C)	50	60	70	60
Extraction time (min)	20	30	40	45

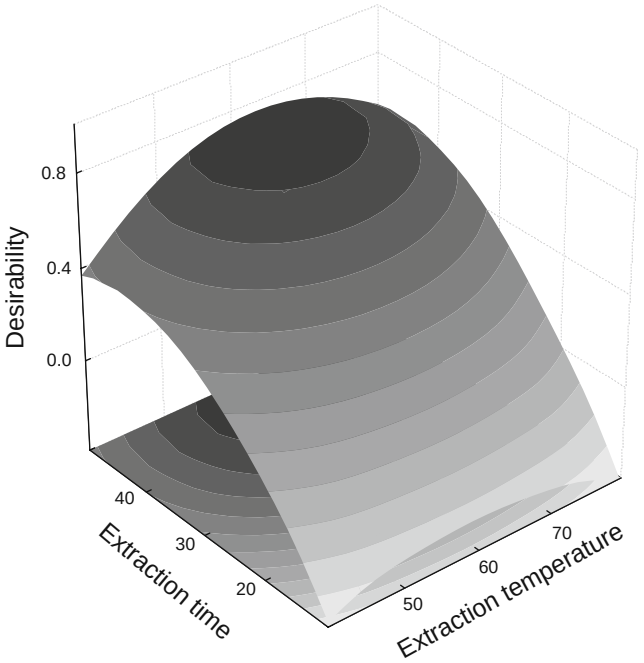


Fig. 1 Desirability surface response from CCD design considering time and temperature extraction for the triazole determination

when the extraction temperature was close to 60 °C and the extraction time around 45 min.

Taking into account the results from the screening and optimization studies, the working extraction conditions to obtain the best response for determination of the triazoles were: 500 rpm for the stirring rate, PDMS/DVB fiber, 60 °C, 45 min for the extraction conditions, and 3 min at 260 °C for desorption conditions.

Method Validation

After establishing the experimental conditions, the procedure was evaluated for each compound with respect to linearity, limit of detection (LOD), recovery, and precision. For these studies, the obtained juices from control apple and wine samples were used.

The calibration concentration ranges are showed in Table 3. These ranges varied among triazole analytes due to the differences in extraction behavior of the PDMS/DVB fiber. The lowest range was for diniconazole in apple (0.38–3.80 μg L^{-1}) and the highest for tebuconazole in grape (190–2,560 μg L^{-1}). Linearity for each analyte, in both apple and grape liquid matrices, showed R^2 values higher than 0.99 except for myclobutanil (0.942 in apple and 0.978 in grape). For each triazole compound, the LOD was determined at a signal-to-noise ratio of 3, considering the chromatograms of apple and grape control samples. In grape and apple samples, the LODs obtained were 0.73 and 0.48 μg L^{-1} for tetraconazole, 7.5 and 4.3 μg L^{-1} for myclobutanil, 0.29 and 0.23 μg L^{-1} for diniconazole, and 162 and 106 μg L^{-1} for tebuconazole. Accuracy was evaluated in terms of recovery at spiking intermediate levels of range concentration. In both matrices, good recoveries were obtained with values between 93.6% and 112.1%.

Table 3 Analytical characteristics of the proposed method for grape and apple liquid samples

Analyte	Sample	Calibration range (μg L^{-1})	R^2	LOD (μg L^{-1})	Recovery		Precision (interday)	
					Spiked level (μg L^{-1})	Mean (%; n=3)	Level (μg L^{-1})	RSD (%)
Tetraconazole	Grape	0.78–10.40	0.9955	0.73	7.80	103.2	0.78	11.7
							7.8	6.8
	Apple	0.78–7.80	0.9919	0.48	3.90	101.7	0.78	6.8
							7.8	3.3
Myclobutanil	Grape	8.7–106.0	0.9780	7.5	87.0	105.0	8.7	17.6
							87	10.2
	Apple	8.7–87.0	0.9416	4.3	43.5	112.1	8.7	11.6
							87	4.1
Diniconazole	Grape	0.38–5.10	0.9947	0.29	2.50	93.6	0.38	18.0
							3.80	6.7
	Apple	0.38–3.80	0.9917	0.23	2.50	96.4	0.38	7.7
							3.80	1.2
Tebuconazole	Grape	190–2,560	0.9917	162	450	94.8	190	10.8
							1,900	7.8
	Apple	190–1,900	0.9919	106	950	97.7	190	10.1
							1,900	5.6

In order to evaluate the precision reproducibility, studies were performed at two concentration levels; at the lowest level of concentration range, and at 10 times the lowest concentration (See Table 3). The results, expressed as relative standard deviation (RSD) in percentage, are also showed in Table 3. The RSDs were better for the highest concentrations in both extracts (<11%), and the apple extract precision was better (1.2–11.6%) than grape extract precision (6.7–18%). All validated parameters were agreed to the SANCO requirements for method validation (SANCO 2009).

Other works that determined triazoles in wine (Trösken et al. 2005; Jiang et al. 2009) found similar recovery and precision results than obtained in our study. However, the LODs presented more variation; we obtained better results for diniconazole and higher values for myclobutanil and tebuconazole than obtained LODs using QuEChERS-GC/MS and LC/MS/MS methodologies.

The comparison with the data obtained for triazoles in wine and fruit juices using SPME-GC/MS (Cortés-Aguado et al. 2008) showed similar precision and recovery results. The LODs obtained in this work were higher for tebuconazole, slightly higher for myclobutanil, and similar for tetraconazole.

Application to Grape and Apple Samples

The developed method was applied to grape and apple samples collected from different vineyards and farms in Basque Country, Navarra, and La Rioja. Standard addition method was used to avoid possible matrix interferences. The additions of three points of the calibration with its duplicates were done. Regression coefficients were between 0.9852 and 0.9990. Figure 2 shows the chromatograms obtained with the control apple liquid sample and one standard addition (tetraconazole, 0.78 μg L^{-1}; myclobutanil, 8.7 μg L^{-1}; diniconazole, 0.38 μg L^{-1}; tebuconazole, 190 μg L^{-1}).

In grapes, diniconazole was found in one sample (9.4 μg L^{-1}), and tetraconazole residues below the limit of detection were detected in other three samples. In apples, three samples were contaminated with myclobutanil (110–122 μg L^{-1}) and tebuconazole residues below the limit of detection were also detected.

Conclusions

A simple and reliable method was developed for the determination of four triazole fungicides: tetraconazole, myclobutanil, diniconazole, and tebuconazole. The extraction was made using SPME in immersion mode, and the separation and determination was carried out with GC/ECD. The adequacy of the method was obtained checking the limit of detection, recovery, and precision studies. Since the ECD detector does not provide unambiguous identification, the use of GC/MS for confirmation of analyte identification in the sample is recommended.

The procedure has some remarkable advantages. The extraction process is simple with practically no sample treatment. It could be assigned as green procedure avoiding the use of organic solvent, since the SPME extraction is solvent free and thermal desorption is used in the GC injector. The good repeatability and reproducibility obtained allows satisfactory quantification. And the cost for analysis and instrumentation can be estimated as low to moderate. The detected drawbacks for the procedure are the influence of sample matrix in the extraction process, and the total analysis time, including extraction and determination, is around 60 min. Therefore, for sample quantification, external calibration with matrix-matched standards or

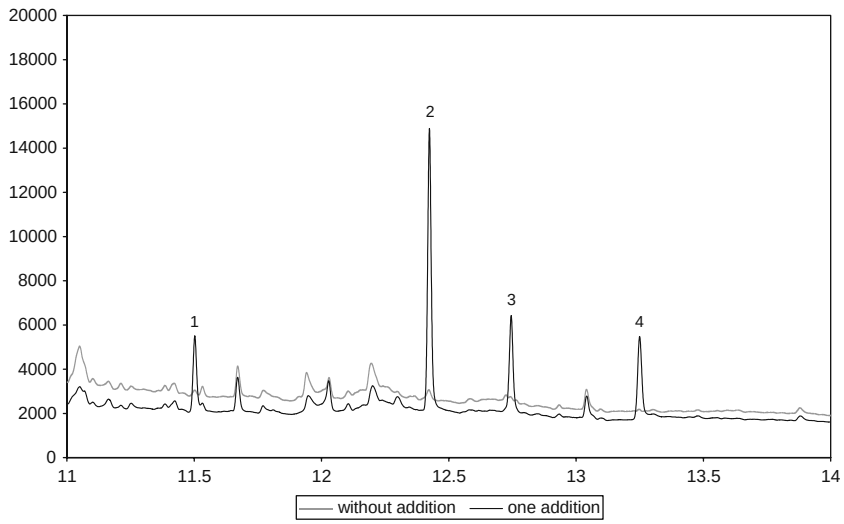


Fig. 2 GC-ECD chromatograms of the control apple sample alone and with the addition of the one mixed standard. Peak assignment: (*1*) tetraconazole, (*2*) myclobutanil, (*3*) diniconazole, and (*4*) tebuconazole

standard addition procedure is advisable. Also, the use of automated systems currently available could make the proposed procedure applicable to large-scale analysis in routine laboratories.

The application of the method to apple and grape liquid samples detected concentrations highest in LOD in myclobutanil in three apple samples and diniconazole in one grape sample. The procedure, besides the apple and grape samples, can be used in other liquid matrices from the blending of fruits.

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References

Beceiro-González E, Concha-Graña E, Guimaraes A, Gonçalves C, Muniategui-Lorenzo S, Alpendurada MF (2007) Optimisation and validation of a solid-phase microextraction method for simultaneous determination of different types of pesticides in water by gas chromatography-mass spectrometry. J Chromatogr A 1141:165–173

Beltran J, López FJ, Hernández F (2000) Solid-phase microextraction in pesticide residue analysis. J Chromatogr A 885:389–404

Bicchi C, Cordero C, Rubiolo P, Occelli A (2001) Simultaneous determination of six triazolic pesticide residues in apple and pear pulps by liquid chromatography with ultraviolet diode array detection. J AOAC Int 84:1543–1550

Blanco C, Font G, Mañes J, Picó Y (2003) Solid-phase microextraction liquid chromatography/tandem mass spectrometry to determine postharvest fungicides in fruits. Anal Chem 75:3606–3615

Cortés-Aguado S, Sánchez-Morito N, Arrebola FJ, Garrido Frenich A, Martínez Vidal JL (2008) Fast screening of pesticide residues in fruit juice by solid- phase microextraction and gas chromatography- mass spectrometry. Food Chem 107:1314–1325

Cunha SC, Fernandes JO, Alves A, Oliveira MBPP (2009) Fast low-pressure gas chromatography-mass spectrometry method for the determination of multiple pesticides in grapes, musts and wines. J Chromatogr A 1216:119–126

Fernández-Álvarez M, Llompart M, Lamas JP, Lores M, Garcia-Jares C, Cela R, Dagnac T (2008) Development of a solid-phase microextraction gas chromatography with microelectron-capture detection method for a multiresidue analysis of pesticides in bovine milk. Anal Chim Acta 617:37–50

Jiang Y, Li X, Xu J, Pan C, Zhang J, Niu W (2009) Multiresidue methods for the determination of 77 pesticides in wine using QuEChERs sample preparation and gas chromatography with mass spectrometry. Food Addit Contam 26:859–866

Lambropoulou DA, Albanis TA (2007) Methods of sample preparation for determination of pesticide residues in food matrices by chromatography-mass spectrometry-based techniques: a review. Anal Bioanal Chem 389:1663–1683

Lasa M, Garcia R, Millán E (2006) A convenient method for epichlorohydrin determination in water using headspace-solid-phase microextraction and gas chromatography. J Chromatogr Sci 44:438–443

López R, Goñi F, Etxandia A, Millán E (2007) Determination of organochlorine pesticides and polychlorinated biphenyls in human serum using headspace solid-phase microextraction and gas chromatography-electron capture detection. J Chromatogr B 846:298–305

Montes R, Rodríguez I, Ramil M, Rubí E, Cela R (2009) Solid-phase extraction followed by dispersive liquid-liquid microextraction for the sensitive determination of selected fungicides in wine. J Chromatogr A 1216:5459–5466

Pawliszyn J (ed) (1997) Solid Phase Microextraction-Theory and Practice. Wiley-VCH, New York

Picó Y, Fernández M, Ruiz MJ, Font G (2007) Current trends in solid-phase-based extraction techniques for the determination of pesticides in food and environment. J Biochem Biophys Methods 70:117–131

Regulation (EC) 396/2005 of 23 February 2005. Off J Eur U 16.3.2005, L 70:1–16. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm

Sagratini G, Mañes J, Giardiná D, Dakiani P, Picó Y (2007) Analysis of carbamate and phenylurea pesticide residues in fruit juices by solid-phase microextraction and liquid chromatography-mass spectrometry. J Chromatogr A 1147:135–143

SANCO document No.10684/2009. Method validation and quality control procedures for pesticide residues analysis in food and feed. Implemented 01/01/2010. http://ec.europe.eu/food/plant/protection/resources/publications_eu.htm

Stalikas C, Fiamegos Y, Sakkas V, Albanis T (2009) Developments on chemometric approach to optimize and evaluate microextraction. J Chromatogr A 1216:175–189

Tomlin CDS (ed) (2000) The pesticide manual, 12th edn. British Crop Protection Council, Surrey

Trösken ER, Bittner N, Völkel W (2005) Quantitation of 13 azole fungicides in wine samples by liquid chromatography–tandem mass spectrometry. J Chromatogr A 1083:113–119

Van den hauwe O, Dumoulin F, Antignac JP, Bouche MP, Elliot C, Van Peteghem C (2002) Liquid chromatographic-mass spectrometric analysis of 11 glucocorticoid residues and an optimization of enzymatic hydrolysis conditions in bovine liver. Anal Chim Acta 473:127–134

Zambonin CG, Cilenti A, Palmisano F (2002) Solid-phase microextraction and gas chromatography- mass spectrometry for the rapid screening of triazole residues in wine and strawberries. J Chromatogr A 967:255–260

Zuazagoitia D, Millán E, Garcia-Arrona R (2009) A screening method for polycyclic aromatic hydrocarbons determination in sediments by headspace SPME with GC-FID. Chromatographia 69:175–178

4. Triazol fungizidak fruituetan determinatzeko fase solidoko mikroerauzketa eta HPLC/DAD bidezko metodoaren garapena

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4. Triazol fungizidak fruituetan determinatzeko fase solidoko mikroerauzketa HPLC/DAD bidezko metodoaren garapena

4.1 Laburpena

Sei triazol fungiziden (dinikonazola, flukinkonazola, flusilazola, miklobutanila, tebukonazola eta tetrakonazola) determinaziorako fase solidoko mikroerauzketa (SPME) eta diodo lerrokatuen detektagailuz hornitutako bereizmen handiko likido kromatografia bidezko metodo bat garatu zen. Aldagai esperimentalak eta beraien balioak bi pausutan emandako diseinu esperimental baten bidez lortu ziren: miaketa (2^{5-1} diseinu faktorial zatikatua) eta optimizazioa (diseinu konposatu zentrala). PDMS/DVB estalduradun zuntzak erabiliz, beste baldintza esperimentalak ondorengoak izan ziren: erauzketa denbora, 90 min; erauzketa tenperatura 60^{o}C; NaCl gehikuntza, 180 g L^{-1}; desortzio estatiko modua eta desortzio denbora, 2 min. Prozedurak eguneko eta egun arteko zehaztasun ona erakutsi zuen (RSD balioak %13.2 baino txikiagoak) eta detekzio-mugak 1.5 eta 5.9 µg L^{-1} artekoak izan ziren lagin likidoetan eta 0.08-tik 0.3 mg kg^{-1} bitartekoak lagin solidoetan. Metodoa aurretik fungizida produktu komertzialez tratatutako mahats eta sagar laginei aplikatu zitzaien. Fruitu laginak bi asteko denbora tartean jaso ziren, eta azala, pulpa eta zukua bereizi ziren. Gainera, fruitu zuhaitzen azpian jasotako euri laginak ere aztertu ziren. Miklobutanila eta tebukonazol arrastoak aurkitu ziren azal eta euri laginetan, baina ez pulpa eta zuku laginetan.

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Development and application of a screening method for triazole fungicide determination in liquid and fruit samples using solid-phase microextraction and HPLC-DAD

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A screening method for determination of six triazole fungicides (diniconazole, fluquinconazole, flusilazole, myclobutanil, tebuconazole and tetraconazole) using the solid-phase microextraction (SPME) technique coupled to a high-performance liquid chromatography-diode array detector (HPLC-DAD) was developed. Experimental variables were chosen and their values were fixed according to an experimental design with the steps of screening (2^{5-1} fractional factorial design) and optimization of the significant variables (central composite design). Using polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers, the other experimental conditions were: extraction time, 90 min; extraction temperature, 60 °C; NaCl addition, 180 g L^{-1}; static desorption mode and desorption time, 2 min. The method showed good inter and intraday precision (RSD values less than 13.2%) and the limits of detection were from 1.5 to 5.9 μg L^{-1} in liquid extracts and from 0.08 to 0.3 mg kg^{-1} in solid products. The method was applied to grape and apple fruit samples previously sprayed with commercial products containing the analytes. Samples were taken from peel, pulp and juice following a pre-harvest interval of two weeks. Also, rain samples under the fruit trees were collected. Myclobutanil and tebuconazole were found in the peel and rain, but not in pulp and juice samples.

Introduction

Triazole pesticides are widely used as antifungal agents to fight against *Oidium*, a common fungus that can affect fruit trees. In general, the action mode of azole fungicides is the inhibition of ergosterol, an essential component in fungal cell membranes, by influencing the cytochrome P450 enzyme activity.[1] Side effects were observed with the exposure to some triazole fungicides in some *in vivo* assays. Also, some studies have shown that those effects in rats lead to disturbances in reproductive systems,[2] or even induce some tumors in livers of mice.[3]

Because of the potential health risks to consumers resulting from acute and chronic dietary exposure, European Union (EU) has published new regulations establishing the Maximum Residual Limits (MRLs) for a wide range of pesticides in different vegetables and fruits.[4,5] Those MRLs for triazole fungicides were ranging from 0.02 to 2 mg kg^{-1} based on the analyte and the type of fruit, including apples and grapes for wine production. Moreover, commercial products establish a pre-harvest interval for safety since fungicide residues may remain in the crops. These fungicide residues may disappear by degradation of the products or by sweeping because of the precipitations. In the Basque Country (North of Spain) the following triazole fungicides diniconazole (D), fluquinconazole (FQ), flusilazole (FS), myclobutanil (M), tebuconazole (TB) and tetraconazole (TT) are widely used in apple orchards and vineyards.

The amount of those analytes in environmental samples is usually in a very low concentration. Thus, very precise and sensitive techniques are needed. Gas or liquid chromatography (GC, LC) techniques coupled to mass spectrometry (MS) are frequently used for pesticide analysis in fruits and vegetables.[6–8] Sample preparation steps previously to chromatographic analysis are required in most of the samples. Historically, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) have been the most used preparative methods, but those techniques are time consuming and need large amounts of solvents. Nowadays, the trends in sample preparation are the solvent-minimized and the solventless techniques.[9] The solid-phase microextraction (SPME) is a useful technique that does not require solvents and can be carried out directly from the liquid phase (direct immersion, DI) or from the headspace (HS) over the samples.[10] It has been used in many different applications including pesticide residue determination.[11–13] Although the pesticide studies often include one triazole fungicide, mainly tebuconazole, few studies considering simultaneously several

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triazoles in fruits and liquid extracts using SPME have been done. SPME coupled to GC-MS for triazole residues in wine and strawberries has been used.[14] Also, SPME and GC with an electron capture detector (ECD) has been shown to be adequate for determining diniconazole, myclobutanil and tetraconazole in juice samples.[15] However, in the latter work the results obtained with tebuconazole were not as good as for the other studied triazoles. Due to the analytical characteristics of the triazole compounds solvent desorption using LC from the SPME fibers could result in a better performance that allows a wider and versatile triazole determination.

There are several experimental variables affecting the SPME procedure such as the type of fiber, temperature, extraction time, salt addition, and desorption conditions. An experimental design that could take into account simultaneously several variables seems to be a convenient approach to selecting the best experimental conditions. A screening design, such as fractional factorial, is recommended to be performed in a previous step to reduce the number of variables. In the next step, the chosen variables, usually two or three, are optimized using response surface methodology. Among the designs, a central composite design (CCD) or Box–Behnken design is frequently used.[16,17]

The aim of the work was to develop a convenient method that allows simultaneous determination of various triazole fungicides in liquid, fruit and related samples using simple equipment (SPME coupled to HPLC-DAD). In order to select the experimental conditions, a planned experimental design with screening and optimization steps was run. The optimized procedure was applied to check and evaluate the triazole residues in the peel, pulp and juice of apples and grapes during a pre-harvest interval of two weeks.

Experimental

Reagents and materials

Diniconazole (99.8%, Pestanal) and tebuconazole (99.6%, Pestanal) were acquired from Sigma-Aldrich (Madrid, Spain), flusilazole (99.3%) and myclobutanil (99.4%) were supplied by LGC Standards (Barcelona, Spain) and tetraconazole (97.5%) and fluquinconazole (98.5%) were obtained from Dr Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were prepared in methanol (superpure solvent, SpS) supplied by Teknocroma (Barcelona, Spain) in a concentration of 1000 mg L^{-1}, and calibration standards were prepared in a range from 5 to 30 mg L^{-1}. Just before each use the dilution of the standards was made by adding 20 μL of these standards to 20 mL of water salted with NaCl. The mobile phase for HPLC was acetonitrile (SpS) acquired from Teknocroma and the buffer used was made by adjusting with 0.01 mol L^{-1} acetic acid–sodium acetate solution to pH 4. The buffer was stored at 4 °C.

Extractions were made using fibers coated with 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) acquired from Supelco (Bellefonte, PA, USA). Sample vials (40 mL amber glass), PTFE-faced silicone septa and holders for extractions were also supplied by Supelco.

Equipment

HPLC analysis was performed using a LC-20AD liquid chromatograph coupled with a SPD-M20A diode array detector (Shimadzu Corporation, Duisburg, Germany). Data were collected and processed using LC Solution software (1.2. version). An interface with a Rheodyne® Valve (Supelco) with 60 ± 10 μL chamber volume was used for desorption. All separations were carried out with a XDB-C18 column (4.6 mm × 250 mm, 5 μm) (Agilent, Wilmington, DW, USA). The mobile phase was a mixture of sodium acetate buffer (0.01 M, pH 4) and acetonitrile (22 : 78, v/v) running at 0.5 mL min^{-1} in isocratic mode. UV-vis absorption spectra were recorded from 190 to 800 nm using 221 nm as a detection wavelength. Under these conditions the sequence and elution time was tetraconazole (7.2 min), myclobutanil (7.5 min), flusilazole (7.8 min), fluquinconazole (8.1 min), tebuconazole (8.4 min) and diniconazole (9.9 min).

The extractions were made in a vessel with a thermostatic jacket joined to a Lauda RE 104 thermo bath (Lauda, GmbH & Co. KG, Lauda-Königshofen, Germany). Agitation was performed using a Heidolph MR 3003 magnetic stirrer (Heidolph, GmbH & Co. KG, Schwabach, Germany). For sample treatment, an ultrasonic bath and an Eppendorf centrifuge with a maximum speed of 14 000 rpm were used.

Experimental designs were performed and the results were evaluated using Statistica software (StatSoft, Tulsa, USA).

Fruit treatment, sample collection and preparation

In order to check and evaluate the occurrence of triazole residues in apples and grapes during a two week pre-harvest interval, a mixture of two triazole pesticides according to the instructions of the manufacturer was applied. Fruit samples were collected the first day, the fourth day, a week and two weeks after the application of the pesticides. Five apples from separate locations of the tree and 2–3 grapes from different bunches until complete 200 g were taken. The samples were divided into 3 groups: peel, pulp and juice and they were separately analyzed. The peel was removed, and the fruit was blended separating the pulp and the juice. The samples were kept in the freezer until the analysis.

The peel or pulp samples (2 g) were covered by acetonitrile (5 mL) and put into an ultrasonic bath over 20 min. Then, the supernatant was transferred to a 2 mL Eppendorf vial and was centrifuged at 14 000 rpm for 10 min. After centrifugation, 1.0 mL was transferred to 40 mL glass vials and diluted to 20 mL with salted water (NaCl, 180 g L^{-1}) before extraction with the SPME fibers. The juice was also analyzed, adding directly salted water to 1 mL of the sample until a volume of 20 mL was reached.

Additionally, rain samples were collected, placing a recipient under the fruit plant. Since in the sampling period the raining days were infrequent, one raining simulation applying tap water to the plant with a spray applicator was used. After filtering the rain samples, just to remove the leaves and branches, the analysis was done by taking 2 to 20 mL of the samples in 40 mL glass vials, completing to 20 mL with double distilled water and adding the amount of needed salt into the vial to obtain 180 g L^{-1} concentration.

SPME procedure

The fibers were previously conditioned over 30 min in the desorption chamber of the HPLC. The thermostatic bath was heated to 60 °C and the magnetic stirrer was fixed at 500 rpm. A 40 mL amber glass vial with 20 mL of liquid extracts as previously prepared and with a magnetic stirrer was placed in the water jacket of the thermostatic bath. In the case of standards adequate volumes of analyte solutions were added to the vial containing 20 mL of salted water. Then, the fibers were immersed into the aqueous solution and after 90 min of exposure time the fibers were withdrawn and put into the SPME-HPLC interface to desorb the analytes. The desorption mode was the static mode, followed by another dynamic mode step of 1 min to sweep all the mobile phase with the analytes of the desorption chamber. After each analysis the fibers were maintained in stirred water for 15 min as a cleaning step and trying to avoid the crystallization of the salt. With these precautions the fibers were reused around sixty times.

Results and discussion

Screening and optimization designs

In order to obtain an appropriate method for SPME-HPLC for triazole compound determination it is important to find the best experimental conditions. There are different variables affecting the SPME process in immersion mode. Extraction time and temperature, desorption time, desorption mode, salt addition, agitation, and the fiber type are some of the most important variables.[13,18–20] Hence, the importance of selecting variables and choosing levels to find the best experimental conditions should be considered. For this purpose screening and optimization design were carried out. The concentration of analytes in the analysis vial to perform the screening and optimization designs were: tetraconazole and myclobutanil, 15.3 μg L^{-1}, flusilazole 15.0 μg L^{-1}, fluquinconazole and tebuconazole, 15.6 μg L^{-1}, and diniconazole 15.9 μg L^{-1}.

Firstly, a screening step was performed. The PDMS/DVB fiber type was chosen considering the previous study with some triazole fungicides.[15] Extraction time and temperature, salt addition, desorption mode and time were the chosen variables. A 2^{5-1} fractional factorial design was made with four quantitative variable levels established as 0–300 g L^{-1} of NaCl in salt addition, 15–60 min for the extraction time, 20–75 °C for extraction temperature, and 1–10 min for desorption time. The only qualitative variable was the mode of desorption, considering the dynamic and static modes. Table 1 shows the variables and levels. The design was completed with twenty runs (16 + 4 central points).

Main effects were visualized using Pareto charts and the data were evaluated by analysis of variance (ANOVA) where the significance of the different factors was evaluated by the *F* test. The Pareto charts of the main effects for tetraconazole and flusilazole are shown in Fig. 1. In the Pareto chart, the bar lengths are proportional to the absolute values of the main effects. An effect that exceeds a vertical reference line (usually 95%) may be considered significant with regard to the response.

Table 1 Variables and levels for screening and optimization of the SPME process

Screening

Variable	Low level	High level	Result
NaCl addition (g L^{-1})	0	300	Significant, for optimization
Extraction time (min)	15	60	Significant, for optimization
Extraction temperature (°C)	20	75	Significant, for optimization
Desorption time (min)	1	10	Not significant, 2 min
Desorption mode	Dynamic	Static	Not significant, static

Optimization

Variable	Low level	Medium level	High level	Optimized values
NaCl addition (g L^{-1})	100	150	200	180
Extraction time (min)	50	65	80	90
Extraction temperature (°C)	60	70	80	60

The positive and negative signs in the Pareto chart indicate that the response is enhanced or reduced, respectively, on passing a given factor from the lowest to the highest level. After the analysis, two of the factors resulted significant for the six triazoles: time of extraction (text) and temperature of extraction (Text). Salt addition (NaCl) showed a significant response at 95% in some of the triazoles. These variables were considered in the optimization steps. Taking into account the non-significant results, the other two variables were fixed. 2 min for desorption time (tdes), since the response decreased at high levels. Static was the chosen desorption mode because it showed less band broadening than the dynamic mode.

The salt addition, extraction time and temperature variables were optimized with a Central Composite Design (CCD). The 2^3 design was increased by $[(2 \times 3) + 1]$ star points located at $+\alpha$ and $-\alpha$ from the center point. The α axial distance was selected to be 1.682 to establish the rotatability condition. Adding three more center points, the total number of experiments was eighteen. Levels of variables are shown in Table 1.

The most common way to summarize the results of a CCD is in the form of a response surface. This can be done in 3D plots representing results and selecting two factors (*i.e.*, temperature and time). Instead of the independent response surfaces for each triazole analytes, the global desirability surface was chosen, since it can provide an overall view of all the considered analytes and variables. The desirability function for each dependent variable was fixed by assigning desirability values of 0.0 (for undesirable, lowest result in this work), 0.5 (for medium) and 1.0 (for very desirable, the highest result in this work). Fig. 2 shows the response surfaces for global desirability obtained from a CCD considering two of the experimental variables. As can be seen, in the experimental domain the best responses were obtained at high extraction times. Hence, 90 min was selected as the extraction time. Although the

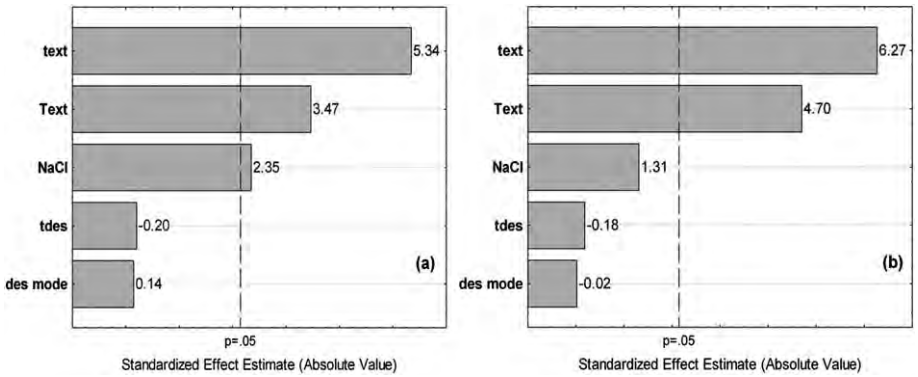

Fig. 1 Pareto charts of the main effects obtained from the 2^{5-1} fractional factorial design for (a) tetraconazole and (b) flusilazole.

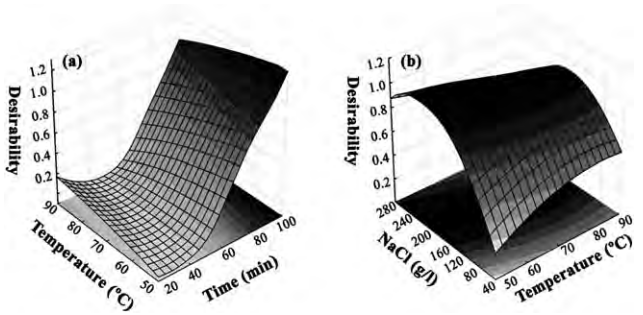

Fig. 2 Response surfaces for global desirability estimated from the central composite design. (a) Considering extraction temperature and extraction time variables and (b) NaCl concentration and extraction temperature variables.

desirability profiles established the optimum value for salt addition in 234 g L^{-1}, 180 g L^{-1} was chosen because there was not remarkable difference in desirability between 180 and 234 g L^{-1} and excessive salt can result in fiber coating damage. Also, there were slight differences in desirability in the temperature range, and 60 °C was chosen as the more manageable temperature.

Method performance

For the quantification of samples an external calibration method was used. The mixtures of standards were randomly analyzed. The linear range and regression coefficients (R^2), limits of detection (LOD), precision and recovery results are shown in Table 2.

The linear range for the calibration curves varied between 5.2 and 50.4 μg L^{-1} and R^2 was between 0.9878 and 0.9980. The number of replicates for each calibration level was at least two. Limits of detection were calculated as three times the deviation of the slope divided by the value of the slope.[21] The limits of detection were from 1.5 to 5.9 μg L^{-1}. With 2 g of solid samples and the dilutions done with the preparation steps, LODs referenced to the solid product were found to be in the range of 0.08 to 0.3 mg kg^{-1}. These values are adequate for the analysis of apples and grapes for the study of following triazole fungicides: diniconazole, tetraconazole, myclobutanil and tebuconazole. The MRLs fixed in the EU regulation for these compounds in apples and grapes are diniconazole (0.1–0.2 mg kg^{-1}), tetraconazole (0.3–0.5 mg kg^{-1}), myclobutanil (0.5–1 mg kg^{-1}), and tebuconazole (1–2 mg kg^{-1}).

The method was also evaluated considering inter and intra-day precision and recovery studies, all of them in two different concentrations (15 and 25 μg L^{-1}) for each analytes. For intra-day precision, $n = 4$ and for inter-day precision, $n = 8$ were considered. The analyses in the latter case were done over four days in two weeks (two consecutive days in each week). Values, expressed as relative standard deviation (RSD) in percentages, ranged between 2.8 and 11.5% in intra-day precision and 5.1 and 13.1% in inter-day precision. For recovery studies, 15 and

Table 2 Analytical characteristics of the SPME-HPLC-DAD method[a]

Triazole fungicides		TT	M	FS	FQ	TB	D
Linear range (μg L^{-1})		5.5–33.0	5.5–33.0	7.0–42.0	5.4–32.4	8.4–50.4	5.2–31.2
R^2		0.9980	0.9968	0.9912	0.9906	0.9878	0.9978
LOD (μg L^{-1})		1.5	1.9	4.2	3.3	5.9	1.5
Inter-day precision (%RSD)	15 μg L^{-1}	7.1	5.1	8.3	9.5	13.1	6.5
	25 μg L^{-1}	9.8	5.8	8.7	7.8	7.7	9.0
Intra-day precision (%RSD)	15 μg L^{-1}	7.2	5.1	11.5	8.2	7.0	10.6
	25 μg L^{-1}	5.4	2.8	5.0	3.3	5.3	5.4
Recovery ± s.d.[b](%)	15 μg L^{-1}	100.0 ± 5.7	96.2 ± 14.8	104.1 ± 11.1	95.3 ± 2.7	108.3 ± 11.3	101.6 ± 1.1
	25 μg L^{-1}	101.8 ± 7.6	123.4 ± 27.1	94.5 ± 0.3	95.0 ± 0.4	97.9 ± 9.9	107.2 ± 0.6

[a] TT, tetraconazole; M, myclobutanil; FS, flusilazole; FQ, fluquinconazole; TB, tebuconazole; D, diniconazole; RSD, relative standard deviation. [b] s.d. standard deviation ($n = 2$).

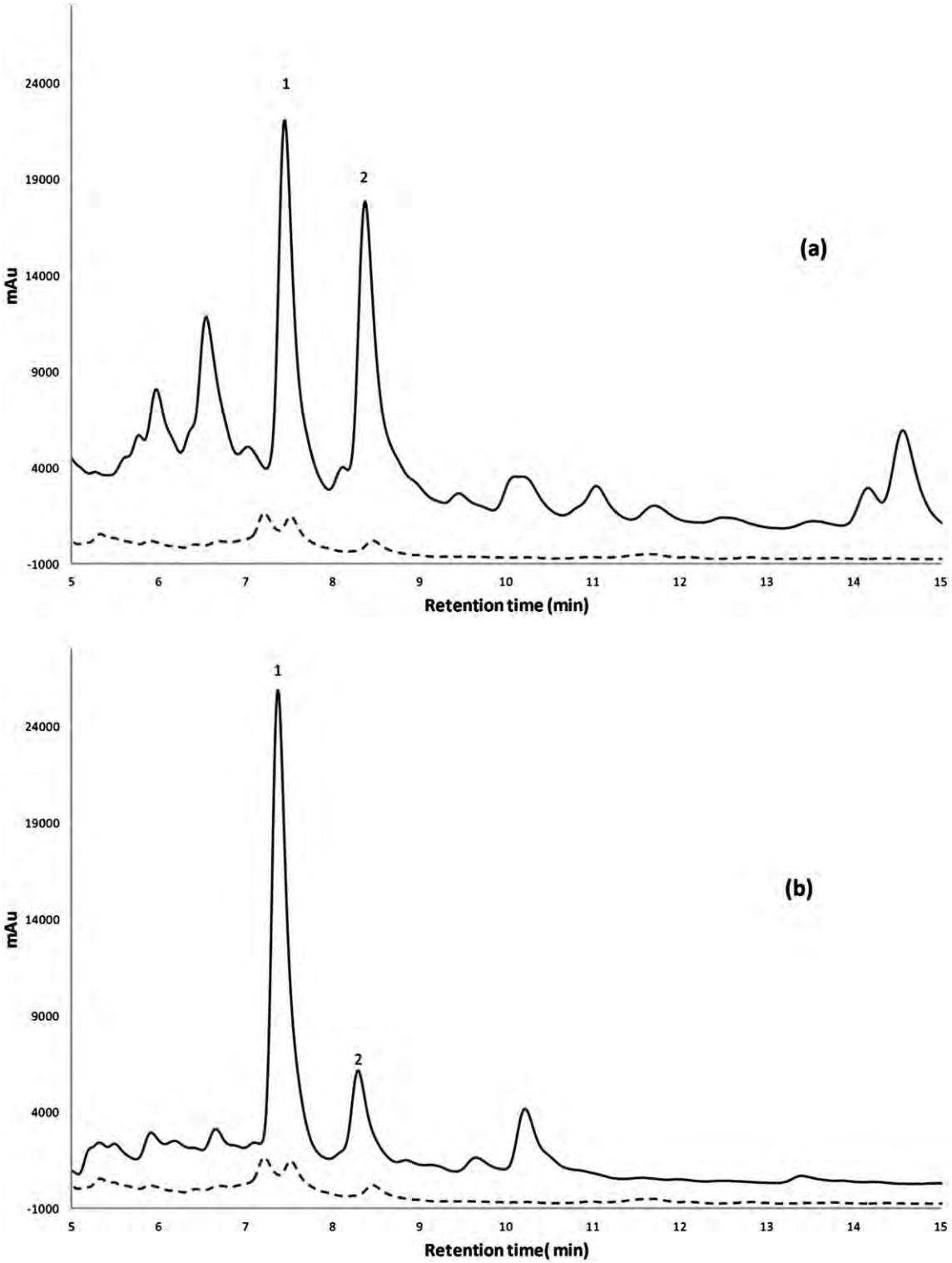

Fig. 3 Chromatograms of (a) one positive peel sample and (b) one positive rain sample. Compound identification: 1, myclobutanil; 2, tebuconazole. The dashed line belongs to the blank procedure.

25 μg L^{-1} were added to aqueous samples and analyzed with the developed technique. With the exception of myclobutanil, for the other triazoles the mean recovery ranged between 95 and 108% (15 μg L^{-1}) and the standard deviation ($n = 2$) between 0.3 and 11.3%.

Comparing with other methods used for triazole determination in liquid samples, the LODs obtained for tebuconazole are lower than LODs showed using HPLC-DAD,[22] and similar to LODs indicated using solid-phase extraction-hollow fiber preconcentration followed by gas chromatography-flame

Table 3 Mean concentration ± s.d.[a] (mg kg^{-1}) of triazole fungicides in collected fruit samples[b]

		Sample collecting days							
		Day 1		Day 4		Day 8		Day 15	
Fruit samples		M	TB	M	TB	M	TB	M	TB
Apple	Peel	12.5 ± 4.0	6.6 ± 0.3	9.6 ± 0.8	10.4 ± 0.1	18.9 ± 1.4	7.8 ± 1.8	4.8 ± 0.1	n.d.
	Pulp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Juice	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Grape	Peel	18.4 ± 1.9	37.2 ± 5.5	23.9 ± 1.0	29.7 ± 2.6	8.7 ± 0.1	24.7 ± 4.4	13.9 ± 8.4	15.9 ± 4.1
	Pulp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Juice	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] s.d. refers to standard deviation ($n = 3$). [b] n.d.: not detected; M, myclobutanil; TB, tebuconazole.

ionization detection (SPE-HF-GC-FID)[23] and using dispersive liquid–liquid microextraction (DLLME) followed by HPLC-DAD.[22] However, it is higher than the 0.012 μg L^{-1} value obtained using quick, easy, cheap, effective, rugged, safe (QuEChERS) sample preparation and the more sensible instrumentation GC-MS.[24]

Referring to precision and recovery the obtained results are comparable to others obtained with different extraction techniques. For diniconazole and tebuconazole in wine the recoveries were from 83 to 110% and the RSDs were from 5 to 10% using QuEChERS coupled to GC-MS.[24] For tebuconazole in grape juices the RSD was 6.5% and the recoveries were from 87 to 117% using SPE-HF-GC-FID.[23] For tebuconazole in water using the DLLME procedure and HPLC-DAD the given values were 93% for recovery and 2.5% for RSD.[22]

Application to real samples

Two different types of fruits, apples and grapes, were studied. Commercial products containing myclobutanil and tebuconazole were applied to fruit trees following the manufacturer's indications. Thus, the fruit samples were collected the first day, the fourth day, a week and two weeks after application. According to the security files of the commercial products, the pre-harvest period is 14 days after application. Fig. 3 shows the chromatograms of two contaminated peel and rain samples and the blank procedure.

In each case three independent samples were analyzed. The mean concentration and the standard deviation, expressed as mg kg^{-1}, are shown in Table 3. Analytes were found in peel samples but were not detected in any inner portion (neither pulp nor juice). This indicates that the analytes have not gone through the peel, and it was concluded that the treatment with these commercial products was superficial.

Rain samples collected on two different days were found to contain analyte residues. The first samples, collected the fourth day after treatment, had 253.1 and 71.8 μg L^{-1} respectively of myclobutanil and tebuconazole under the apple tree. Under the vineyard, lower concentrations of 114.6 and 14.1 μg L^{-1} for myclobutanil and tebuconazole respectively were found. On the ninth day, myclobutanil residues were found but there were not any tebuconazole residues. The myclobutanil values were 42.2 and 34.4 μg L^{-1} respectively for the samples collected under the apple tree and the vineyard.

Conclusions

A reliable screening method, using SPME and HPLC simple equipment, has been developed for the determination of triazole fungicides in liquid and fruit samples. The best experimental conditions for SPME extraction were obtained using an experimental design with two steps. Good results were obtained regarding limits of detection, inter and intra-day precisions, and recoveries. The proposed method could be easily incorporated to more sensible and confirmatory techniques such as LC-MS.

Due to the use of triazole fungicides in grape and apple trees, the peel, pulp and the juices of the samples were analyzed. It was concluded that the treatment was superficial because no detectable levels of analytes were found in pulp and juice samples within the pre-harvest period established by the commercial product. However, some residues of analytes were found in the peel; hence, fruit cleaning before consumption is advisable. Finally, some analyte concentrations were also found in rain samples. Being a superficial treatment, the rain would clean the fruits sweeping the analytes.

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References

1 D. R. Ekman, H. C. Keun, C. D. Eads, C. M. Furnish, R. N. Murrell, J. C. Rockett and D. J. Dix, *Metabolomics*, 2006, **2**, 63.
2 C. Taxvig, U. Hass, M. Axelstad, M. Dalgaard, J. Boberg, H. R. Andeasen and A. M. Vinggaard, *Toxicol. Sci.*, 2007, **100**, 464.
3 J. W. Allen, D. C. Wolf, M. H. George, S. D. Hester, G. Sun, S. Thai, D. A. Delker, T. Moore, C. Jones, G. Nelson, B. C. Roop, S. Leavitt, E. Winkfield, W. O. Ward and S. Nesnow, *Toxicol. Pathol.*, 2006, **34**, 853.

4 EC Regulation No. 396/2005 of 23 February 2005 on maximum residue levels of pesticides in or on food and feed plant and animal origin and amending Council Directive 91/414/EEC, OJ L 70, 16.3.2205, p. 1.
5 EC Commission Regulation 149/2008 of 29 January 2008 amending regulation (EC) No 396/2005 of the European Parliament and of the Council establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto, OJ L 58, 1.3.2008, p. 1, MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.
6 M. Araoud, W. Douki, A. Rhim, M. F. Najjar and N. Gazzah, *J. Environ. Sci. Health, Part B*, 2007, **42**, 179.
7 S. Walorczyk, *Rapid Commun. Mass Spectrom.*, 2008, **22**, 3791.
8 B. Kmellár, L. Abrankó, P. Fodor and S. J. Lehotay, *Food Addit. Contam.*, 2010, **27**, 1415.
9 M. Tankiewicz, J. Fenik and M. Biziuk, *Talanta*, 2011, **86**, 8.
10 J. Pawliszyn, *Applications of solid phase microextraction*, The Royal Society of Chemistry, Cambridge, 1999.
11 J. S. Aulakh, A. K. Malik, V. Kaur and P. Schmitt-Kopplin, *Crit. Rev. Anal. Chem.*, 2005, **35**, 71.
12 Y. Picó, M. Fernández, M. J. Ruiz and G. Font, *J. Biochem. Biophys. Methods*, 2007, **70**, 117.
13 G. Sagratini, J. Mañes, D. Giardiná, P. Damiani and Y. Picó, *J. Chromatogr., A*, 2007, **1147**, 135.
14 C. G. Zambonin, A. Cilenti and F. Palmisano, *J. Chromatogr., A*, 2002, **967**, 255.
15 A. Bordagaray, R. Garcia-Arrona and E. Millán, *Food Anal. Methods*, 2011, **4**, 293.
16 C. González-Barreiro, M. Lores, M. C. Casais and R. Cela, *J. Chromatogr., A*, 2000, **896**, 373.
17 T. Zou, D. Wang, H. Guo, Y. Zhu, X. Luo, F. Liu and W. Ling, *J. Food Sci.*, 2012, **71**, C46.
18 A. Sánchez-Ortega, M. C. Sampedro, N. Unceta, M. A. Goicolea and R. J. Barrio, *J. Chromatogr., A*, 2005, **1094**, 70.
19 P. Parrilla Vázquez, A. R. Mughari and M. Martinez Galera, *Anal. Chim. Acta*, 2008, **607**, 74.
20 P. Viñas, N. Campillo, M. Hernández-Pérez and M. Hernández-Córdoba, *Anal. Chim. Acta*, 2008, **611**, 119.
21 P. Konieczka and J. Namiesnik, *Quality Assurance and Quality Control in the Analytical Chemical Laboratory*, CRC Press, Boca Raton, 2009.
22 L. M. Ravelo-Pérez, J. Hernández-Borges, M. Asensio-Ramos and M. A. Rodríguez-Delgado, *J. Chromatogr., A*, 2009, **1216**, 7336.
23 M. A. Farajzadeh, L. Khoshmaram and M. R. A. Mogaddam, *J. Sep. Sci.*, 2012, **35**, 121.
24 Y. Jiang, X. Li, J. Xu, C. Pan, J. Zhang and W. Niu, *Food Addit. Contam., Part A*, 2009, **26**, 859.

5. Ultrasoinuz lagundutako emultsifikazio mikroerauzketa tanta organiko flotatzailearen solidifikazioarekin eta HPLC/DAD bidezko metodo baten garapena triazol fungiziden determinaziorako lagin likidotan

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5. Ultrasoinuz lagundutako emultsifikazio mikroerauzketa tanta organiko flotatzailearen solidifikazioarekin eta HPLC/DAD bidezko metodo baten garapena triazol fungiziden determinaziorako lagin likidotan

5.1 Sarrera

Triazol fungizidak onddoen tratamendurako laborantzetan erabiltzen diren pestizida sistemikoak dira. Konposatu horiek, ergosterolaren biosintesia ekiditen dute eta horren ondorioz onddoen zelula-mintzen egituran eta beraien funtzioak kaltetzen dira [Tomlin, 2000]. Triazol fungizida taldearen barnean daude dinikonazol (D), tetrakonazol (TT), tebukonazol (TB), flusilazol (FS), flukinkonazol (FQ) eta miklobutanil (M) izeneko konposatuak. Gero eta zabalagoa den pestiziden erabileraren ondorioz, gizakiengan eta ekosisteman eragin dezaketen kaltearen inguruan arreta piztu da. Izan ere, hainbat ikerketa toxikologikok erakutsi dute saguetan ondorio tumorgenikoak [Wolf *et al.*, 2006] eta sistema endokrinoaren funtzionamendu normala oztopatu dezaketela [Goetz *et al.*, 2007]. Fungizida arrastoak gizakiaren kontsumorako diren jakietan topatu direnez, segurtasun eta erregulazio arrazoiengatik, beharrezkoak dira pestizida arrastoak determinatzea metodo zehatzen bidez. Horregatik, kontsumitzaileen osasuna bermatzeko, Europar Batasunak, baimendutako gehienezko hondakin-mugak (MRL, *maximum residue limit*) definitu ditu. MRL hauek 0.01 eta 1 mg kg^{-1} bitartekoak dira lan honetan aztertutako analitoentzat sagar eta mahats laginetan [Regulation EC 149/2008; Regulation EC 459/2010; Regulation EU 34/2013; Regulation EU 1317/2013; Regulation EU 61/2014].

Lagin prestaketak berebiziko garrantzia du pestizida arrastoen determinazioan. Azken urteetan lagin prestaketa teknikak garatzeko ahalegin handiak egin dira, prozedura analitikoen kalitatea eta sentsibilitatea hobetuz eta denbora eta ingurumenarentzat kaltegarriak izan litezkeen disolbatzaile organikoak aurrezteko helburuarekin [Tankiewicz *et al.*, 2011]. Sortzio erauzketa tekniken barnean daude solido faseko erauzketa (SPE), solido faseko mikroerauzketa (SPME) eta barratxo-nahasgailuaren sortzio erauzketa (SBSE). SPE oso erabilia izan da lagin prestaketa

teknika moduan eta honen garapenerako helburu nagusiak eraginkortasuna hobetzean eta adsorbatzaile berriak garatzean datza. Desabantaila nagusiak, ordea, kartutxoen kostua eta erabili beharreko disolbatzaile kopuru handiak dira. Disolbatzaile bolumen handiaren arazoari aurre egiteko, SPEren bertsio miniaturizatuak sortu ziren. SPME eta SBSE teknikak disolbatzailerik gabeko erauzketa teknikak dira eta beste ezaugarri batzuen artean sentsibilitate ona eta aplikazio eremu zabala eskaintzen dute. Halere, erauzketa bakar baterako beharrezko denbora nahiko luzea izan liteke, aldagaien optimizazioan eta konfiantzako kalibratu bat lortzeko zailtasunak egon daitezke eta zuntzen eta nahasgailuen prezio altuak eragozpenak izan daitezke [Hyötyläinen, 2009].

Likido faseko mikroerauzketak (LPME) likido-likido erauzketaren disolbatzaile kopuru handiaren erabilerak dakartzan ohiko arazoak ekiditen ditu. LPME burutzeko modu ezberdinak daude, hala nola, uretan disolbaezina den bolumen oso txikiko tanta erauztailea laginarekin kontaktuan jarriz edo bi faseen artean selektiboa izan den mintz baten bidez [Stocka *et al.*, 2011]. Likido-likido mikroerauzketa dispertsiboak, aldiz, tanta organiko erauztailea tantatxo ñimiñoetan banatzen du, ur faseari laino itxura emanez eta likido-likido erauzketa klasikoaren alternatiba baliagarri moduan sortu zen. Hiru osagai dituen sistema da: analito edukia duen fase akuosoa, uretan disolbaezina den tanta organikoa eta uretan disolbagarria den dispertsatzailea. Tanta erauztailea laino egoeratik tanta formara zentrifugazio bidez itzuliko da erauzketaren ondoren determinazioa burutu dadin. DLLME beraz, azkarra, merkea, sinplea, erabilerraza, disolbatzaile organiko bolumen minimoa eta aberastasun-faktore altuak eskaintzen dituen teknika da [Bosch-Ojeda eta Sánchez-Rojas, 2011]. Edonola ere, DLLME kasuan erabiltzen diren disolbatzaile organikoak toxikotasun altua izan dezakete. LPME teknikaren barnean tanta organiko flotatzailearen solidifikazioa (SFO) proposatu zen tanta jasotzeko teknika bezala [Khalili Zanjani *et al.*, 2007]. Metodo honen funtsa tantaren urtze-puntuan dago, hau giro tenperaturaren ingurukoa bada, izotz bainu batean solidifikatu daiteke eta lagin akuosoaren gainazaletik espatula baten laguntzaz jaso eta eraman daiteke. Izoztutako tanta, laborategiko tenperaturan urtuko denez, berriz ere likido egoeran aurkituko da ondorengo determinazio kromatografikorako. Teknika hau merkea, azkarra eta sentibera izan daiteke, baina erauzketa denboran zehar tanta moduan badago, prozesua mantsoa izan daiteke. Beraz DLLME eta SFOren arteko konbinazio bat proposatu zen (DLLME-SFO) bi tekniken abantailak bateratuko zituena, masa transferentzia azkarragoen bidez denbora murrizketak lortuz [Leong eta Huang, 2008]. Aurrez aipatu bezala, DLLMEren desabantaila nagusienetako bat dispertsatzailea erabili beharra da, horregatik ingurumenarentzat mingarriak izan

litezkeen disolbatzaileak ekiditeko, ultrasoinuz lagundutako emultsifikazio mikroerauzketa (USAEME) sortu zen. Teknika honen bidez, hirugarren disolbatzaile baten beharrik gabe tanta organikoaren dispertsioa lortu zen [Regueiro *et al.*, 2008].

Aurrez aipaturiko erauzketa teknika batzuk triazolen arrastoak determinatzeko erabiliak izan dira ur mota eta fruitu zuku ezberdinetan. Oro har, erauzketa bidez prekontzentratu ondoren, gehienbat gas kromatografia (GC) edota likido kromatografia (LC) teknikak erabili izan ohi dira detektagailu mota ezberdinez baliatuz: sugar ionizazio detektagailua (FID), lerrokatutako diodoen detektagailua (DAD) edo masa espektrometria (MS) esaterako. SPE prozedura hobetu baten bidez, triazolez gain, kutsatzaile ezberdinak (organofosforo pestizidak, ftalatoak etab.) determinatu ziren uretan [Baugros *et al.*, 2008]. Ingurumeneko ur mota ezberdinean ere triazolak determinatu ziren grafenoan oinarritutako nanopartikula magnetikoen adsortzio bidez [Wang *et al.*, 2012]. Beste sortzio teknikak ere erabiliak izan dira ur laginetan triazolak aztertzeko: hala nola, SPME [Bordagaray *et al.*, 2013] eta SBSE eta DLLME bateratuz [Farajzadeh *et al.*, 2010]. DLLME oinarritzat hartuta beste adar batzuk ere garatu dira, esaterako likido ionikoak gehituz [Ravelo-Pérez *et al.*, 2009], hodi zulatu estuak erabiliz [Farajzadeh *et al.*, 2012] edota metanola dispertsatzaile bezala eta SFO teknika erabiliz [Wang *et al.*, 2011]. Orain arte, ez dago ezagutzarik triazolen determinaziorako dispertsatzaile gabeko USAEME-SFO teknika erabili denik ur eta fruitu-zuku laginetan.

Hainbat aldagai daude erauzketan eragiten dutenak. Esaterako, erauztaile mota eta bolumena, gatz gehikuntza, agitazio abiadura, lagin bolumena, erauzketa tenperatura eta denbora [Ghambarian *et al.*, 2013]. Erauzketa prozesuaren baldintza onenak lortu ahal izateko erabiltzen da diseinu esperimentala, izan ere, aldagai ezberdinak batera optimizatu daitezke eta berauen artean gerta daitezkeen elkarrekintza-efektuak ikus daitezke esperimentu kopuru murritz batekin. Erantzun azaleren diseinuen bidez (RSM, *response surface methodology*) eta diseinu konposatu zentrala kontuan hartuz, interesatzen zaigun erantzunean gehien eragingo duten aldagaien balio hoberenak bilatzen dira [Stalikas *et al.*, 2009]. Hurbilketa hau IL-DLLME-HPLC/DAD metodoan erabili da pestiziden determinaziorako [Ravelo-Pérez *et al.*, 2009] eta baita ere karbamato eta organofosforo pestizidak uretan aurkitzeko DLLME ikerketan ere [Sousa *et al.*, 2013].

Lan honen helburua uretan eta fruitu zukuetan triazolak determinatzeko USAEME-SFOn oinarritutako metodo baten garapena izan zen bereizmen handiko

kromatografia likidoaren laguntzaz (HPLC/DAD). Metodoak tanta organiko ñimiño bat besterik ez du erabili, dispertsatzaile arriskutsuak ekidinez. Aldagai garrantzitsu batzuk finkatu ondoren (erauztailea bera eta bolumena, edota lagin kantitatea esaterako) erauzketa tenperatura eta denbora, sodio kloruro gehikuntza aztertu ziren diseinu konposatu zentralaren bidez. Ondoren metodoa balidatu eta azkenik lagin mota ezberdinetan aplikatu zen.

5.2 Esperimentala

5.2.1 Erreaktiboak eta ekipamendua

Ikerketa honetan erabilitako dinikonazola (%99.8, Pestanal®) eta tebukonazola (%99.6, Pestanal®) Sigma-Aldrich-en (Madril, Espainia) eskuratu ziren, flusilazola (%99.3) eta miklobutanila (%99.4) LGC Standards-en (Bartzelona, Espainia) eta tetrakonazola (%97.5) eta flukinkonazola (%98.5), aldiz, Dr. Ehrenstorfer-ek (Augsburg, Alemania) hornitua izan zen. Banakako 6000 mg L^{-1}-ko disoluzio-amak Teknokroma (Bartzelona, Espainia) etxeko metanoletan (SpS) prestatu ziren. Horietatik abiatuz, erdiko estandarrak 100 mg L^{-1}-ra diluitu ziren eta ondoren analito ezberdinen patroiak nahasi ziren amaierako bialetan 20 eta 890 µg L^{-1} bitarteko kontzentrazioak lortuz (ikus 5.1. taula). HPLC-rako fase-mugikorra egiteko HPLC kalitateko Teknokroma etxeko metanola erabili zen eta tanpoia egiteko erabilitako sodio azetatoa (PA kalitatea) eta azido azetikoa Panreac-ek (Bartzelona, Espainia) hornituak izan ziren. Tanpoia egiteko 0.01 mol L^{-1} azido azetiko 0.01 mol L^{-1} sodio azetatorekin pH 4-raino doitu eta hozkailuan gorde zen. 1-undekanola (purutasuna %99), hexadekanoa (purutasuna %99), eta 1-bromohexadekanoa (purutasuna %97) Sigma-Aldrich-en eskuratu ziren; 1-dodekanola, aldiz, Panreac-en, gatz disoluzioak egiteko sodio kloruroa bezala.

5.1. Taula. *Kalibraturako prestatutako estandar disoluzioetako analitoen kontzentrazio zehatzak.*

Kalibratuko estandarren kodeak	Kontzentrazioa (µg L^{-1})					
	M	**FQ**	**TT**	**FS**	**TB**	**D**
kal20	20.7	19.0	20.5	20.9	22.2	20.6
kal50	51.9	47.6	51.2	52.2	55.4	51.5
kal80	83.0	76.2	81.9	83.5	88.7	82.4
kal100	103.7	95.2	102.3	104.4	110.8	103.0
kal150	155.6	142.8	153.5	156.6	166.3	154.5
kal200	207.4	190.4	204.7	208.8	221.7	206.0
kal400	414.8	380.8	409.4	417.5	443.4	412.1
kal600	622.2	571.2	614.0	626.3	665.0	618.1
kal800	829.6	761.6	818.7	835.0	886.7	824.2

M: Miklobutanila
FQ: Flukinkonazola
TT: Tetrakonazola
FS: Flusilazola
TB: Tebukonazola
D: Dinikonazola

HPLC analisiak LC-20AD instrumentu batean egin ziren, SPD-M20A DAD detektagailua erabiliz (Shimadzu Corporation, Duisburg, Alemania). Datuak LC solution-en 2.1 bertsioa zuen softwarearekin jaso ziren. Banaketa XDB-C18 zutabe batean eman zen (250 mm x 4.6 mm x 5µm) Agilent-ek (Wilmington, DW, EEBB) hornitua, analisia giro tenperaturan egin eta injekzio bolumena 20 µL izan zelarik. Sodio azetato/azido azetiko eta metanolez osatutako nahasteak 0.5 mL min^{-1}-ko fluxua izan zuen. Erabilitako gradiente konposizioak ondorengo programarekin burutu zen: hasieran, minutu batez %78 metanola mantendu ondoren 13 minutura arte metanol proportzioa jaitsi zen %71.5raino iritsi arte. 13tik 14 minutura bitartean %85ra igo eta bertan 10

minutuz mantendu zen. 24 minututik 30 minutura zirkuitua hasierako %78ko konposizioarekin homogeneizatu zedin 1.5 mL min^{-1}-ko fluxura igo zen bi minutu eta erdiz. Baldintza hauetan triazolak ordena eta denbora hauetan eluitu ziren: miklobutanila [M] (9.9 min), flukinkonazola [FQ] (10.6 min), tetrakonazola [TT] (11.1 min), flusilazola [FS] (12.2 min), tebukonazola [TB] (15.3 min) eta dinikonazola [D] (22.8 min). UV espektroa 190 eta 500 nm tartean jaso zen, nahiz eta determinazioak egiteko uhin-luzera aukeraketa egin: dinikonazola 249 nm-tan eta gainerakoak 221 nm-tan determinatu ziren.

Erauzketak Bandelin Sonorex Digitec DT100H (ALLPAX GmbH &Co. KG, Papenburg, Alemania) ultrasoinu bainuan egin ziren (35 kHz-ko ultrasoinu maiztasunarekin); solidifikazioak, aldiz, Julabo F26 (GmbH, Ausgburg, Alemania) bainu termostatikoan. Diseinu esperimentala eta datuen analisia Statistica softwarea (StatSoft, Tulsa, EEBB) erabiliz egin zen.

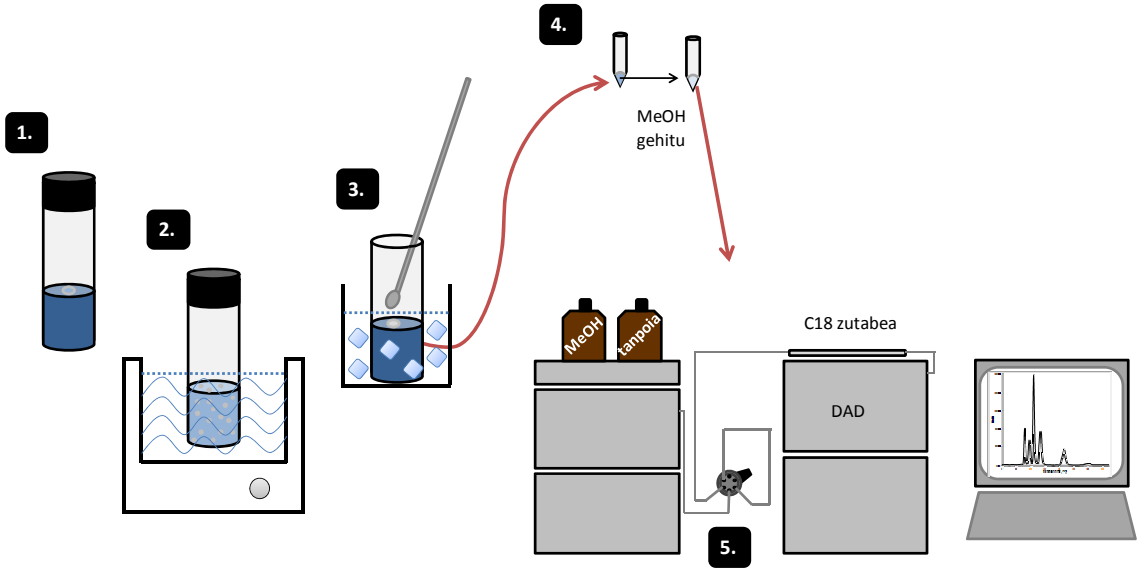
5.2.2 Laginak

Garatutako metodoa frogatzeko benetako laginetan burutu ziren erauzketak. Horretarako sagardoa, zuku komertzialak eta freskoak hautatu ziren. Lagin komertzialak (sagardoa, sagar-zukua eta mahats-muztioa) supermerkatuetan eskuratu ziren eta zuku freskoak aldiz, zuku-makinekin egin ziren eta ondoren paper-iragazkien bidez iragazi ziren, pulpa soberakina kentzeko.

5.2.3 USAEME-SFO prozedura

250 g L^{-1} NaCl disoluzio 10 mL 40mL-ko tapoidun ontzi batean isuri eta bertan triazolen nahasketa disoluzioa gehitu zen. Ondoren 50 µL 1-undecanol erauztailea gehitu zitzaion, ultrasoinuan ezarri aurretik eskuz lehen astindu bat emanez. Ultrasoinu bainua aurretik 30^{o}C-tara (±1^{o}C) berotuta zegoelarik, 18 minutuz mantendu zen erauzketa-ontzia bertan. Laino egoeratik berriz ere erauztailea tanta formara eraman

eta jaso ahal izateko 10 minutuz 4200 rpm-tan zentrifugatu eta 3ºC-tan zegoen ur-bainuan sartu zen 5 minutuz, bertan tanta izoztuz. Solidifikatutako tanta kontu handiz espatula batez jaso eta Eppendorf bial batera eraman zen giro tenperaturan urtu zedin. Urtutako tanta horretatik 25 µL hartu ziren xiringa baten laguntzaz eta HPLC-n injektatu aurretik 20 µL metanol gehitu zitzaizkion fase-mugikorrarekin bateragarria izateko. 5.1 irudian ikus daiteke prozedura osoaren irudikapen grafikoa.



5.1. Irudia. *USAEME-HPLC/DAD prozeduraren irudikapen grafikoa: (1) disoluzioaren prestaketa eta undekanolaren gehikuntza; (2) ultrasoinuz lagundutako erauzketa; (3) zentrifugatu ondorengo tantaren izoztea; (4) tantaren urtzea eta metanolaren gehikuntza; (5) HPLC injekzioa eta determinazioa.*

Laginen analisia egiterako orduan lagin/ur disoluzio proportzioa erabakitzeko tantaren portaera egokia aztertu zen. Disoluzioak lagin kantitate handiegia izan ezkero, tantaren formakuntzan arazoak zeuden, horregatik, eta frakzio ezberdinekin frogak egin ondoren, 0.5 mL zuku eta 9.5 mL ur erabili ziren, betiere bukaerako gatz kontzentrazioa 250 g L^{-1} mantenduz. Gainerako prozedura, aurretik aipatutako baldintza berberetan egin zen.

5.2.4 Aberastasun-faktorea

Aberastasun-faktoreak (EF, *Enrichment factor*) erauzketaren arrakastaren ideia bat ematen du. Analito bakoitzaren erauztaileko orekako kontzentrazioaren eta hasierako kontzentrazioaren arteko erlazioa da aberastasun-faktorea 5.1 ekuazioan ikus daitekeen bezala.

$$EF = \frac{C_d^{eq}}{C_0} \qquad (5.1)$$

Hasierako kontzentrazioa (C_0) fase akuosoko analito bakoitzaren kontzentrazioa da, hau da, geuk prestatzen dugun disoluzioaren hasierako kontzentrazioa. Erauztailearen orekako kontzentrazioa (C_d^{eq}) erauzketa denboraren ondoren tanta organikoak duen analito kontzentrazioa izango da. Azken kontzentrazio hau HPLC-n egindako kanpo-kalibratu baten laguntzaz egin zen, patroi kontzentrazio ezberdinak zuzenean kromatografoan injektatuz lortutakoa. Erauzketa egin ondoren (optimizatu ondorengo baldintzak errespetatuz), tanta injektatu eta bere azalera injekzio-zuzenez lortutako kalibrazio-zuzenean ordezkatuz kalkulatu zen kontzentrazioa.

5.3 Emaitzak eta eztabaida

5.3.1 Erauztailearen aukeraketa

Mikroerauzketa prozesuan zehar, erauztailearen aukeraketak berebiziko garrantzia izan zuen. USAEME eta SFO bateratzen zirenean, erauztaileak hainbat baldintza bete behar zituen. Alde batetik, analitoak erauzi ahal izateko afinitatea izatea, urarekin disolbaezina izatea eta erauzketa denboran zehar fase organikoak egonkorra izateaz gain analitoekin ez erreakzionatzea. Ondoren, kromatografia bidez determinatuko zenez, portaera kromatografiko egokia izatea beharrezkoa zen, hau da, analitoekin gainezartzea ekidin eta analisi bakoitzaren ondoren erauztaile organikoa guztiz garbitu beharko zen zutabe kromatografikotik. Horrez gain, SFO aplikatu ahal

izateko, tantaren fusio tenperaturak giro tenperaturaren ingurukoa izan beharko zuen, izan ere, 3^{o}C-tan izoztu zedin eta ondoren laborategiko tenperaturan berriz erraz urtu [Ganjali *et al.*, 2010; Han eta Row, 2012]. Ezaugarri hauek beteko zituzten lau erauztaile hautatu ziren: 1-bromohexadekanoa (BRHEX, T_{fus}= 17^{o}C), hexadekanoa (HEX, T_{fus}= 18^{o}C), 1-dodekanola (DOD, T_{fus}= 22^{o}C) eta 1-undekanola (UND, T_{fus}= 14^{o}C).

Lehenik eta behin erauztaileen eta HPLC-ren fase-mugikorraren bateragarritasuna aztertu zen. Horretarako azetonitrilo/ur eta metanol/ur bolumen/bolumen ehuneko ezberdinak frogatu ziren. 5.2. taulan ikus daitekeen bezala, 1-bromohexadekanoak eta hexadekanoak disolbagarritasun guztiz urria erakutsi zuten azetonitrilo/ur eta metanol/ur disoluzioetan. 1-undekanolak eta 1-dodekanolak, aldiz, disolbagarritasun hobea zuten, fase-mugikorraren disolbatzaile ehunekoa 60 baino handiagoa zenean. Azetonitriloak baino metanolak bateragarritasun hobea erakutsi zuen, izan ere, azetonitrilo nahastean 70/30 eta 80/20 nahasteetatik aurrera disolbatu ziren 1-undekanola eta 2-dodekanola. Metanolaren kasuan 60/40 ehunekotik aurrera disolbatzen hasten ziren tanta organikoak. Hasierako frogak ikusita beraz, 1-undekanola eta 1-dodekanola hautatu ziren ikerketarekin aurrera egiteko.

pH azido eta basikoetan zuten portaera ikusteko azido klorhidriko eta sodio hidroxido tantak gehitu zitzaizkien disolbaezinak ziren 5.2. taulako disoluzioei, baina ez zen aldaketarik nabari disolbagarritasunean.

5.2. Taula. *Erauztaileen bateragarritasun froga HPLC-ko fase-mugikor aukerekin. Bolumen totala 2 mL + 20 µL erauztaile.*

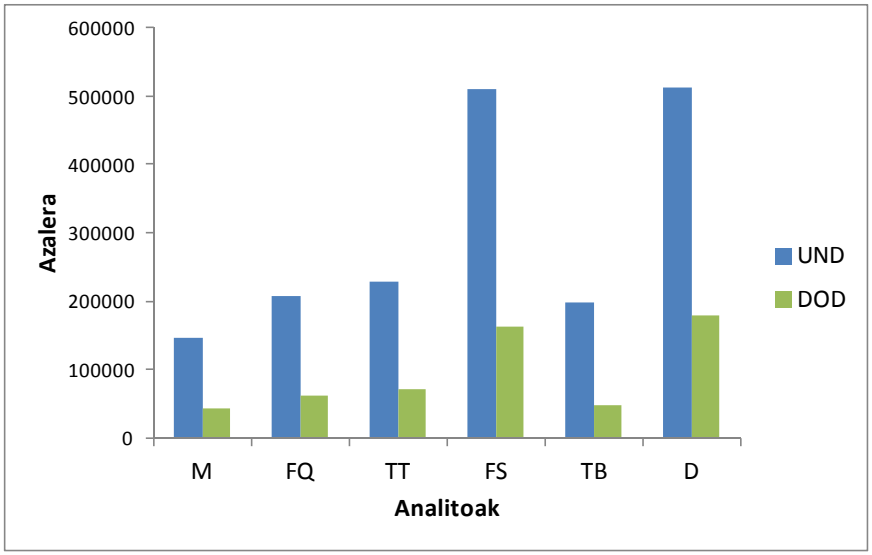
Erauztailea	**ACN/URA (v/v)**					
	25/75	**50/50**	**60/40**	**70/30**	**80/20**	**90/10**
BRHEX	x	x	x	x	x	x
HEX	x	x	x	x	x	x
UND	x	x	~	√	√	√
DOD	x	x	x	x	√	√

5.2. Taula (jarraipena). *Erauztaileen bateragarritasun froga HPLC-ko fase-mugikor aukerekin. Bolumen totala 2 mL + 20 µL erauztaile.*

Erauztailea	MeOH/URA (v/v)					
	25/75	50/50	60/40	70/30	80/20	90/10
BRHEX	x	x	x	x	x	x
HEX	x	x	x	x	x	x
UND	x	x	~	√	√	√
DOD	x	x	~	√	√	√

x: ez da bateragarria, disoluzio
~: ez da guztiz bateragarria, disoluzio uherra
√: bateragarria

Erauztaileen aukera murriztu ondoren, 1-undekanola eta 2-dodekanolarekin erauzketa frogak egin ziren, bien arteako ezberdintasunak ikusteko. Froga hauetarako erabilitako baldintzak ondorengoak izan ziren: Zuzenean ezarritako tantaren mikroerauzketa (DSDME) erabili zen hasierako froga hauetarako; triazol bakoitzaren kontzentrazioa 80 µg L^{-1}-koa izanik; erauztaile bolumena 15 µL; erauzketa denbora 30 minutu; erauzketa tenperatura 60^{o}C, gatz gehikuntzarik ez eta 500 rpm-ko agitazioa. Solidifikazioa 3^{o}C-tan eman zen 10 minutuz. 5.2. irudian ikus daitekeen bezala, analito guztien kasuan, 1-undekanola erabiliz erauzketak eraginkorragoak izan ziren 1-dodekanola erabiliz baino. Beraz, aurrerantzeko erauzketa guztiak 1-undekanolarekin egin ziren.

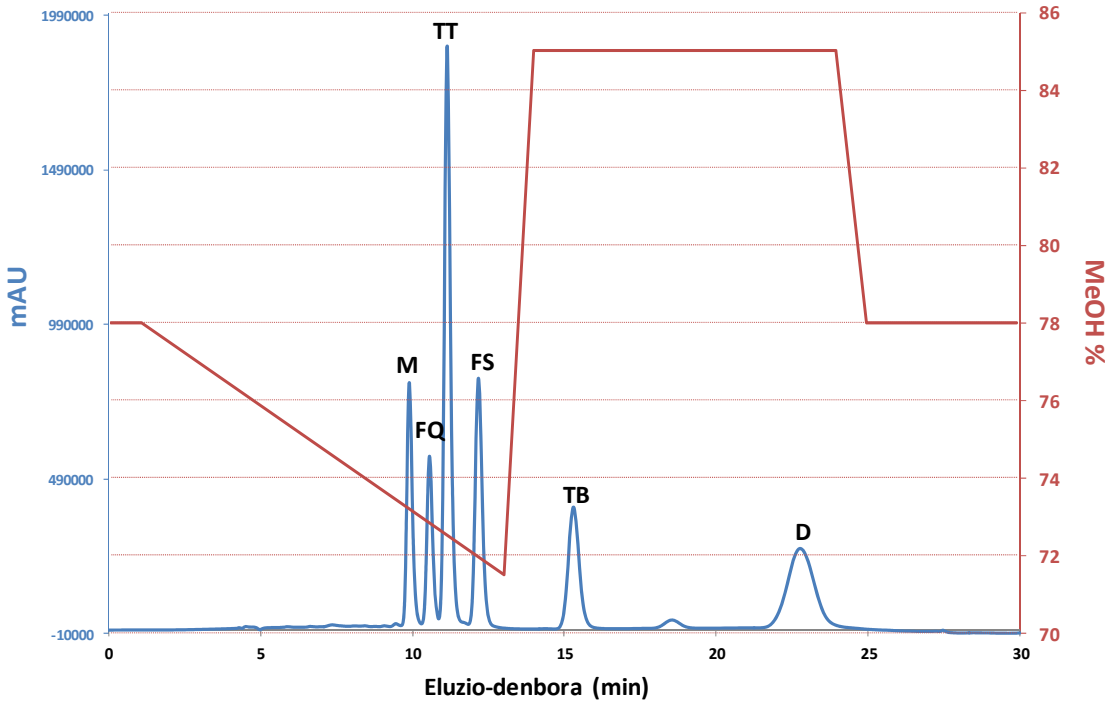


5.2. Irudia. *1-undekanola (UND) eta 1-dodekanolaren (DOD) erauzketa konparazioa. . M: miklobutanila; FQ: flukinkonazola; TT: tetrakonazola; FS: flusilazola; TB: tebukonazola eta D: dinikonazola.*

5.3.2 Banaketa kromatografikoa. Fase-mugikorra

Erauztailearen aukeraketa egin eta gero, lehen pausua tantak ondoren izango zuen fase-mugikorraren bateragarritasuna behatu zen. Azetonitriloa eta metanola erabili zitezkeen, metanolak portaera zertxobait hobea bazuen ere. Aurreko ikerketa batean sei analito horien banaketa egin zenean [Bordagaray *et al.*, 2013], fase-mugikorra osatuko zuen disolbatzaile organikoaren erabilera mugatua zegoen, SPME zuntzengatik. Kasu honetan muga hori ez zegoenez eta azetonitriloarekin lortutako banaketa hoberenak apur bat gainezartzen zirenez, metanola erabili zen.

Hainbat gradienteekin frogak egin ondoren, banaketa hoberena metanolarekin lortu zen 5.3. irudian adierazten den gradientearekin. Bertan ikus daiteke metanolaren ehunekoaren aldaketa eta gradiente horrekin lortzen den banaketa kromatografikoa. Fase-mugikorra osatu zuen beste disoluzioa pH 4-ra doitutako azetiko/azetato tanpoia izan zen, aurrez izandako eskarmentuan oinarritua egokitzat jotzen zelako.



5.3. Irudia. *Fase-mugikor gradientearekin (gorrian metanol ehunekoa adierazten da) lortutako sei triazolen banaketa kromatografikoa. Kromatograma 221 nm-tan jasotakoa da.*

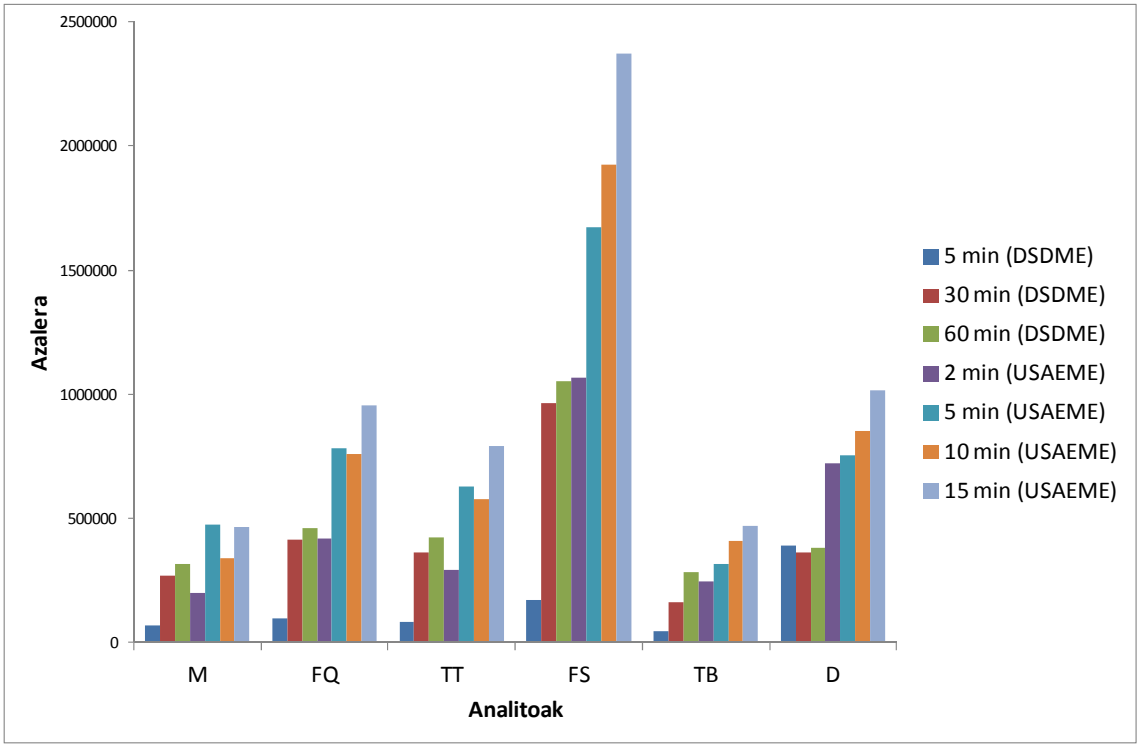
Fluxua 0.5 mL min^{-1}-tan mantendu zen denbora kromatografikoan zehar, azken bi minutu eta erdiak ezik. Fluxu aldaketak oinarrizko lerroan jitoak sor ditzake, horregatik ez da gomendagarria analitoak eluitzen dutenean aldatzea, baina behin analito guztiak zutabetik eta detektagailutik pasa badira, zirkuitu osoa azkarrago homogeneizatzeko erabilgarria da.

Baldintza horietan, ondorengoa izan zen analitoen eluzio-ordena: miklobutanila (9.9 ± 0.1 min), flukinkonazola (10.6 ± 0.1 min), tetrakonazola (11.1 ± 0.1 min), flusilazola (12.2 ± 0.1 min), tebukonazola (15.3 ± 0.2 min) eta dinikonazola (22.8 ± 0.7 min). Atxikipen-denbora aldaketak egon daitezke kromatograma batetik bestera, parentesi artean ageri dira analito hauen denborak jasan dituen desbideraketak. Ikus daitekeen bezala, eluzio-denbora luzeetan jasaten den desbideratzea handiagoa da. Izan ere, analitoak zutabean igaroko duen denbora zenbat eta handiagoa izan, zutabeko partikulekin interakzio kopuru gehiago ematen dituenaren ezaugarri da eta horren ondorioz, askotan gailur-kromatografikoa zabaldu eta mugitu ohi da.

5.3.3 DSDME-SFO eta USAEME-SFOren arteko konparazioa

Analitoen izaeraren ondorioz eraginkorragoa izan liteke erauzketa modu bat ala beste. Erauzketan zehar tanta osorik mantentzea edo dispertsatzearen aukeraketa egin behar da. Adibidez, analitoen lurrunkortasunak zerikusia izan dezake, izan ere gas fasean (buru-gunean) analito kontzentrazio garrantzitsua badago, erauztailea likidoaren gainazalean egotea mesedegarria litzateke; beste kasu batean, ordea, analitoak batez ere likido fasean badaude, tantak likidoarekin ahalik eta kontaktu gehien badu izango da erauzketarik eraginkorrena. Ultrasoinuak tanta ehunka tanta txikiagotan banatuko duenez, kontaktu gainazala handiagotzen da fase akuoso eta organikoaren artean, masa-transferentzia lagunduz, eta horren ondorioz eraginkortasuna hobetzen da.

Analito hauen lurrunkortasuna nahiko mugatua denez, eraginkorragoa izan zen USAEME bidezko erauzketa DSDME bidezkoa baino 5.4. irudian ikus daitekeen bezala. Analisi hauek ondorengo baldintzetan burutu ziren: amankomuneko baldintzak triazolen kontzentrazioa (80 μg L^{-1}), lagin akuoso bolumena (10 mL), erauztailea eta bere bolumena (UND, 20 μL), erauzketa tenperatura (25^{o}C), gatz gehikuntza (180 g L^{-1}) eta solidifikazio denbora eta tenperatura (5 min, 3^{o}C) izan ziren. Teknika bakoitzarenak diren ezaugarriak ondorengoak izan ziren: DSDME-rako erabilitako agitazioa 500 rpm eta USAEMEren ondoren erabili beharreko zentrifugazioa 10 minutu eta 4200 rpm-ko abiadura. Grafikoan ikus daitekeen bezala, denbora ezberdinen konparazioa egin zen. Argi ikus daiteke USAEME askoz eraginkorragoa dela, izan ere, ordu betez emandako DSDME-ren azalera kromatografikoak ia USAEME bidez 2 minututan lortutako azaleren parekoak baitira eta bi erauzketa motetan denbora bera mantenduz (5 min) ikus daiteke kasu guztietan USAEME erauzketa hobeagoa dela. Zehatzago esanda, ia 10 aldiz handiagoa flusilazolaren kasuan eta bikoitza dinikonazolaren kasuan.



5.4. Irudia. *DSDM-SFO eta USAEME-SFO metodoen konparazioa denbora ezberdinetan zehar sei analitoentzako.*

Behin erauztailea eta erauzketa-mota hautatuta, beste aldagaiak optimizatzeari ekin zitzaion. Jarraian egindako analisi guztiak 1-undekanola eta USAEME erabiliz egin ziren.

5.3.4 Diseinu esperimentala

Metodo bat garatu ahal izateko, ahalik eta erantzun kromatografiko hoberena bermatzen duten baldintzak optimizatzea beharrezkoa da. Aldagai ezberdinak banan-bana azter daitezke, baina aldagai kopurua handia izanez gero, lan nekagarria izan daiteke. Bestalde, hainbat aldagai batera aztertzeko metodoak daude eta horretarako diseinu esperimentalaren barnean hainbat erreminta daude eskuragarri: miaketa tresnak, aldagai batek metodoan duen eragina ikusi ahal izateko, edota optimizazio teknikak, garrantzitsuak diren aldagaien balio optimoak jakiteko.

Aurrez eginiko hainbat saioen ondoren aldi berean optimizatu beharreko aldagaiak hautatu ziren: erauzketa denbora eta tenperatura eta sodio kloruro gehikuntza. Gainerako baldintzak aurrez finkatu ziren ikertzailearen esperientziaren arabera. Esaterako lagin akuoso bolumena 10 mL-tan finkatu zen, bolumen handiagoak zentrifugan eragiten zuen indar zentripetuak ontzia puskatzen baitzuen. Bestalde, aurrez eginiko frogetan undekanola hautatu eta bere bolumena 50 µL-tan finkatu zen eroso lan egin ahal izateko. Tanta txikiagoekin lan egitea zaila zen, ondoren jaso beharreko tantaren sorkuntza nekez ematen zelako. Zentrifugazio baldintzak tanta berriz ondo osatu ahal izateko baldintza minimoetan finkatu ziren, hau da, 10 minutu eta 4200 rpm-ko abiadura. Izozte baldintzak ere 3^{o}C eta 5 minututan finkatu ziren, tenperatura eta denbora hori aski baitziren solidifikazioa egoki emateko. HPLC-rako bateragarria egiteko gehitu zitzaion metanol bolumena ahalik eta txikiena izan zen (20 µL), baina gutxienezko kantitate hori behar izan zen disoluzio guztiz garden bat lortzeko.

Optimizatu beharreko aldagaiak hiru bakarrik zirenez, zuzenean diseinu konposatu zentral (CCD) bat proposatu zen, miaketaren urratsa jauzi eginez, analisi kopuru totala (miaketa + optimizazioa) asko handiagotuko baitzen eta hiru aldagaiko CCD baten analisi kopurua ez baita oso altua.

5.3.4.1 Diseinu konposatu zentrala

Ahalik eta erantzun kromatografiko hoberena lortze arren, optimizazio diseinu bat burutu zen diseinu konposatu zentral baten bidez. Horretarako hiru aldagai aukeratu ziren, gainerakoak aurrez finkatu baitziren. Optimizaziorako proposaturiko aldagaiak erauzketa tenperatura eta denbora eta gatz gehikuntza izan ziren.

Diseinua 2^3 diseinu faktorial batez eta [(2 x 3) + 1] izar puntuez osatu zen, puntu zentralez gain. Esperimentuaren puntu faktorial eta axial bakoitza bi aldiz errepikatu zen. Hortaz, guztira 30 esperimentu izan ziren eta hiru puntu zentral gehiago gehitu ziren. Puntu axialak hautatzeko erabilitako baldintza errotabilitatea izan zen ($\alpha = \sqrt[4]{N_f} = 1.682$; $N_f=2^3$). 5.3. taulan ageri dira diseinu honetako aldagaiak eta berauen balioak.

Aldagai tarteak, beraz, ondorengoak izan ziren: erauzketa tenperaturaren tartea 25tik 35^{o}C-ra bitartean finkatu zen, eta izar puntuak 21 eta 38^{o}C-tan. Denborari dagokionez, 5 eta 15 minutuko tartea aukeratu zen eta izar puntuak 2 eta 18 minutuetan kokatu ziren. Azkenik, sodio kloruro gehikuntza 130 eta 230 g L^{-1} bitartekoa izan zen eta izar puntuak 96 eta 264 g L^{-1} izan ziren.

5.3. Taula. *CCD-rako hautatutako aldagaiak eta beraien mailen balioak.*

Aldagaiak	**Maila**			
	Baxua [-1]	**Altua [+1]**	**[-α]**	**[+α]**
Erauzketa tenperatura [Er T] (^{o}C)	25	35	21	38
Erauzketa denbora [Er t] (min)	5	15	2	18
NaCl gehikuntza [NaCl] (g L^{-1})	130	230	96	264

Esperimentu ordena ausazkoa izan zen, 5.4. taulan ikus daitekeen bezala. Bertan aldagai bakoitzaren balio kodifikatua [-α, -1, 0, +1 edo +α] eta aurrera eramandako esperimentuen ordena ageri dira. 5.5. taulan, berriz, esperimentuaren erantzunak ikus daitezke. Erantzun hori, azalera kromatografikotan islatzen da, hain zuzen ere 221 nm-tan jasotako kromatogramen gailurren azalera da optimizatu nahi den erantzun maximoa.

5.4. Taula. *CCD diseinua bere aldagai kodifikatuekin eta ausazko ordenarekin.*

Esperimentua	Aldagaiak α		
	Er T (ºC)	Er t (min)	NaCl (g L^{-1})
13	0	+α	0
5	+1	-1	-1
19	-1	+1	-1
16 (C)	0	0	0
21	+1	-1	-1
30	0	0	-α
4	-1	+1	+1
15	0	0	+α
18	-1	-1	+1
9 (C)	0	0	0
11	+α	0	0
7	+1	+1	-1
25 (C)	0	0	0
2	-1	-1	+1
8	+1	+1	+1
17	-1	-1	-1
3	-1	+1	-1
27	+α	0	0
10	-α	0	0
20	-1	+1	+1
26	-α	0	0
22	+1	-1	+1
28	0	-α	0
12	0	-α	0
14	0	0	-α
23	+1	+1	-1
24	+1	+1	+1
29	0	+α	0
32 (C)	0	0	0
31	0	0	+α
1	-1	-1	-1
6	+1	-1	+1
33 (C)	0	0	0

(C): puntu zentrala

5.5. Taula. *CCD diseinuan lortutako analitoen azalera kromatografikoak.*

Esperimentua	Azalerak					
	M	**FQ**	**TT**	**FS**	**TB**	**D**
13	735103	728034	2097653	1549233	571112	1494757
5	719030	758690	2016598	1557274	531821	1022966
19	910073	751868	2079796	1580599	590643	1153723
16 (C)	737504	760393	2061979	1589878	532765	1004423
21	690373	707533	1992041	1587163	577538	1412607
30	781349	722576	2037206	1584125	589798	1430800
4	763899	750416	2195582	1634068	598419	1484520
15	767111	725774	2131325	1542750	568866	1360141
18	797267	730267	2119212	1530695	568035	1343438
9 (C)	785767	792603	2155597	1634932	555707	1311018
11	757699	768782	2133107	1794588	554879	1092962
7	697082	717831	2054310	1596952	583388	1143580
25 (C)	782065	738729	2114198	1583286	610491	1389217
2	758541	764908	2065959	1601087	535944	1020278
8	758817	741773	2156140	1613883	593318	1145262
17	819439	828122	2211654	1805352	582469	1461784
3	766836	736297	2003889	1582637	579061	1113181
27	778136	759934	2075546	1526284	589957	1530291
10	887740	837642	2350107	1827153	656042	1243770
20	749174	753634	2120660	1628680	601069	1498336
26	896170	841231	2176186	1679985	568574	1155942
22	766548	734046	2082176	1525272	588531	1424109
28	771531	711050	2015644	1536705	585771	1746323
12	705895	693373	1977589	1472447	552769	1042229
14	768034	773762	2085880	1638853	547073	965614
23	720236	719484	2008127	1609721	572064	1513036
24	800072	786484	2280209	1788034	606155	1431937
29	1009943	847876	2264475	1984968	584182	1616679
32 (C)	796205	711458	2059134	1543403	584884	1134552
31	915935	763095	2129599	1515268	588882	1441173
1	785479	805805	2115226	1606017	555994	1039442
6	700240	690240	1984585	1476899	542672	994878
33 (C)	761538	687025	1930512	1499539	536061	1051115

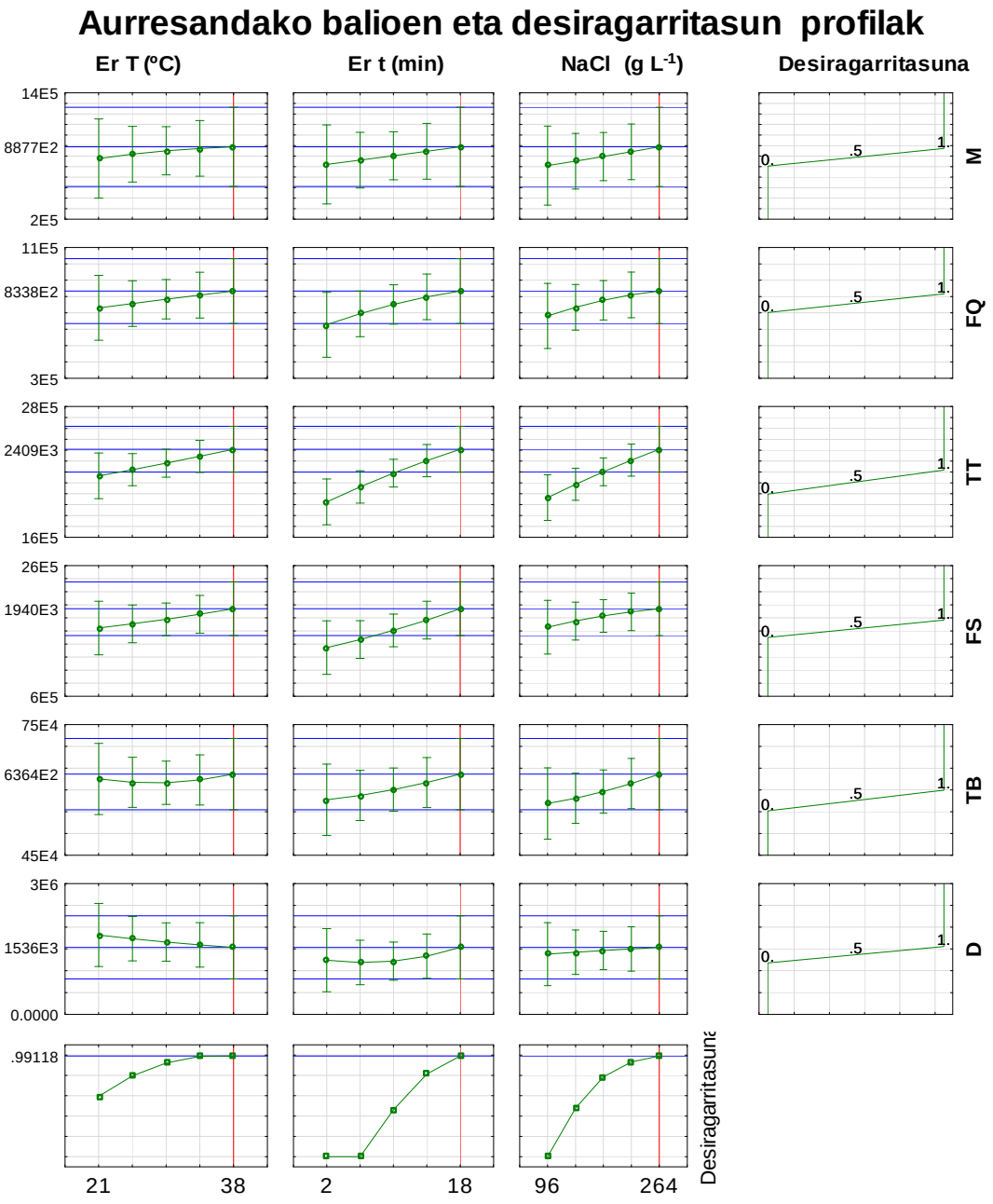
(C): puntu zentrala

Emaitza hauek Statistica softwarearekin aztertu ziren. Emaitzak irudikatzeko modurik ohikoena erantzun azalera bidezkoa da. Irudikapen horietan analito bakoitzarentzat eta bi aldagai hautatuz 3D gainazal bat marrazten da, non beraien joera ikusten den. Hau da, ardatzetan hautatutako bi aldagaien balioak jartzen dira eta

balio batetik besterako bide horretan erantzunaren aldaera ikusten da. Irudikapen honen desabantaila, ordea, lortzen diren irudikapen kopurua da. Izan ere analito bakoitzarentzat, kasu honetan, hiru irudikapen beharko genituzke.

Irudikatzeko beste modu bat desiragarritasun-funtzioaren bidez lortutakoa litzateke [Costa *et al.*, 2011]. Desiragarritasun-funtzioarekin aldagai bat baino gehiago optimizatu daitezke aldi berean [Derringer eta Suich, 1980]. Funtzioak, erantzun hoberenari (desiragarritasun handienari, hau da, azalera kromatografiko handienari) 1.0 balioa ematen dio eta, aldiz, erantzunik okerrenari (desiragarritasun txikiena duena, azalera kromatografiko txikia) 0.0 balio ematen dio. Beraz, desiragarritasun-funtzioak erantzun guztiak 0.0 eta 1.0 balioen artean eskalatzen dituela esan daiteke. Desiragarritasun-funtzioa aplikatu ondorengo irudikapena ikus daiteke 5.5. irudian. Lerro bakoitzean analito ezberdin bakoitza ageri da eta aldagaiek berauengan izango duten eragina. Azken lerroan, orotara lortutako desiragarritasuna ikusten da, hots, aldagai bakoitzak analitoengan oro har izango duen efektua.

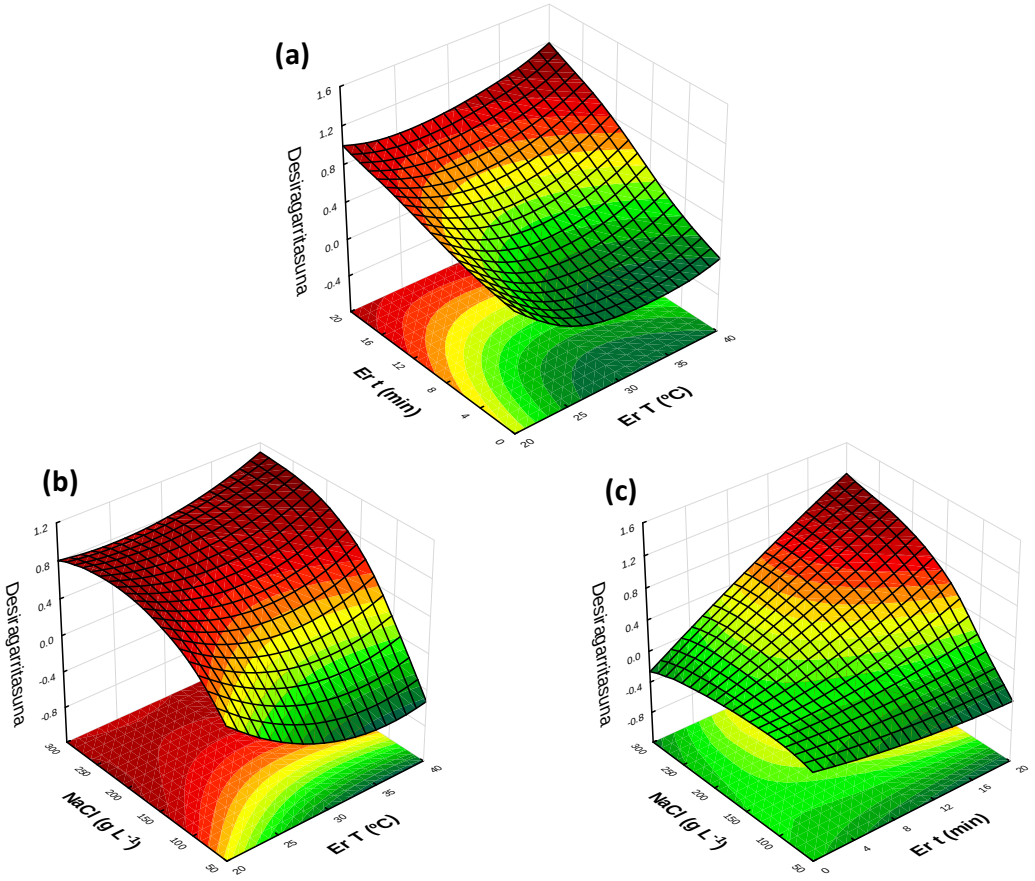
Zutabeetan, aldagai ezberdinak ikus daitezke: lehen zutabean erauzketa tenperaturaren eragina ikusten da, bigarrenean erauzketa denbora eta hirugarrenean NaCl kontzentrazioa. Lehen zutabean ikus daitekeen bezala, kasu gehienetan, tenperaturaren igoerak erauzketaren lagungarri direla ikusten da, baina dinikonazolarentzat kontrako efektua gertatzen da, tenperatura handiagotan, erauzketaren eraginkortasuna txikiagoa da. Balio optimoa 38^{o}C-tan aurkitzen da, baina balio honek puntu zentraletik aurreragoko balioekin ez dirudi alde esanguratsurik duenik. Bigarren zutabean erauzketa denbora ikus daiteke, eta bertan denbora luzeagoek eragin positiboa dutela ikusten da analito guztietan. Bada, balio optimoa 18 minututan ezar daiteke. Azkenik, hirugarren zutabean, gatz gehikuntza ikusten da. Bertan, NaCl erauzketaren lagungarria dela ikus daiteke, analito guztiek erantzun positiboagoa baitute gatz kontzentrazio altuekin.



5.5. Irudia. *Aurresandako balio eta desiragarritasun profilak optimizazioan kontuan hartutako hiru aldagaiekin: Erauzketa tenperatura eta denbora eta gatz gehikuntza.*

Analito bakoitza independenteki aztertu beharrean, desiragarritasun gainazal globalak ere azter daitezke. Gainazal mota horiek analito guztien efektua batera hartzen dute aintzakotzat. Halere, aldagaiak binan-bina ikus daitezke eta beraz, konbinazio guztiak kontsideratu ahal izateko beharrezkoak dira hiru grafiko. Horiek, ondorengo 5.6. irudian ikus daitezke. Kolore eskala berdetik (desiragarritasun txikiena)

gorrira (desiragarritasun handiena) doa. Erauzketa tenperaturari behatuz gero, tenperatura altuagoek erauzketa zertxobait laguntzen dutela ikus daiteke 5.6. irudiko (a) eta (b) grafikoetan, baina efektua ez da oso nabarmena. Denborari dagokionez, denbora luzeek eraginkortasun handiagoa erakusten dute (a) eta (c) grafikoetan. Azkenik, (b) eta (c) grafikoetan gatz gehikuntzari dagokionez, bere eragin positiboa ikus daiteke.



5.6. Irudia. *Desiragarritasun globalaren erantzun-gainazalak: (a) Erauzketa denbora eta tenperatura kontuan hartuz; (b) gatza eta erauzketa tenperatura kontsideratuz eta (c) gatza eta erauzketa denbora aintzakotzat hartuz.*

Bi irudiak kontuan hartuz (5.5. eta 5.6. irudiak), ondorengoa ondorioztatu daiteke: baldintza optimoak 38^{o}C, 18 minutu eta 264 g L^{-1} NaCl lirateke, baina, ikerketarekin jarraitzeko aukeratutako baldintzak apur bat aldatu ziren. Izan ere,

tenperaturarekiko alde esanguratsurik ez zegoenez eta analito batzuei tenperatura altuek mesederik egiten ez zienez, tenperatura 30^{o}C-tara jaistea erabaki zen. Bestalde, gatz gehiegi erabiltzeak zentrifugatu ondoren tanta berriz sortzeak kaltetu lezake eta beraz, kontzentrazioa pixka bat jaitsi zen 250 g L^{-1}-ko balioa hautatuz.

Ondorioz, laborategiko esperientziagatik, aldagai bakarreko analisian edo aldagai anitzeko analisian lortutako emaitzekin ondorengo baldintzak finkatu ziren erauzketa prozesurako:

- **Erauztailea: 1-undekanola**
- **Erauztaile bolumena: 50 µL**
- **Lagin bolumena: 10 mL**
- **Gatz gehikuntza: 250 g L^{-1}**
- **Erauzketa tenperatura: 30±1^{o}C**
- **Erauzketa denbora: 18 min**
- **Zentrifugazio abiadura: 4200 rpm**
- **zentrifugazio denbora: 10 min**
- **Izozte tenperatura: 3^{o}C**
- **Izozte denbora: 5 min**
- **Urtutako tantatik hartutako bolumena: 25 µL**
- **Gehitutako amaierako metanola: 20 µL**

Puntu horretatik aurrera eginiko erauzketa guztietan baldintza hauek finkatu eta errespetatu ziren.

5.3.5 Metodoaren balidazioa

Metodoaren baldintzak behin optimizatu eta finkatu ondoren, metodoaren balidazioari ekin zitzaion. Metodo baten egokitasuna ikusteko beharrezkoa da zenbait ezaugarri analitiko jakin ahal izatea [Ruiz-Angel *et al.*, 2014]. Horrela, gure metodoa nahi den helbururako egokia, zehatza eta fidagarria den ala ez erabaki baitezakegu [Taverniers *et al.*, 2004; Rambla-Alegre *et al.*, 2012]: tarte lineala eta linealtasuna, detekzio-muga, errepikakortasuna eta zehaztasuna dira horietako batzuk. Aberastasun-faktorea metodo balidaziotik kanpo geratzen bada ere, ezaugarri interesgarria da erauzketaren eraginkortasuna ikusteko eta atal honetan kalkulatu zen.

Kalibratua egiterako orduan uhin-luzera ezberdinak hartu ziren aintzakotzat. Oro har, ultramore espektroak maximoak erakusten dituen uhin-luzera aukeratzen da determinazioa egiteko, baina 4. atalburuan azaldu zen moduan, analito hauen uhin-luzera maximoa balio txikietan zegoen (190- 200 nm bitartean) eta balio horietan fase-mugikorreko disolbatzaileek eragin zuzena izan dezakete. Kasu horretan, metanola erabili zen eta honen uhin-luzera mozketa 211 nm-koa da [Burgess eta Frost T., 1999]. Ondorioz, uhin-luzera altuagoetara jo zen. Analito gehienak 221 nm inguruan beste maximo edo inflexio-puntu bat erakusten zuten, dinikonazolak ezik (honek bigarren maximoa 249 nm-tan zuen). Hori ikusita, errepikakortasuna aztertu zen bi uhin luzeretan eta emaitza hoberenekin hartu zen erabakia. 5.6. taulan ikus daitekeen bezala, 221 nm-tan egin ziren neurketak miklobutanil, flukinkonazol, tetrakonazol, flusilazol eta tebukonazol kasurako eta 249 nm-tan dinikonazolaren kasurako.

Linealtasuna gutxi gorabehera 20-800 µg L^{-1}-ko tartean aztertu zen kontzentrazio ezberdineko bederatzi estandarren bitartez (analito bakoitzaren balio zehatzak 5.1. taulan ageri dira). Kalibrazioa egiterako orduan bi tarte hartu ziren kontuan, tarte baxua (20-200 µg L^{-1}) eta altua (100-800 µg L^{-1}); eta bakoitza eraikitzeko 5.1 taulako sei estandar erabili ziren. Tarte horietan lortutako erregresio koefizientearen balioak (R^2) 0.9931 baino altuagoak izan ziren kasu guztietarako (ikus 5.6. taula). Kalibrazio zuzen hauek eraikitzeko estandar bakoitzarekin egindako hiru erauzketen batezbestekoak erabili ziren.

Tarte altuko erregresio koefizienteak (R^2), oro har, baxukoak baino hobeagoak dira eta kasu batean ia 1 baliora ere iristen da (M). Linealtasun balioak ikusita, kalibratu denak hartzen dira ontzat.

5.6. Taula. *USAEME-SFO-HPLC/DAD metodoarekin lortutako kalibrazioaren ezaugarriak. Tarte baxua eta altua hartu dira kontuan.*

Analitoa	Uhin-luzera (nm)	Tartea	Tarte lineala (μg L^{-1})	Kalibrazio ekuazioa	R^2
M	221	Baxua	20.7-207.4	y=17468x +185871	0.9971
		Altua	103.7-830.0	y=17044x +251703	0.9999
FQ	221	Baxua	19.0-190.4	y=13592x +79406	0.9960
		Altua	95.2-762.0	y=14191x -49435	0.9987
TT	221	Baxua	20.5-204.7	y=46579x +158017	0.9971
		Altua	102.3-819.0	y=48072x -187345	0.9993
FS	221	Baxua	20.9-208.8	y=22865x +128490	0.9931
		Altua	104.4-835.0	y=23144x +20504	0.9991
TB	221	Baxua	22.2-221.7	y=15288x +117321	0.9947
		Altua	110.8-887.0	y=16544x -138380	0.9993
D	249	Baxua	20.6-206.0	y=21387x +90349	0.9948
		Altua	103.0-824.0	y=23143x -256896	0.9990

Kalibrazioaren ondoren baliagarria den beste ezaugarri bat da metodoaren detekzio-muga (LOD, *Limit of Detection*). Ezaugarri honek, garatutako metodoak neur dezakeen kontzentraziorik txikiena emango digu. Detekzio-muga kalkulatzeko hainbat modu daude, oro har, desbideratze estandar bat koefiziente batez biderkatzen lortzen da [Miller eta Miller, 2002]. IUPAC-ek [Currie, 1995] hainbat zuri neurtu ondoren kalkulatzen den desbideratze estandarra hiru aldiz biderkatuz lortzen da. Metodo zuriak lortzea, ordea, zaila izan daiteke kasu batzuetan. Kromatografian adibidez, kromatograma luzeak egonez gero, denbora asko beharko litzateke. Gainera, kasu

jakin horretan, eluzio-denboren mugikortasuna dela eta, tarte zehatz batean neurketak egitea okerreko aukera izan daiteke. Horren ordez, kalibrazio maldaren desbideratzea erabili zen eta ondorengo moduan kalkulatu ziren metodoaren detekzio-mugak [Konieczka eta Namieśnik, 2009]:

$$s_{y/x} = \sqrt{\frac{\sum(\hat{y}_i - y_{i,ref})^2}{I-2}} \qquad (5.2)$$

$s_{y/x}$ maldaren desbideratze estandarra izango da eta $\sum(\hat{y}_i - y_{i,ref})^2$, aldiz, maldak duen hondar-balioa, non $y_{i,ref}$ erreferentziazko y balioa den eta $\hat{y}_i$ kalibrazio zuzenetik lortutako y balioa. (*I-2*) askatasun graduak dira, non *I* dugun lagin kopurua izango den. Azkenik *b*, maldaren balioa izango da.

$$LOD = \frac{3 \cdot s_{y/x}}{b} \qquad (5.3)$$

Kalkulatutako kalibrazioaren maldaren desbideratzetik ($s_{y/x}$) kalkulatu zen detekzio-muga (LOD), hiru aldiz maldaren desbideratzea eginez eta maldaren balioaz zatituz. Ekuazio horiek kontuan hartuz kalkulatu ziren detekzio-mugak eta lortutako balioak 10.9 eta 17.2 µg L^{-1} bitartekoak izan ziren 5.7. taulan ikus daitekeen bezala.

Europar Batasunak (EB) ezarritako gehienezko hondakin mugak (MRL) mg kg^{-1} unitateko emanak daudela eta metodo honen garapenean lagin likidoak erabili dira. Halere lagin hauen dentsitatea 1 kg L^{-1} ingurukoa dela kontuan hartuz eta guztiz konparagarriak ez badira ere, lortutako detekzio mugak EB-k ezarritako mugak baino balio baxuagoak dira.

Bestalde, triazolen determinazioan erabilitako antzerako teknikekin konpara daitezke lan honetan lortutako emaitzak. Ur laginetan eta IL-DLLME-HPLC/DAD bidez [Ravelo-Pérez *et al.*, 2009] determinatutako tebukonazolaren detekzio-muga 3.9 µg L^{-1}-koa izan zen. Aldiz, Luok DLLME-HPLC/DAD bidez ur-laginetan lortutako tebukonazolaren enantiomero ezberdinen detekzio-mugak 19.8 eta 25.4 µg L^{-1} bitartekoak izan ziren [Luo *et al.*, 2013]. Bestalde, fruitu laginetan lortutako detekzio-mugak ere ezberdinak dira. Esaterako SBSE-DLLME-GC/FID bidez dinikonazola eta tebukonazola determinatu ziren eta lortutako balioak 1.3 eta 1.6 µg L^{-1} artekoak izan ziren [Farajzadeh *et al.*, 2010]. Ultrasoinuz lagundutako emultsifikazio mikroerauzketa magnetikoa (USAEMME) GC/FID bidez determinatutako miklobutanil eta

tebukonazolarentzat fruitu zukuetan lorturiko detekzio-mugak 1.8 eta 2.3 µg L^{-1} bitartekoak izan ziren [Li *et al.*, 2014].

5.7. Taula. *USAEME-SFO-HPLC/DAD metodoarekin lortutako detekzio-mugak.*

Analitoa	LOD (µg L^{-1})
M	11.2
FQ	11.9
TT	10.9
FS	17.2
TB	16.0
D	14.8

Datuen fidagarritasuna ikusteko errepikakortasuna aztertzen da, hau da, analisi batetik bestera zein desbideratze jasan dezaketen behatzen da. Horretarako desbideratze estandar erlatiboa erabiltzen (DEE) da. Bi errepikakortasun mota aztertu ziren: lehena egun baten errepikatutako sei erauzketa burututa (n=6) eta bigarrena, aldiz, egun ezberdinetako errepikakortasuna zen eta hamabost neurketa hartu ziren kontuan (n=15). Bigarren honetarako hamabost neurketa horiek bi astetan zehar burututako hiru egun ezberdinetan egin ziren. Errepikakortasun bakoitza bi kontzentrazio ezberdinetan ikusi zen, tarte baxua eta altua kontuan hartuz. Horiek horrela, lortutako balioak % 3.1 eta 6.0 (50 µg L^{-1}) bitartekoak izan ziren eta %1.9 eta 4.6 (400 µg L^{-1}) artekoak egun barneko errepikakortasunean. Aldiz, egun ezberdinetako errepikakortasuna %5.7 eta 10.6 (50 µg L^{-1}) bitartekoa eta %3.1 eta 6.1 (400 µg L^{-1}) artekoa izan zen. Balio guztiak 5.8. taulan ikus daitezke.

5.8. Taula. *USAEME-SFO-HPLC/DAD metodoarekin bi kontzentrazio ezberdinetan (tarte baxuan eta altuan) lortutako errepikakortasun balioak desbideratze estandar erlatiboaz(DEE) adierazita.*

Analitoa	Egun barneko errepikakortasuna (n=6) (DEE%)		Egun arteko errepikakortasuna (n=15) (DEE%)	
	50 µg L^{-1}	400 µg L^{-1}	50 µg L^{-1}	400 µg L^{-1}
M	6.0	3.1	9.4	6.1
FQ	4.1	4.6	8.6	5.2
TT	4.4	3.0	5.7	4.3
FS	3.1	1.9	10.6	4.3
TB	5.1	2.6	6.7	3.1
D	5.4	3.7	10.2	6.1

Lortutako errepikakortasun datuak AOAC (*Official Methods of Analysis*) onartutako balioekin konparatu ziren kontzentrazio tartea kontuan hartuz [AOAC International, 2012]. 5.9 taulan ikus daitekeen bezala, metodo honen bidez lorturiko balioak onak kontsidera daitezke.

5.9. Taula. *AOAC metodo bat balidatzerakoan onargarriak diren balioak.*

Analito frakzioa	Unitatea	RSD (%)	Berreskuratzea (%)
1	100 %	1.3	98-102
10^{-1}	10 %	1.9	98-102
10^{-2}	1 %	2.7	97-103
10^{-3}	0.1 %	3.7	95-105
10^{-4}	100 ppm	5.3	90-107
10^{-5}	10 ppm	7.3	80-110
10^{-6}	1 ppm	11	80-110
10^{-7}	100 ppb	15	80-110
10^{-8}	10 ppb	21	60-115
10^{-9}	1 ppb	30	40-120

Azkenik, nahiz eta parametro hau metodoaren balidaziotik kanpo dagoen, likido mikroerauzketa kasuetarako aberastasun-faktorea aztertzen da. Aberastasun-faktoreak erauzketaren arrakastaren ideia bat ematen digu. Hasierako disoluzioak duen analito edukia (C_0) tanta organikoan zenbateraino kontzentratzen den (C_d^{eq}) esaten digu.

$$EF = \frac{C_d^{eq}}{C_0} \qquad (5.4)$$

Horretarako 80 µg L^{-1}-ko disoluzio bat prestatu zen eta erauzketa aurrez finkatutako baldintzetan eman zen. C_d^{eq} kalkulatzeko, aurrez injekzio-zuzena erabiliz kalibratu bat egin zen HPLC erabiliz eta horrekin kalkulatu ziren balioak. Sei neurketa egin ziren guztira eta lortutako balioak beren desbideratzearekin 5.10. taulan ageri dira. Balioak 226-etik 255-era bitartekoak izan ziren.

5.10. Taula. *USAEME-SFO-HPLC/DAD metodoarekin 80 µg L^{-1}-ko kontzentrazioan lortutako aberastasun faktorea.*

Analitoa	**Aberastasun-faktorea (EF) (n=6) (± DE)**
	80 µg L^{-1}
M	246 ± 5
FQ	253 ± 5
TT	239 ± 2
FS	226 ± 4
TB	255 ± 1
D	249 ± 1

Metodo honekin lortutako EF balioak triazolentzako beste metodoekin lortu direnekin konpara daitezke. DLLME erabiliz miklobutanil eta tebukonazolarentzat 83-289 bitarteko balioak lortu ziren ur laginetan [Wang *et al.*, 2011; Ye *et al.*, 2012]. Fruitu laginetan barratxo nahasgailuaren sortzio erauzketa-likido likido mikroerauzketa dispertsibo (SBSE-DLLME) eta Sililatutako erauzketan ontziko likido likido mikroerauzketa dispertsibo (SEV-DLLME) bidez dinikonazol eta tebukonazolarentzat lortutako balioak altuagoak izan ziren, 482-710 bitartekoak izan ziren [Farajzadeh *et al.*, 2010; Farajzadeh *et al.*, 2011]

Balidazioko azken parametro bezala, zehaztasuna aztertu zen. Horretarako, ordea, benetako laginetan burutu ziren berreskuratze-frogak. Garatutako metodoa lagin ezberdinetan aplikatu zen: sagardoa, sagar-zuku naturala, sagar-zuku komertziala, mahats-zuku naturala eta mahats-muztio komertzialak erabili ziren horretarako.

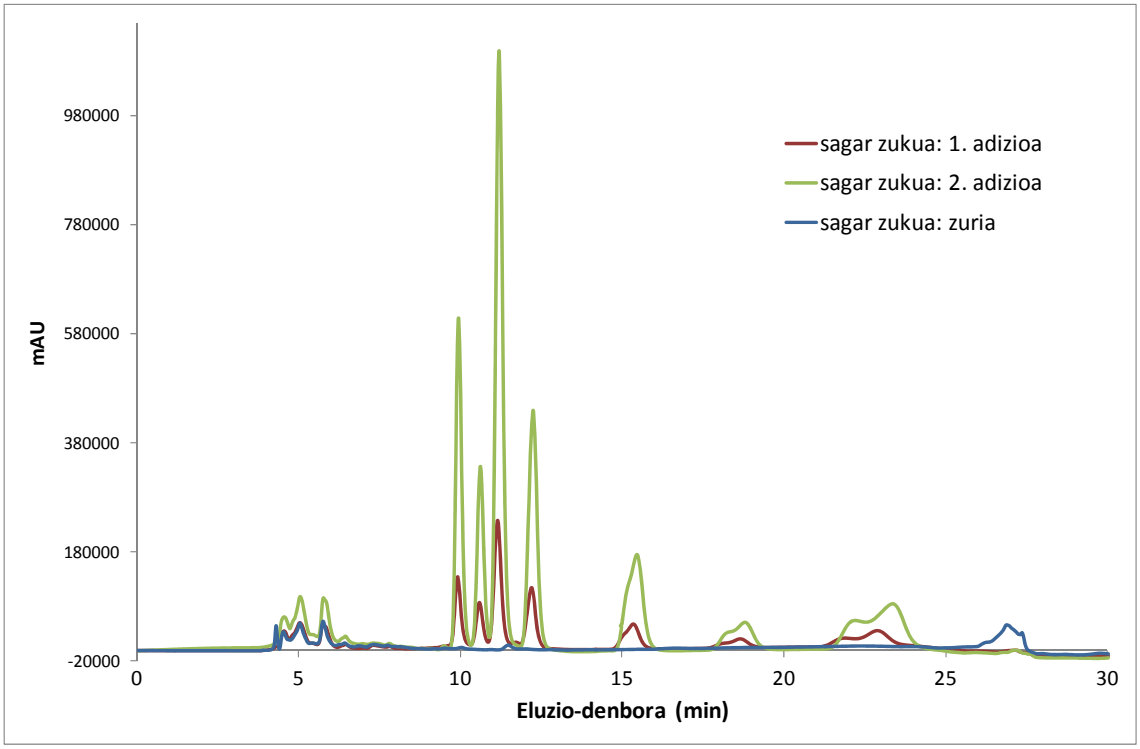
Hori horrela, lagin ezberdinen matrizeen portaera ikusteko estandarrak gehitu zitzaizkien eta beraien berreskuratze-ehunekoa aztertu zen. Berreskuratze hau aurkitutako balioa benetako balioaz zatituta lortu zen eta ehunekotan eman ohi da. Bi kalibrazio tarte ezberdinetan ikusi zen berreskurapena, beraz, gehitutako estandarrak analisirako bialean 80 eta 400 µg L^{-1}-ko kontzentrazioa eman zuten. 5.11. taulan ikus daitekeen berreskuratze balio bakoitza lau erauzketen ondoren lortutako batezbestekoa da eta desbideratze estandar erlatiboarekin batera aurkezten dira.

Matrizeak eragin zuzena zuen erauztailearen portaeran, ondorioz, froga batzuen ondoren ikusi zen laginetik gehitu beharreko bolumena 0.5 mL-koa zela (10 mL bolumen totala urarekin osatu zen).

5.11. taulan ikus daitekeen lehen kontua sagardoarekin lortutako berreskuratze ehuneko kaxkarrak eta bere DEE handiak dira. Sagardoarekin ikusi zen arazo nagusienetariko bat tantaren birformakuntza izan zen. Zentrifugazioaren ondoren tanta osoa ez zen berriz ondo sortzen, tanta asko baizik. Hori izoztu eta jasotzerako orduan jaso zitekeen ur kantitatea nahiko handia zen eta erauztaile horretatik hartu beharreko 25 µL-ek ur kantitate nahiko handia izan zezaketen. Sagardoarekin batera gehitutako alkoholak zerikusia zuela izan zitekeen horren arrazoia. Izan ere DLLME kasuetarako oso erabilia da etanola dispertsatzaile moduan eta USAEME prozedura honi alkohola gehitzeak tantaren portaeran eragin zuzena izan lezakeela uste izan zen.

Mahats-zukuarekin ere arazo garrantzitsuak egon ziren tantaren portaeran. 5.11. taulan ikus daitekeen bezala, tarte txikiko kontzentrazioan ez zen emaitza onargarririk lortu. Tantak beste hainbat konposatu erauzten zituen tantaren portaeran eragiten zutenak. Likido kromatografian sartu aurretiko baldintza garrantzitsu bat bezala ezarri zen, injektatuko zen frakzioa guztiz gardena izatea, fase-mugikorrarekin guztiz bateratuko zela ziurtatzeko eta zutabea bera ez buxatzeko. Bateragarritasun hori errazteko gehitzen zitzaion metanola HPLC-n sartu aurretik. Disoluzio hori uherra bazen, ez injektatzea erabaki zen. Lagin mota honekin batez ere arazo hori azpimarra zitekeen, injektatu zitezkeen tanta kopurua murritza izan zen eta injektatu zen tanten emaitzak ez ziren onak izan.

Beste hiru laginetan ez zen horrenbeste arazo ikusi eta analisiak aurrera eramatea lortu zen arazo berezirik gabe, nahiz eta noizean behin tantaren portaeran arazoren bat edo beste egon. Sagar-zuku komertzialean lortutako emaitzak onak izan ziren aztertutako bi kontzentrazioetan; %94.6-111.8 eta %97.5-108.7 bitartekoak izan ziren 80 eta 400 μg L^{-1}-ko kontzentrazioetan hurrenez hurren. 5.7. irudian ikus daitezke sagar zuku komertzialetan egindako bi adizioen erauzketak eta prozesuaren zuria lagin horretan. Sagar-zuku naturalean emaitzak eskasagoak izan ziren, %82.2-84.3 eta %88.1-93.8 bitartekoak 80 eta 400 μg L^{-1} adizioentzako. Azkenik, mahats-muztioan lortutako emaitzak, hiruetatik kaskarrenak, %64.5 eta 92.2 bitartekoak izan ziren 80 μg L^{-1}-ko kontzentrazioarentzat eta %68.8 eta 112.4 artekoak 400 μg L^{-1}-ko adizioentzat.



5.7. Irudia. *Sagar zuku komertzialean lortutako kromatogramak. Urdinez, zukuaren mikroerauzketaren zuria, gorriz, 80 µg L^{-1}-ko adizioa egin ondorengo erauzketa (1. adizioa) eta orlegiz, 400 µg L^{-1}-ko adizioa egin ondorengo erauzketa (2. adizioa).*

5.11. Taula. *Lagin ezberdinetan lortutako berreskuratzea 80 eta 400 µg L^{-1}-ko kontzentrazioetan desbideratze estandar erlatiboarekin.*

	80 µg L^{-1}						**400 µg L^{-1}**					
	221nm					**249nm**	**221nm**					**249nm**
	M	**FQ**	**TT**	**FS**	**TB**	**D**	**M**	**FQ**	**TT**	**FS**	**TB**	**D**
SAGARDOA												
DEE (%)	28.0	23.3	23.0	7.7	19.5	10.9	11.8	17.3	14.9	21.2	18.6	15.5
Berreskuratzea (%)	59.9	49.3	54.5	51.9	48.3	46.7	85.6	68.4	71.7	64.5	63.7	44.3
SAGAR-ZUKU KOMERTZIALA												
DEE (%)	9.7	7.9	2.6	5.8	1.5	2.7	3.5	1.5	2.4	1.7	1.8	4.5
Berreskuratzea (%)	111.4	109.9	94.6	111.8	102.9	100.6	108.7	108.6	97.5	108.6	107.7	101.2
SAGAR-ZUKU NATURALA												
DEE (%)	4.3	6.7	6.1	8.4	5.5	4.6	9.0	9.8	9.9	9.0	9.6	6.5
Berreskuratzea (%)	83.6	84.0	82.2	82.3	84.3	82.8	91.1	92.8	90.6	93.8	90.8	88.1
MAHATS-MUZTIO KOMERTZIALA												
DEE (%)	10.7	11.5	11.3	10.7	10.8	13.6	8.4	12.1	9.9	12.5	10.3	14.6
Berreskuratzea (%)	92.2	81.0	73.8	78.8	77.8	64.5	112.4	94.1	88.2	88.3	87.8	68.0
MAHATS-ZUKUA												
DEE (%)							10.5	14.9	16.1	14.0	14.3	8.0
Berreskuratzea (%)							90.8	69.6	64.7	63.3	63.6	42.8

5.4 Ondorioak

Ultrasoinuz lagundutako emultsifikazio mikroerauzketa teknika bat garatu da lan honetan. Erauzketa prozesuaren ondoren erauztailea eta bertan erauzitako analitoak HPLC/DAD bidez determinatu dira, banaketa eta detekzioa burutzeko asmoz. Metodoak linealtasun, errepikakortasun eta aberastasun-faktore oso onak eman ditu eta lortutako detekzio-mugak mikrogramo-litroko mailakoak izan ziren.

Berreskuratze-ehunekoak laginaren araberakoak izan ziren. Parametro hori ona izateko ezinbesteko baldintza izan zen tantaren portaera egokia eta lagin batzuek eragin zuzena izan zuten erauztailearen jokatzeko moduan. Horren ondorioz, lagin mota batzuetan ez ziren berreskuratze onak lortu, baina tanta ondo formatu zen kasuetan berreskuratze eta beraien desbideratze estandar erlatiboak onak izan ziren.

Beste erauzketa teknika batzuekin konparatuz, teknika honek hainbat abantaila eskaintzen ditu. Hasteko, bere erauzketa denbora laburrak: ultrasoinuak eragiten duen dispertsioagatik, erauztailea mikrotanta bihurtu eta ondorioz, kontaktu-gainazala areagotzen denez, masa-transferentzia azkarrago gertatzen zen, denbora murriztuz. Bestalde, linealtasun-tarte zabalak eskaintzen ditu, erabiltzen den tantaren bolumenak hala bermatzen duelako. Gainera, beste teknika batzuekin alderatuz, merkea eta erabilerraza da, 1-undekanol ontzi bakoitzarekin milaka erauzketa burutu baitaitezke eta analisi bakoitzeko tanta berri bat erabiltzen denez, ez dago memoria efekturik. Horrez gain, likido-likido erauzketa klasikoarekin konparatuz gero, ingurumenarekiko errespetagarriagoa dela esan daiteke, erabiltzen diren disolbatzaile organiko bolumenak askoz txikiagoak baitira.

5.5 Erreferentziak

AOAC INTERNATIONAL, **2012.** AOAC Official Methods of Analysis, Guidelines for standard method performance requirements. Appendix F, .

Baugros, J.B., Giroud, B., Dessalces, G., Grenier-Loustalot, M.F. and Cren-Olivé, C.; *Multiresidue analytical methods for the ultra-trace quantification of 33 priority substances present in the list of REACH in real water samples.* **2008.** Analytica Chimica Acta, 607(2), 191-203.

Bordagaray, A., García-Arrona, R. and Millán, E.; *Development and application of a screening method for triazole fungicide determination in liquid and fruit samples using solid-phase microextraction and HPLC-DAD.* **2013.** Analytical Methods, 5(10), 2565-2571.

Bosch-Ojeda, C. and Sánchez-Rojas, F.; *Separation and Preconcentration by Dispersive Liquid–Liquid Microextraction Procedure: Recent Applications.* **2011.** Chromatographia, 74(9-10), 651-679.

Burgess, C. and Frost T.; *Standards and best practice in absorption spectrometry.* **1999.** 1 edn. Blackwell Science Ltd. Oxford (UK).

Costa, N.R., Lourenço, J. and Pereira, Z.L.; *Desirability function approach: A review and performance evaluation in adverse conditions.* **2011.** Chemometrics and Intelligent Laboratory Systems, 107(2), 234-244.

Currie, L.A., *Nomenclature in evaluation of analytical methods including detection and quantification capabilities (IUPAC Recommendations 1995).* **1995.** Pure and Applied Chemistry, 67, 1699-1723.

Derringer, G. and Suich, R.; *Simultaneous optimization of several response variables.* **1980.** Journal of Quality Technology, 12(4), 214-219.

Farajzadeh, M.A., Bahram, M., Jafary, F. and Bamorowat, M.; *Combination of Extraction by Silylated Vessel-Dispersive Liquid–Liquid Microextraction as a High-Enrichment Factor Technique: Optimization and Application in Preconcentration of Some Triazole Pesticides from Aqueous Samples Followed by GC-FID Determination.* **2011.** Chromatographia, 73(3), 393-401.

Farajzadeh, M.A., Djozan, D. and Khorram, P.; *Development of a new dispersive liquid–liquid microextraction method in a narrow-bore tube for preconcentration of triazole pesticides from aqueous samples.* **2012.** Analytica Chimica Acta, 713, 70-78.

Farajzadeh, M.A., Djozan, D., Nouri, N., Bamorowat, M. and Shalamzari, M.S.; *Coupling stir bar sorptive extraction-dispersive liquid liquid microextraction for preconcentration of triazole pesticides from aqueous samples followed by GC-FID and GC-MS determinations.* **2010.** Journal of Separation Science, 33(12), 1816-1828.

Ganjali, M.R., Sobhi, H.R., Farahani, H., Norouzi, P., Dinarvand, R. and Kashtiaray, A.; *Solid drop based liquid-phase microextraction.* **2010.** Journal of Chromatography A, 1217(16), 2337-2341.

Ghambarian, M., Yamini, Y. and Esrafili, A.; *Liquid-phase microextraction based on solidified drops of organic solvents.* **2013.** Microchimica Acta, 180, 519-535.

Goetz, A.K., Ren, H., Schmid, J.E., Blystone, C.R., Thillainadarajah, I., Best, D.S., Nichols, H.P., Strader, L.F., Wolf, D.C., Narotsky, M.G., Rockett, J.C. and Dix, D.J.; *Disruption of Testosterone Homeostasis as a Mode of Action for the Reproductive Toxicity of Triazole Fungicides in the Male Rat.* **2007.** Toxicological Sciences, 95(1), 227-239.

Han, D. and Row, K.; *Trends in liquid-phase microextraction, and its application to environmental and biological samples.* **2012.** Microchimica Acta, 176(1), 1-22.

Hyötyläinen, T., Critical evaluation of sample pretreatment techniques. **2009.** *Analytical and Bioanalytical Chemistry,* 394(3), 743-758.

Khalili Zanjani, M.R., Yamini, Y., Shariati, S. and Jönsson, J.A.; *A new liquid-phase microextraction method based on solidification of floating organic drop.* **2007.** Analytica Chimica Acta, 585, 286-293.

Konieczka, P. and Namieśnik, J.; *Quality Assurance and Quality Control in the Analytical Chemical Laboratory.* **2009.** 1 edn. CRC Press, Taylor & Francis Group. Boca Raton (USA).

Leong, M.I. and Huang, S.D.; *Dispersive liquid–liquid microextraction method based on solidification of floating organic drop combined with gas chromatography with electron-capture or mass spectrometry detection.* **2008.** Journal of Chromatography A, 1211(1–2), 8-12.

Li, Y., Yang, X., Zhang, J., Li, M., Zhao, X., Yuan, K., Li, X., Lu, R., Zhou, W. and Gao, H.; *Ultrasound-assisted emulsification magnetic microextraction: a fast and green method for the determination of triazole fungicides in fruit juice.* **2014.** Analytical Methods, 6, 8328-8336.

Luo, M., Liu, D., Zhou, Z. and Wang, P.; *A New Chiral Residue Analysis Method for Triazole Fungicides in Water Using Dispersive Liquid-Liquid Microextraction (DLLME).* **2013.** Chirality, 25(9), 567-574.

Miller, J.N. and Miller, J.C.; *Estadística y quimiometría para química analítica.* **2002.** Pearson Educación, S.A. Madrid (Spain).

Rambla-Alegre, M., Esteve-Romero, J. and Carda-Broch, S.; *Is it really necessary to validate an analytical method or not? That is the question.* **2012.** Journal of Chromatography A, 1232, 101-109.

Ravelo-Pérez, L.M., Hernández-Borges, J., Asensio-Ramos, M. and Rodríguez-Delgado, M.A.; *Ionic liquid based dispersive liquid–liquid microextraction for the extraction of pesticides from bananas.* **2009.** Journal of Chromatography A, 1216(43), 7336-7345.

Regueiro, J., Llompart, M., Garcia-Jares, C., Garcia-Monteagudo, J.C. and Cela, R.; *Ultrasound-assisted emulsification–microextraction of emergent contaminants and pesticides in environmental waters.* **2008.** Journal of Chromatography A, 1190(1–2), 27-38.

Regulation EC 149/2008 of 29 January 2008 amending Regulation (EC) No 396/2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto. Official Journal of the European Union. **(1.3.2008)** L 58/1-398. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EC 459/2010 of 27 May 2010 amending Annexes II, III and IV to Regulation (EC) No 396/2005 of hte European Parliament and of the council as regards maximum residue levels for certain pesticides in or on certain products. Official Journal of the European Union. **(28.5.2010)** L 129/3-49. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 34/2013 of 16 January 2013 amending Annexes II, III and IV to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residuel levels for 2-phenylphenol, ametoctradin, *Aureobasidium pullulans* strains DSM 14940 and DSM 14941, cyproconazole, difenoconazole, dithiocarbamates, folpet, propamocarb, spinosad, spirodiclofen, tebufenpyrad and tetraconazole in or on certain products. Official Journal of the European Union. **(26.1.2013)** L 25/1-48. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 1317/2013 of 16 December 2013 amending Annexes II, III and V to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for 2,4-D, beflubutamid, cyclanilide, diniconazole, florasulam, metolachlor and S-metolachlor, and milbemectin in or on certain products. Official Journal of the European Union. **(17.12.2013)** L 339/1-43. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Regulation EU 61/2014 of 24 January 2014 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for cyromazine, fenopropidin, formetanate, oxamyl and tebuconazole in or on certain products. Official Journal of the European Union. **(24.1.2014)** L 22/1-32. MRLs available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

Ruiz-Angel, M.J., García-Alvarez-Coque, M.C., Berthod, A. and Carda-Broch, S.; *Are analysts doing method validation in liquid chromatography?* **2014.** Journal of Chromatography A, 1353(0), 2-9.

Sousa, R., Homem, V., Moreira, J.L., Madeira, L.M. and Alves, A.; *Optimisation and application of dispersive liquid-liquid microextraction for simultaneous determination of carbamates and organophosphorus pesticides in waters.* **2013.** Analytical Methods, 5(11), 2736-2745.

Stalikas, C., Fiamegos, Y., Sakkas, V. and Albanis, T.; *Developments on chemometric approaches to optimize and evaluate microextraction.* **2009.** Journal of Chromatography A, 1216(2), 175-189.

Stocka, J., Tankiewicz, M., Biziuk, M. and Namieśnik, J.; *Green Aspects of Techniques for the Determination of Currently Used Pesticides in Environmental Samples.* **2011.** International Journal of Molecular Sciences, 12(11), 7785-7805.

Tankiewicz, M., Fenik, J. and Biziuk, M.; *Solventless and solvent-minimized sample preparation techniques for determining currently used pesticides in water samples: A review.* **2011.** Talanta, 86, 8-22.

Taverniers, I., De Loose, M. and Van Bockstaele, E.; *Trends in quality in the analytical laboratory. I. Traceability and measurement uncertainty of analytical results.* **2004.** Trends in Analytical Chemistry, 23(7), 480-490.

Tomlin, C.D.S.; *The Pesticide Manual.* **2000.** 12 edn. The British Crop Protection Council. Farnham (UK).

Wang, C., Wu, Q., Wu, C. and Wang, Z.; *Application of dispersion–solidification liquid–liquid microextraction for the determination of triazole fungicides in environmental water samples by high-performance liquid chromatography.* **2011.** Journal of Hazardous Materials, 185(1), 71-76.

Wang, W., Ma, X., Wu, Q., Wang, C., Zang, X. and Wang, Z.; *The use of graphene-based magnetic nanoparticles as adsorbent for the extraction of triazole fungicides from environmental water.* **2012.** Journal of Separation Science, 35(17), 2266-2272.

Wolf, D.C., Allen, J.W., George, M.H., Hester, S.D., Sun, G., Moore, T., Thai, S., Delker, D.A., Winkfield, E., Leavitt, S., Nelson, G., Roop, B.C., Jones, C., Thibodeaux, J. and Nesnow, S.; *Toxicity profiles in rats treated with tumorigenic and nontumorigenic triazole conazole fungicides: propiconazole, tridimefon and myclobutanil.* **2006.** Toxicologic Pathology, 34(7), 895-902.

Ye, C., Liu, Q., Wang, Z. and Fan, J.; *Dispersive liquid-liquid microextraction combined with high-performance liquid chromatography-ultraviolet detection for the determination of three triazole derivatives in environmental water samples.* **2012.** International Journal of Environmental Analytical Chemistry, 92(10), 1176-1186.

6. Partzialki gainezarritako gailur kromatografikoen aldagai anitzeko kalibrazioa PARALIND bidez

TrAC, Trends in Analytical Chemistry-en onartua (2015ko otsaila): Ane Bordagaray, José Manuel Amigo. Modelling highly co-eluted peaks of analytes with high spectral similarity.

6. Partzialki gainezarritako gailur kromatografikoen aldagai anitzeko kalibrazioa PARALIND bidez

6.1 Sarrera

Kromatografian gerta litekeen arazoetariko bat gailurren gainezarpena da. Batzuetan, banaketa kromatografiko perfektua lortzea ezinezkoa delako, laginaren konplexutasunagatik edota denbora kromatografiko luzeak desiragarriak ez direlako. Hori aldagai bakarreko kalibrazio klasikoak eraikitzeko orduan eragozpena izan daiteke eta aldagai anitzeko metodoak erabilgarriak izan daitezke. Metodo hauekin informazio kuantitatiboa ez ezik, informazio eta modelo kualitatibo ugari lor daitezke (sailkapena, hatz-marka eta jatorria moduko informazioa eman dezaketenak) [Bro, 2006].

Gaur egungo instrumentazioak kanal bat baino gehiagoko detektagailuz hornituak daude, eta horrek lagin edo analisi bakoitzeko datu-matrize bat eskuratzeko ahalmena ematen du. HPLC-DAD kasuan esaterako, hiru dimentsioko kromatogramak lortzen dira, eluzio-denbora bakoitzeko ultramore espektro osoa jasotzen baita. Bigarren-mailako datu-matrizeak deritze eta eluzio-denbora bakoitzeko espektro bat gordetzen da (hots, UV espektroa edo masa espektroa). Propietate horrek abantaila nagusi bat du, izan ere, konposatu bakoitzak espektro konkretu eta bakar bat duenez, analitoen kuantifikazioa ahalbidetzen du, eta egon litezkeen interferentzien eragina minimizatzen du [Goicoechea *et al.*, 2011]. Propietate honek gailurren dekonboluzioa baimendu dezake hainbat metodo erabiliz, esate baterako, PARAFAC eta PARAFAC2 (*PARAllel FACtor analysis*) edo MCR (*Multivariate Curve Resolution*) [Skov eta Bro, 2008; Amigo *et al.*, 2010; Kumar *et al.*, 2014]. Metodo hauek, espektroaren izaera bakarraz baliatzen dira gailurren ebazpena egiteko, baina espektroek beraien artean korrelazio handia badute, metodo hauek hein urritasuna dutela esaten da. Heina, matrizeei dagokienez, propietate matematiko bat da, non datu matrizeko errenkadak (edota zutabeak) linealki independenteak diren. Kasu horietan, hein-urritasuna ebazteko beste metodo bat garatu zen PARAFAC-en oinarritua: PARALIND (*PARAllel FActor analysis with LINear Dependence*) [Bahram eta Bro, 2007; Bro *et al.*, 2009].

Dimentsio anitzeko datuak tratatzerakoan erabili daitezkeen metodoen bidez arrakasta izateko egoera ezin hobea litzateke ondorengoa: ko-eluzio eta espektroen arteko nahikoa alde egotea [Manne eta Grande, 2000; de Juan eta Tauler, 2007]. PARAFAC2 eta MCR bezalako metodoekin datu kromatografikoak ebatzi ahal izateko analito bakoitzaren eraginez sortutako datuak linealki independenteak izan beharko dute beste analitoen datuekin. Beraz, kasu batzuetan, arazoak egon litezke bi analitok profil kromatografiko bera badute (guztiz gainezarrita daudenean), edota profil espektralean nahikoa alde ez badute.

Metodo hauekin lan egiterako orduan garrantzitsua da faktore edo osagai kopurua zehaztea. Hori egiteko aurrez jakina den informazioaz baliatu daiteke, baina baita PCA (*Principal Component* Analysis) [Bro eta Smilde, 2014] edo EFA (*Evolving Factor Analysis*) [Maeder, 1987; Maeder eta de Juan, 2009] bezalako metodoen bidez. Hala eta guztiz ere, hauek ez dira hein urritasuna aurkitzeko gai, eta dimentsioren batean faktore kopuruarekin asmatu dela badirudi ere, gerta liteke beste dimentsio batean hala ez izatea.

Atal honetan, 4. kapituluan lortutako datuak erabili dira, gailur kromatografikoen gainezarpen partziala ikusten denez, datuen ebazpena egin nahi izan da metodo kimiometrikoen bidez. Kasu honetarako PARALIND-en gaitasuna erakutsi nahi izan da ultramore espektroen antzekotasun gradua oso altua den kasu batetarako. Horretarako, kromatografian oso erabiliak izan diren PARAFAC2 eta MCR metodoekin konparatu dira emaitzak [Manne eta Grande, 2000; de Juan eta Tauler, 2007; Amigo *et al.*, 2008; Marini *et al.*, 2011; Salvatore *et al.*, 2013; Boeris *et al.*, 2014].

6.1.1 Oinarri teorikoa

PARALIND-en oinarria PARAFAC metodoan dago, ezberdintasuna modu edo ardatz batean izango duen hein-urritasuna izango da. Hein-urritasuna eluzio profilean edo espektro profilean ageri daiteke oro har. Kontuan izan beharreko datua da, izan ere profil hori lehenengo moduan jarri beharko da modeloa egiteko erabilitako matrizean. Beraz, eragin hori kontuan hartuko duen mugatzaile batean egongo da

ezberdintasun nagusia. PARAFAC-en bidez matrizea hurrengo osagaietan banatzen da [Smilde *et al.*, 2004]:

$$\boldsymbol{X}_k = \boldsymbol{A}\boldsymbol{D}_k\boldsymbol{B}^T + \boldsymbol{E}_k \qquad (k = 1 \ldots K) \qquad (6.1)$$

Hein-urritasun hori modeloan sartzeko, dependentzia-matrize bat, **H**, gehituko dugu ekuazioan eta, beraz, hein urritasuna izango duen dimentsioa, $\overline{\boldsymbol{A}}$, dependentzia matrizea barnean duen matrize berria izango da [Bahram eta Bro, 2007; Bro *et al.*, 2009]:

$$\overline{\boldsymbol{A}} = \boldsymbol{A}\boldsymbol{H} \qquad (6.2)$$

H matrizeak ez balu hein-urritasunik matrize karratu diagonal bat izango da, eta kasu horretan $\overline{\boldsymbol{A}} = \boldsymbol{A}$ izango da. Beraz PARAFAC modeloa bera lortuko genuke. **H** matrizeak hein urritasuna badu, zutabe baino lerro gutxiagoko matrize bat izango da. Adibidez, demagun 5 faktoreko modelo bat aurreikusi dugula (R=5), baina, hein urritasuna duela lehenengo moduan, hau da, lehenengo moduko faktore kopurua 4 dela. Kasu horretan osatuko den dependentzia-matrizea ondorengoa izango da:

$$\boldsymbol{H} = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 1 & 1 \end{bmatrix} \qquad (6.3)$$

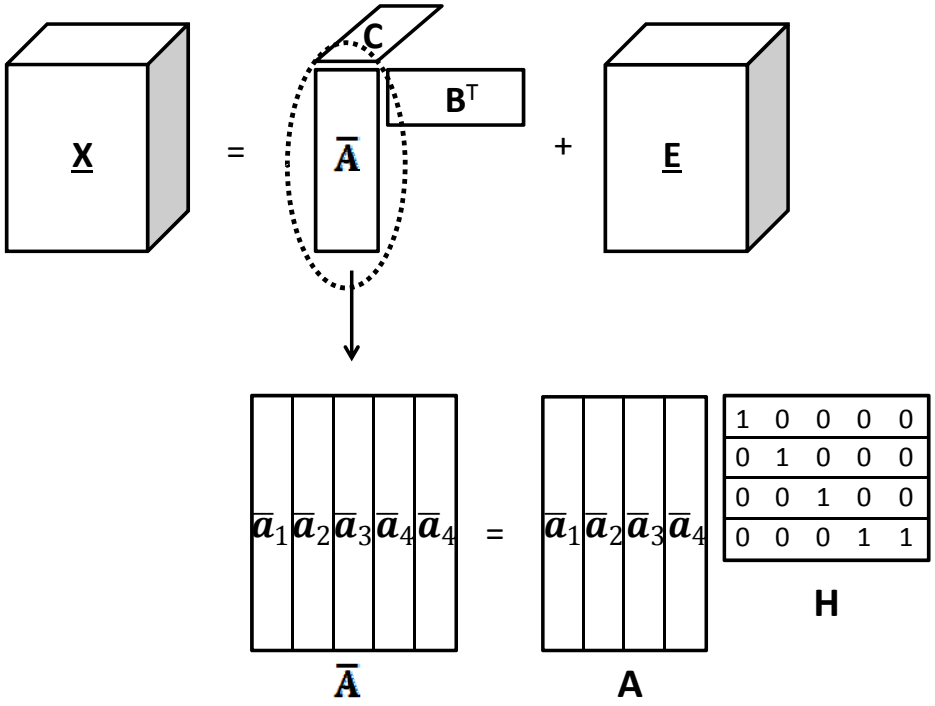
Hori dela eta, 6.1 ekuazioa PARALIND kasuan honela geratuko litzateke:

$$\boldsymbol{X}_k = \boldsymbol{A}\boldsymbol{H}\boldsymbol{D}_k\boldsymbol{B}^T + \boldsymbol{E}_k \qquad (k = 1 \ldots K) \qquad (6.4)$$

Edo beste era batera:

$$\boldsymbol{X}_k = \overline{\boldsymbol{A}}\boldsymbol{D}_k\boldsymbol{B}^T + \boldsymbol{E}_k \qquad (k = 1 \ldots K) \qquad (6.5)$$

Horrela bada, PARALIND modeloa aplikatuta gure hasierako matrizea $\boldsymbol{X}_k$ (I x J) informazioa duten beste hiru matrizeetan banatzen da, errore-matrizeaz gain: $\overline{\boldsymbol{A}}$ (I x R), hein urritasuna duen profila (eluzio edo espektro profila), besteak baino osagai gutxiago izango dituena; $\mathbf{D}_k$ (R x R), kontzentrazioaren inguruko informazioa emango digun profila eta **B** (J x R) hirugarren profila (eluzio edo espektro profila).



6.1. Irudia. *PARALIND modeloaren matrizeen irudikapen grafikoa.*

6.1. irudian ikus daiteke 6.5 ekuazioaren irudikapena matrizeei dagokionez. Hasiera batean PARAFAC modelo arrunt bat dirudi, baina $\bar{\boldsymbol{A}}$ matrizean dago ezberdintasuna: matrize honek hein-urritasuna badu, $\bar{\boldsymbol{A}}$ matrizea osatzen duten $\bar{\boldsymbol{a}}_i$ bektore ezberdinak linealki askeak ez direla esan nahi du. Hots, zutabe bat, beste baten konbinazio lineal bezala adierazi daitekeela. Beraz, 6.1. irudiko adibide horri jarraituz, modeloa deskribatzeko hasieran pentsa genezakeen 5 ekarpenen ordez, benetako 4 ekarpen daude. Hau da, 4 osagaiekin deskriba daiteke modu hori ($\bar{\boldsymbol{a}}_1$, $\bar{\boldsymbol{a}}_2$, $\bar{\boldsymbol{a}}_3$ eta $\bar{\boldsymbol{a}}_4$). Ondorioz, $\bar{\boldsymbol{A}}$ matrizea beste bi matrizeetan bana daiteke: **A** (osagai esanguratsuekin osatutako matrizea) eta **H** (dependentzia-matrizea, **A** eta $\bar{\boldsymbol{A}}$-ren erlazioa adieraziko duena). 6.1 irudian ikus daitekeen **H** dependentzia-matrizeak **A**-ren azken zutabea ($\bar{\boldsymbol{a}}_4$) errepikatzen dela adierazten du.

PARAFAC2 eta MCR modeloak askotan azaldu dira kromatografiaren aplikazio metodoetan [Roma, 1995; de Juan *et al.*, 1998; Bro *et al.*, 1999; de Juan eta Tauler, 2003; Bro, 2006; de Juan eta Tauler, 2006; Amigo *et al.*, 2008; Amigo *et al.*, 2010; Salvatore *et al.*, 2013], beraz, bi algoritmoen ideien zertzelada batzuk besterik ez dira aipatuko hemen.

PARAFAC2 PARAFAC-en oinarritzen da, baina abantaila nagusia eluzio-denboran egon daitezkeen kromatogramen arteko ezberdintasunak modela ditzakeela da. PARAFAC modeloak guztiz trilinearra izan behar duen bitartean, PARAFAC2-an eluzio-denboren profilean malgutasuna onartzen da. Horregatik, PARAFAC modeloan mugatzaileak edozein modutan jarri balitezke ere, PARAFAC2 modeloak ezin dira mugatu eluzio profilaren moduan. Horregatik, PARAFAC2 modeloan lagin kopurua bezainbeste eluzio-profil lortzen dira. Aldiz, PARAFAC-en profil bakar bat lortuko litzateke lagin guztientzat:

PARAFAC:

$$\boldsymbol{X}_k = \boldsymbol{A}\boldsymbol{D}_K\boldsymbol{B}^T + \boldsymbol{E}_k \qquad (6.6)$$

PARAFAC2:

$$\boldsymbol{X}_k = \boldsymbol{A}\boldsymbol{D}_k\boldsymbol{B}_k^T + \boldsymbol{E}_k \qquad (6.7)$$

MCR-k, aldiz, datu trilenearrak nahiz ez trilinearrak modelatzeko gaitasuna erakutsi du. Hau da, teknika ezberdinez lortutako datuak ere modela daitezke lerroen zentzuan datu matrizea osatuz gero. Kontuan izan behar da, berez MCR modelo bilinear bat dela 6.8 ekuazioa jarraitzen duena, baina datu trilinearrak izanez gero zutabe-norabidean matrizea moldatu daiteke, modelo bilinearraren bidez ebatzi dadin.

$$\boldsymbol{D} = \boldsymbol{C}\boldsymbol{S}^T + \boldsymbol{E} \qquad (6.8)$$

MCR-ren askatasun errotazionala dela eta mugatzaileak erabiltzea beharrezkoa da soluzio bakar eta zentzudun bat lortzeko.

*OHARRA: Atalburu honetan ageritako formulei dagokienez letra larriak beltzez daudenean bi dimentsioko matrizeei erreferentzia egiten die (adb: **X**), hiru dimentsioko matrizeak, aldiz, letra larriz, beltzez eta azpimarratuta adieraziko dira (adb: $\underline{\boldsymbol{X}}$). $\boldsymbol{X}_k$ kasu honetan $\underline{\boldsymbol{X}}$ matrizearen k xafla izango da. Letra xeheak beltzez daudenean bektoreei egiten zaie erreferentzia (adb: **b**) eta letra xehe normalak, eskalarrak izango dira. (I x J x K) moduko adierazpenak, matrizeen dimentsioak zehazten dituzte, modu edo ardatz bakoitzean. Ager daitezkeen bestelako ikurrak testuan definituak daude.*

6.2 Esperimentala

6.2.1 Erreaktiboak eta ekipamendua

Ikerketa honetan erabilitako tebukonazola [TB] (%99.6, Pestanal®) Sigma-Aldrich-en (Madril, Espainia) eskuratu zen, flusilazola [FS] (%99.3) eta miklobutanila [M] (%99.4) LGC Standards-en (Bartzelona, Espainia) eta tetrakonazola [TT] (%97.5) eta flukinkonazola [FQ] (%98.5), aldiz, Dr. Ehrenstorfer-ek (Augsburg, Alemania) hornitua izan zen. Banakako 1000 mg L^{-1}-ko disoluzio-amak Teknokroma (Bartzelona, Espainia) etxeko metanoletan (SpS) prestatu ziren. Horietatik abiatuz, estandar disoluzio nahasteak prestatu ziren 5 eta 30 µg L^{-1} bitarteko kontzentrazioekin (ikus 6.1. taula). HPLC-rako fase mugikorra egiteko HPLC kalitateko Teknokroma etxeko metanola erabili zen eta tanpoia egiteko erabilitako sodio azetatoa (PA kalitatea) eta azido azetikoa Panreac-ek (Bartzelona, Espainia) hornituak izan ziren, baita gatz disoluzioak egiteko sodio kloruroa ere.

Erauzketak egiteko 65 µm-ko lodieradun polidimetilsiloxano/dibinilbentzeno (PDMS/DVB) zuntzak, erabilitako ontziak eta PTFE-silikona septak Supelcok hornituak izan ziren (Bellenfonte, PA, USA).

HPLC ekipamendua LC-20AD kromatografo batez eta SPD-M20A lerrokatutatko diodo detektagailu batez osatua zegoen (Shimadzu Corporation, Duisburg, Alemania). Datuak LC Solution (1.2. bertsioa) softwarearekin jaso ziren. HPLC-ko fase mugikorra azetonitriloz (SpS, Teknokroma) eta pH 4-ko tanpoi batez osatu zen. Tanpoia egiteko 0.01 mol L^{-1} azido azetiko disoluzioa 0.01 mol L^{-1} sodio azetato disoluzioarekin doitu zen pH 4-raino eta hozkailuan gorde zen analisi momentura arte. Fase mugikorra modu isokratikoan eluitu zen 0.5mL min^{-1}-ko fluxuan 78/22 frakzioa osatuz (v/v, azetonitrilo/tanpoia). Baldintza horietan analitoak ondorengo ordenan eluitu ziren: tetrakonazola (7.0 min), miklobutanila (7.3 min), flusilazola (7.7 min), flukinkonazola (8.0 min) eta tebukonazola (8.3 min).

6.1. Taula. *Kalibrazio eta balidazio estandarren kontzentrazioak (µg L^{-1}).*

	Estandarra	TT	M	FS	FQ	TB
Kalibrazio laginak	**1 estandarra**	5.1	20.4	10.0	31.2	15.6
	2 estandarra	10.2	25.5	15.0	5.2	20.8
	3 estandarra	15.3	30.6	20.0	10.4	26.0
	4 estandarra	20.4	5.1	25.0	15.6	31.2
	5 estandarra	25.5	10.2	30.0	20.8	5.2
	6 estandarra	30.6	15.3	5.0	26.0	10.4
Balidazio laginak	**7 estandarra**	15.3	15.3	15.0	15.6	15.6
	8 estandarra	25.5	25.5	25.0	26.0	26.0

TT: Tetrakonazola
M: Miklobutanila
FS: Flusilazola
FQ: Flukinkonazola
TB: Tebukonazola

Datuak MATLAB (Mathworks, Natick, EEBB) softwarearen bidez aztertu ziren. PARAFAC eta PARAFAC2 algoritmoak PLS-Toolbox-a (731 bertsioa) erabiliz egin ziren (Eigenvector Research Incorporated, Wenatchee, EEBB). MCR/ALS algoritmoa www.mcrals.info [Tauler*, et al*. [7/2014]-ean ikusia] web orrian dago eskuragarri eta PARALIND algoritmoa www.models.life.ku.dk/algorithms [Bro*, et al*. [7/2014]-ean ikusia] web orrian aurki daiteke.

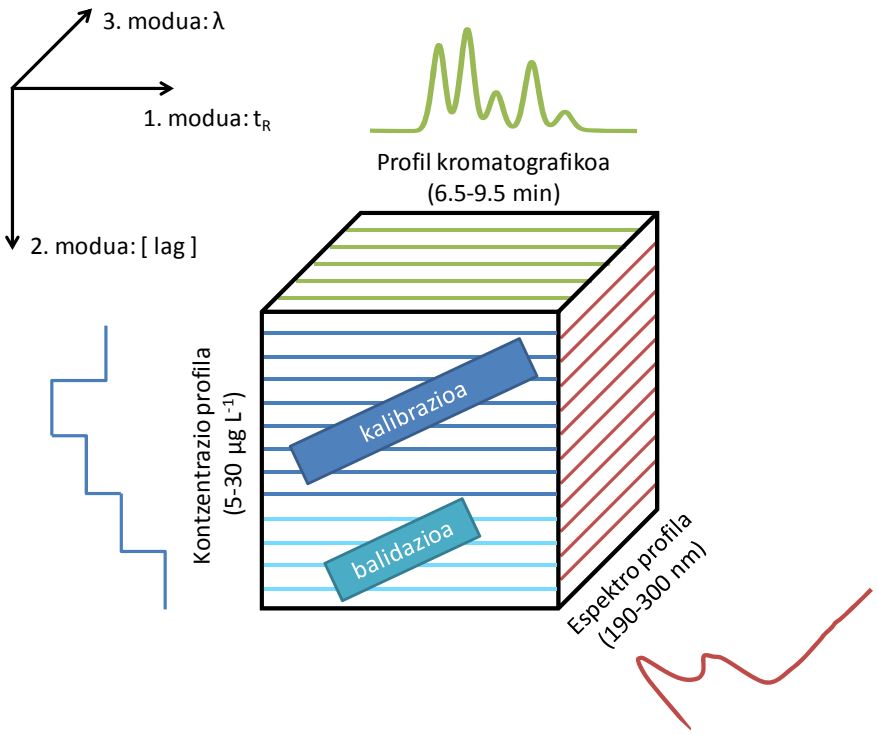
Kapitulu honetan zehar MATLAB jatorriko hainbat grafiko aurkituko dira eta argitzea komeni da MATLAB-ek erabiltzen duen kolore kodea. Hala bada, ordena honetan ageri dira koloreak: 1) urdina; 2) berdea; 3) gorria; 4) urdin argia; 5) magenta; 6) horia (mostaza).

Atal honetarako erabilitako datuen oinarria 4. atalburuan dago. Datu hauek SPME-HPLC/DAD metodotik datoz, beraz, xehetasun esperimentalak atal horretan deskribatzen dira. Atal honetan datuen tratamendua egin da, partzialki gainezarriak dauden metodo kimiometrikoen bidezko gailurren ebazpena.

6.2.2 Datu matrizeen egitura

Lerrokatutako diodoen detektagailuak 190 nm-tik 800-raino bitarteko uhin luzerak jaso ditzake eluzio-denbora unitate bakoitzeko, analisi bakoitzeko hiru dimentsioko kromatogramak lortuz. Lortutako kromatogramak bigarren mailako abantaila deiturikoa betetzen dute; hau da, eluzio-denbora (t_R) bakoitzeko espektro (λ) oso bat jasotzen da. Beraz estandar bakoitzak datu-matrize bat badu ($\mathbf{t_R}$ x **λ**), n lagin talde batek hiru dimentsioko matrize bat osatuko dute ($\mathbf{t_R}$ x **λ** x **n**). hiru dimentsioko matrize hau 6.2 irudian ikus daiteke. Aldagai anitzeko kalibrazioaren portaera egokia dela ikusteko balidazio multzo bat banatzen da kalibrazioko lagin taldetik. Horrela bada, kalibrazio eta balidazio-multzoak bereiz daitezke 6.2 irudian.

Lehen lana, matrizea osatzea izango da, eta ondoren intereseko tartea aukeratu beharko da. Kontzentrazio profila osatzeko 12 kalibrazio (n_{kal}) lagin eta 6 balidazio (n_{bal}) lagin hautatu ziren (18 aldagai guztira). Profil kromatografikoa eraikitzeko analitoak eluitutako denbora tartea bakarrik aukeratu zen, 6.5 minututik 9.5 minutura (282 aldagai). Tarte honi zabalera nahikoa eman zitzaion gerta daitezkeen eluzio-denbora aldaketak menpean hartzeko. Azkenik, espektro profila, 190-300 nm (91 aldagai) bitarteko uhin luzerak hautatuz osatu zen. Beraz matrize osoaren dimentsioa <18 x 282 x 91> da.



6.2. Irudia. *Aldagai anitzeko kalibrazioa burutzeko erabilitako hiru dimentsioko datu-matrizearen irudikapena eta matrizearen ardatzak: lehenengo moduak erretentzio-denboren norabidea adierazten du; bigarren moduak laginen kontzentrazioena eta hirugarren moduak, uhin-luzeren norabidea datu-matrizean.*

6.3 Emaitzak eta eztabaida

6.3.1 Aurre-tratamenduak

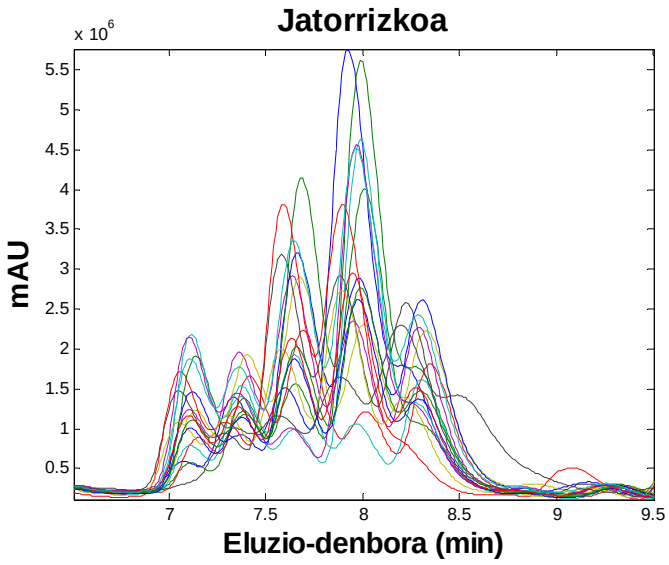
Seinale kromatografikoak hiru osagai ditu: seinale analitikoa (analitoaren kontzentrazioaren araberakoa), hondoa (analitoari ez dagokion seinale sistematikoa, oinarrizko lerroa) eta soinua (oinarrizko lerroaren bariazio ez-sistematikoa) [Amigo *et al.*, 2010]. Interesgarria den osagaia lehenengoa da, baina beste biak eragin zuzena

dute gerta daitekeen errorean. Horien efektuak gutxitzeko hainbat trikimailu eskaintzen ditu kimiometriak.

Esanguratsuak diren modeloak eraikitzeko hainbat aurre-tratamendu aplika daitezke, hala nola, oinarrizko lerroaren jitoaren zuzenketa, gailurren leuntzea, deribatuak, lerrokatzea, etab. Lan honetako datuekin ikusitako arazo nagusiena eluzio-denboren aldaketa izan zen, beraz, lerrokatze tratamenduak aplikatu ziren. Beste aurre-tratamendurik ez zitzaien aplikatu datu hauei, izan ere, aurre-tratamenduak berez denbora-inbertsioa eskatzen du eta gainera ondorio ezegokietara eraman gintezke [Engel *et al.*, 2013].

6.3.1.1 Lerrokatzea: COSHIFT eta COW

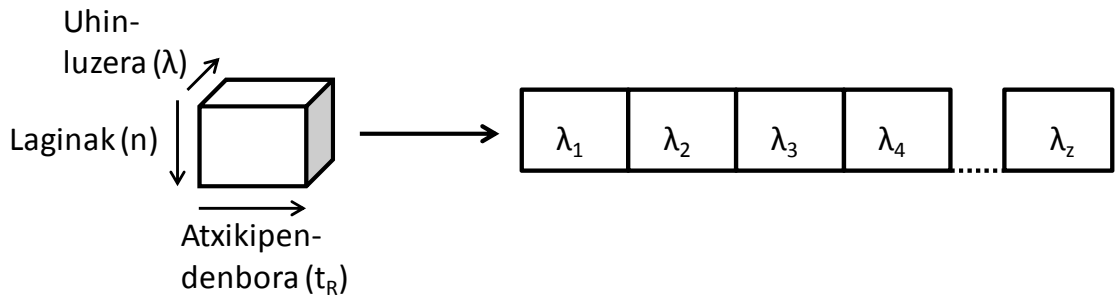
Likido kromatografian, presio aldaketa txikiak egon daitezke kromatograma batetik bestera. Horrek, eragin zuzena du eluzio-denboretan eta aldaketak gerta daitezke. 6.3 irudian ikus daiteke lan honetako erabili diren estandarren kromatograma ezberdinak.



6.3. Irudia. *221 nm-tan jasotako estandarren kromatogramak.*

Hainbat modelo aplikatu ahal izateko beharrezkoa da gailurrak lerrokatuta egotea, hau da, denbora tarte berdinean egotea eta analito bakoitzaren gailurrak lagin ezberdinetan zehar bat egitea. Helburu horrekin bi zuzenketa aplikatu dira, lehenengoa COSHIFT (*Correlation Shifting*) [Van Mispelaar *et al.*, 2003; Van den Berg *et al.*, 2005] izenekoa eta bigarrena COW (*Correlation Optimised Warping*) [Nielsen *et al.*, 1998; Tomasi *et al.*, 2004]. COSHIFT erabiliz egon litezkeen eluzio-denbora aldaketa handiak zuzenduko dira, COW ez baita aldaketa handiak konpontzeko gai. Ikus 6.3 irudiko 7-2 laginaren desplazamendua, besteekin alderatuz nabarmentzen da.

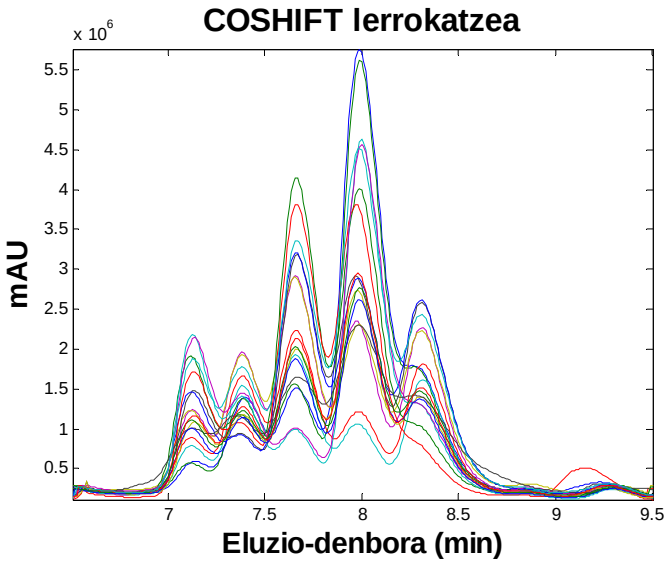
Aurre-tratamendu hauek bi dimentsioko matrizeetan aplikatzen dira, beraz lehenengo lana hiru dimentsioko matrizea bi dimentsiokoa bihurtzea izan zen. Horretarako, uhin-luzerei dagokien dimentsioa deuseztatu zen uhin luzera ezberdinetan jasotako kromatogramak bata bestearen ondoren lerratuz 6.4 irudian ikus daitekeen bezala. Modu horretan, lagin bakoitzeko bektore bat lortzen da. Lerrokatu ondoren, berriz alderantzizko eragiketa egin zen hiru dimentsioko matrizea berreskuratzeko.



6.4. Irudia. *hiru dimentsioko matrizearen deskonposaketaren irudikapena.*

COSHIFT aplikatu ahal izateko kontuan hartu beharreko sarrera-datuak ondorengoak dira: lerrokatu beharreko datu matrizea, erreferentzia bektorea eta aldaketa aplikatuko dugun datu-puntu kopuru maximoa (zenbat puntu mugi daitezkeen gehienez lagin bakoitza erreferentziarekin bat egiteko). Erreferentziazko bektore bezala edozein aukera daiteke, baina betiere gomendagarria da gutxi gorabehera erdialdean kokatuta dagoena aukeratzea. Kasu honetan 7-4 lagina aukeratu zen. eta puntu kopuru maximoa grafikoen azterketa bisualaren bidez egin zen. Datu kopuru txikietatik hasi eta aldaketa maximoa n=21 erabili lortu zen, puntu horretatik aurrera ez zen zuzenketa gehiagorik nabari, beraz, hori hautatu zen COSHIFT

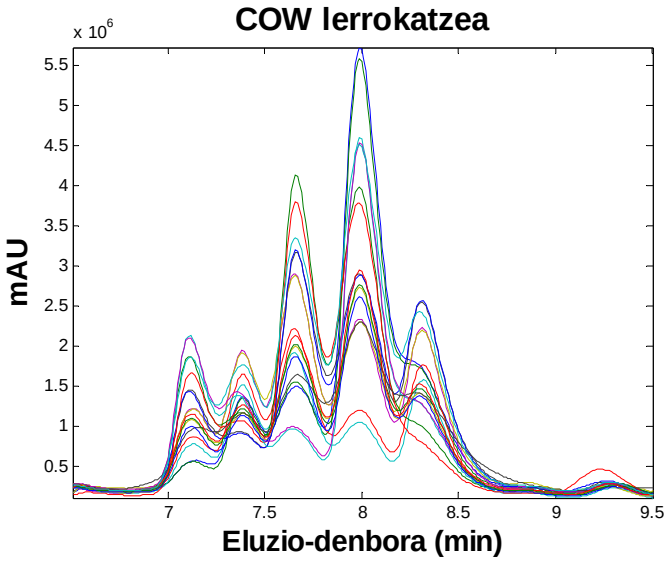
egiteko parametro bezala. 6.5 irudian ikus daiteke zuzenketa horrekin lortutako emaitza.



6.5. Irudia. *COSHIF aplikatu ondorengo zuzenketa: aldaketa puntu kopuru maximoa 21.*

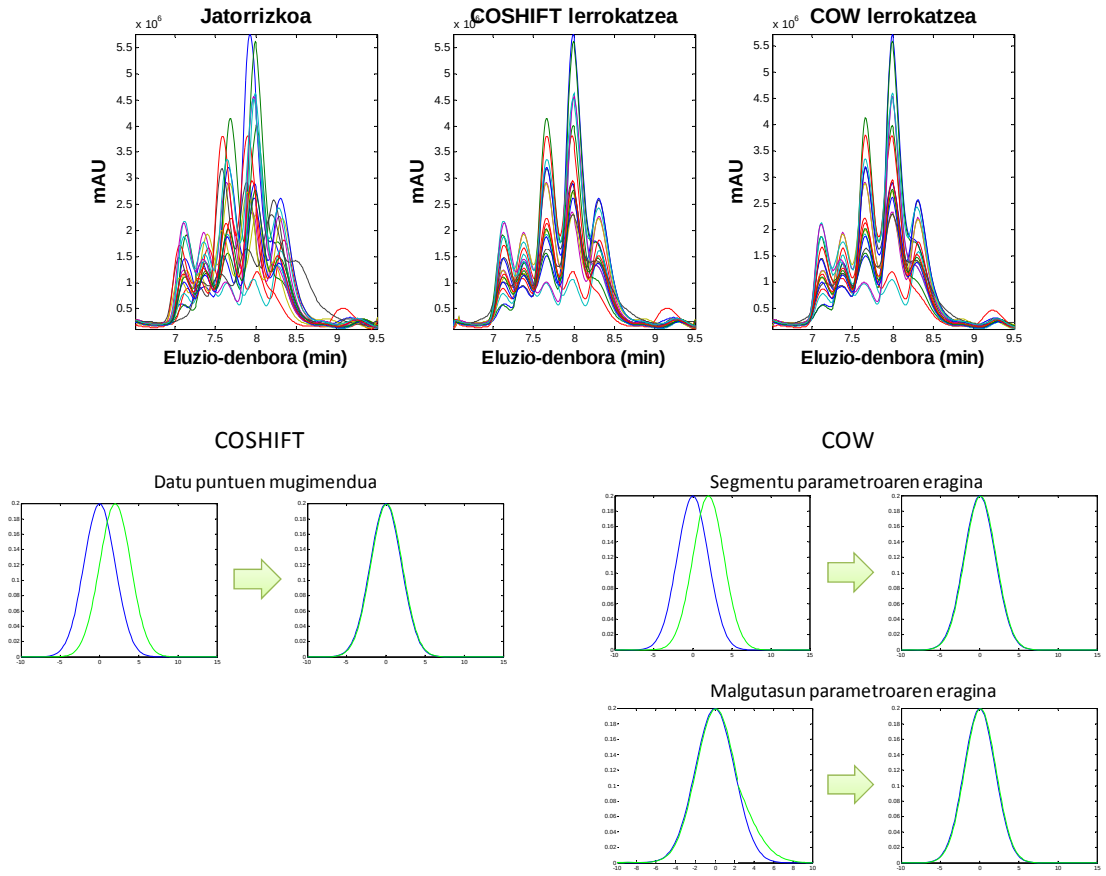
COSHIFT aplikatu ondoren lerrokatze finago bat lortu liteke COW aplikatuz. COW-en laguntzaz lagin batetik bestera gerta litezkeen kromatogramen denborak zuzentzeaz gain, gailurren forma ere moldatzen da. Horrela, forma aldaketa txikiak ere zuzendu litezke.

COW aplikatzeko sarrera-parametroak ondorengoak dira: lerrokatu beharreko matrizea, erreferentziazko bektorea, segmentu luzera eta malgutasuna. Parametro hauek aukeratzeko *optim_cow* algoritmoa erabil daiteke (*www.models.life.ku.dk/algorithms*). Algoritmo honek deformazio-efektua (*warping effect*) aztertuko du, sinpletasuna eta gailur-faktorearen arteko adostasunaren bitartez [Skov *et al.*, 2006]. Skov-en arabera sinpletasunak lerrokatzea ondo egin den esaten du eta gailur-faktoreak, aldiz, lerrokatu aurreko eta ondorengo gailurraren forma aztertuko du. Bi eragin hauek aztertzen dira elkarrekin eta horien arteko adostasuna izango da deformazio-efektua. Beraz, COW egiterakoan kontuan hartu beharreko parametroak *optim_cow*-ren laguntza erabiliz lortuko dira. Lerrokatzeak bisualki aztertzea ere beharrezkoa da, hautatutako parametroak egokiak diren baieztatzeko. Lerrokatze honetan segmentu tamaina 15 hautatu zen eta malgutasuna 4. Horrela lortutako lerrokatzea 6.6 irudian ikus daiteke.



6.6. Irudia. *COW lerrokatzea aplikatu ondorengo kromatograma: segmentu-luzera, 15; malgunatsuna, 4.*

Lerrokatze prozesu osoa ikus daiteke 6.7. irudian. Aldaketa handiena jatorrizko kromatogramatik COSHIFT aplikatu ondorengo kromatogramara ikus daiteke, baina, detaile txikiak behatuz gero, COW aplikatu ondoren beste zuzenketa txikiak ikus daitezke.



6.7. Irudia. *Jatorrizko kromatograma eta lerrokatze prozesuaren faseak: COSHIFT eta COW. Azpialdean, pausu bakoitzaren parametro ezberdinek gailur bakar batean izango luketen eragina.*

6.3.2 Modeloen eraikuntza

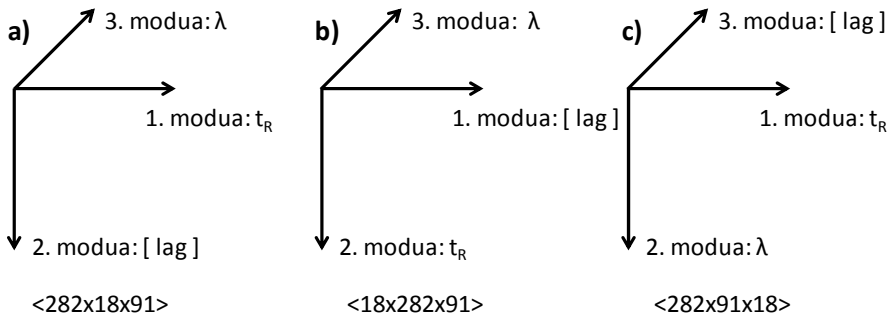
Datu berdinak erabiliz, hainbat modelo matematiko proposatu ziren datuak deskribatu eta deskonposatzeko. Deskonposizio metodoak modelo matematiko bat jarraitzen badute ere, aurrez dakigun informazioa erabilgarria eta aberasgarria izan liteke modelo egonkor, esanguratsu eta egokiagoak lortzeko orduan. Modelo-mugatzaile batzuen bitartez erabili daiteke informazio hori [Bro, 1997; de Juan eta Tauler, 2003; de Juan eta Tauler, 2006]. Hauek, matrizea osatzen duten edozein ardatzeko profiletan aplika daitezke.

Mugatzaileen artean, ezagunenak ez-negatibotasuna, unimodalitatea, eta masa-balantzea dira. Ez-negatibotasuna profil batean aplikatzen dugunean, matrize horrek balio negatiborik ezin izan dezakeela esaten diogu modeloari. Esaterako, kontzentrazio bat ez da sekula negatiboa izango, gutxienez zero izango da. Unimodalitatea aplikatzen dugunean, aurrez mahai gainean jartzen dugun informazioa ondorengoa izango da: osagai batek profil hori deskribatzerakoan maximo bakar bat izango duela. Adibidez, profil kromatografikoetan, osagai batek analito bakar bat deskribatu ohi duenez, unimodalitatea aplikatu daiteke, aldiz, espektro ultramore baten kasuan bi maximo edo gehiago aurkitzea posible denez, unimodalitatea aplikatu ezkero okerreko informazioa emango genuke. Masa balantzearen baldintza erreakzioetan erabili ohi da: A + B → C bada; (1:1:1 estekiometria errespetatuz), kontzentrazio profilak ematen dizkigun osagaien batura konstante izango da zinetika osoan zehar.

Mugatzaile hauek modeloari egonkortasuna eman diezaiokete, eta adibidez MCR kasuan, lortzen diren emaitzak askatasun errotazionala dutenez, mugatzaileen bidez anbiguotasun hori murrizten da modelo bakarrak lortuz. Izan ere, modelo hauek orokorrean iterazio metodoen bidez askatzen direnez, ebazpen matematiko bat baino gehiago izan litezke matematikoki zuzenak, baina esanahi kimiko zentzugabeak izan ditzake iterazioaren hasiera puntuaren arabera. Mugatzaile hauen bitartez, modelo esanguratsuago batera bideratzen dira ebazpen matematikoak.

Ondorengo modeloetan ez-negatibotasuna erabili da batez ere, baina unimodalitatea ere aplikatu da kasu batean, baldintza honekin edo gabe lortutako emaitzak alderatzeko. Betiere, modelo bakoitzak bere baldintza espezifikoak dituela kontuan izanda, mugatzaileak modu batean edo bestean jarriko dira. Esaterako; PARAFAC2 modeloa eraikitzerakoan, eluzio-denboren profilaren aldaketengatik, ardatz horretan ez da mugatzailerik jarri behar, profil hori ezin baita mugatu.

Horrez gain, kontuan izan behar da modelo mota bakoitza burutzeko, datu-matrizeak era batera edo bestera antolatu behar direla (ikus 6.8.irudia). Hots, moduak edo ardatzak modu konkretu batean jarri beharko dira. Atal bakoitzean adieraziko da zein eratan jarri diren matrizearen ardatzak.



6.8. Irudia. *Matrize-datua antolatzeko modu ezberdinak: a) eluzio-denborak lehenengo moduan (t_R), laginak bigarrenean ([lag]) eta uhin-luzerak hirugarrenean (λ). b) laginak lehenengo moduan ([lag]), eluzio-denborak bigarrenean (t_R) eta uhin-luzerak hirugarrenean (λ). c) eluzio- denborak lehenengo moduan (t_R), uhin-luzerak bigarrenean (λ) eta laginak hirugarrenean([lag]). Kakotxen artean modu bakoitzeko aldagai kopurua agertzen da matrizea adibidean esaten den bezala antolatzen denean.*

6.3.2.1 Parallel Factor Analyisis 2 (PARAFAC2)

PARAFAC2 algoritmoak gaitasun handia erakutsi du HPLC-DAD instrumentuarekin lortutako datuekin [Skov eta Bro, 2008; Marini *et al.*, 2011; Johnsen *et al.*, 2014]. Izan ere eluzio-denboren jasandako aldaerak ongi modelatzea lortzen baitu. PARAFAC2 modeloa eraikitzeko HPLC-tik lortutako datuak erabili dira, inolako aurre-tratamendurik gabe. Modeloa eraiki aurreko aldakuntza bakarra ardatzak antolatzea izan da. Horrela bada, eluzio-denborak lehenengo moduan kokatu dira, hori baita atxikipe-denboren aldakuntzak baimenduko dituen modua. Bigarrenean, uhin-luzerak eta azkenik, hirugarren moduan laginak jarriko dira.

Modelo-mugatzaileak kasu honetan lehenengo moduan ezin dira jarri, modu horri nolabaiteko malgutasuna eman behar baitzaio eta mugatzaileak jarriz gero modeloa nahasiko genuke. Kasu honetan ez-negatibotasuna aplikatu zen beste bi moduetan, laginen kontzentrazioak eta ultramore espektroak logikaz balio positiboak bakarrik izan ditzakete eta.

Aukeratutako eluzio-denbora tartean, 5 analitoen gailurrak ageri dira, beraien artean partzialki gainezarriak. Beraz, aurreikusi daiteke PARAFAC2 modeloak 5 osagai

izan ditzakeela printzipioz, baina 6 izan liteke, bestelako ezpurutasun edo ekarpenik badago (oinarrizko lerroarena adibidez).

Modeloaren egokitasuna ikusteko hainbat parametro eta grafiko ikus daitezke. Hasteko, garrantzitsuak izango dira 3 moduen irudikapenak eta berauek emandako informazioa. Azaldutako bariantza ehunekoa ere garrantzitsua da (6.9 ekuazioa), aukeratutako modeloek datuak zenbateraino deskribatze dituen azaltzen baitu.

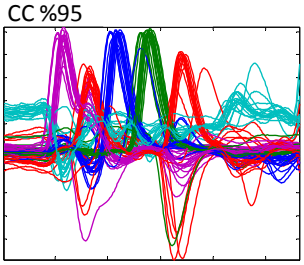
$$Azaldutako\ bariantza\ (\%) = 100 \cdot \left(1 - \frac{SSE}{SSX}\right) \qquad (6.9)$$

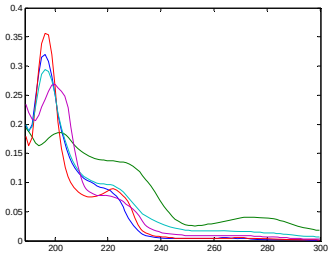
Non, *SSE* (*Sum of the squares of the residuals*) hondar-balio karratuen batura den, eta *SSX* (*Sum of the squares of the matrix elements*) datu matrizeko elementuen karratuen batura.

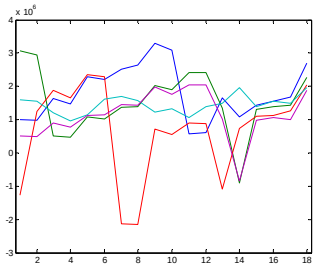
Azkenik, nukleo-egonkortasunak (CC, *Core Consistency*) PARAFAC (1 eta 2) modeloetan hautatutako osagai kopuruaren egokitasuna ematen digu [Bro *et al.*, 1999; Kamstrup-Nielsen *et al.*, 2013]. CC ehunekoa baxua bada modeloa gaindoituta dagoela esaten da. Hau da, osagai kopurua gehiegizkoa dela eta soinua ere modelatu nahian dabilela modeloa.

Ondorengo irudietan (6.9. irudia) zenbait modelo ikus daitezke. Izenak modeloa nola egina dagoen adierazten du, hau da, PARAFAC2_5unc kasuan, 5 osagaiz eginiko PARAFAC2 modeloa dela adierazten du. “unc” zatiak mugatzailerik ez dela erabili adierazten du eta “nn”-k, aldiz, ez-negatibotasuna erabili dela. Modelo bakoitzaren irudikapen bakoitzean lehenik ezkerretan lortutako eluzio profila ikus daiteke, erdian, ultramore espektroaren profila, eta azkenik, eskuinean laginen kontzentrazio profilak. Kolore kodeak osagai zenbakien arabera ondorengoak dira: 1) urdina; 2) berdea; 3) gorria; 4) urdin argia; 5) magenta; 6) horia (mostaza). Ikus daitekeen bezala, eluzio profila lagin bakoitzeko eluzio-profilek osatzen dute, beste bi moduak ordea, osagai bakoitzak modu hori deskribatzeko erabilitako joera deskribatzen dute.

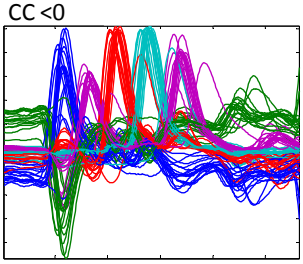
PARAFAC2_5unc
Az bar. %99.973
CC %95

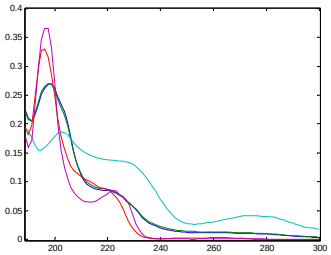

1. modua: eluzio profila

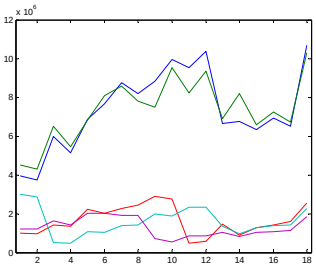

2. modua: espektro profila


3. modua: kontzentrazio profila

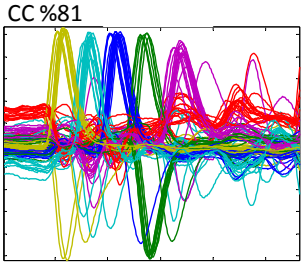
PARAFAC2_5nn
Az bar. %99.974
CC <0

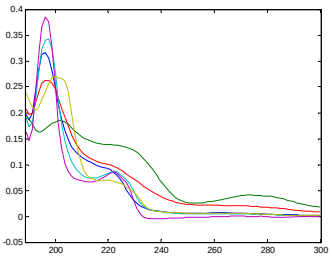

1. modua: eluzio profila

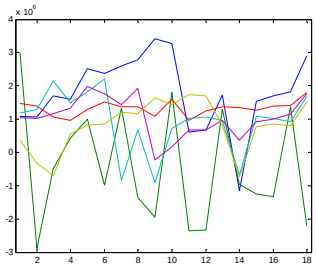

2. modua: espektro profila


3. modua: kontzentrazio profila

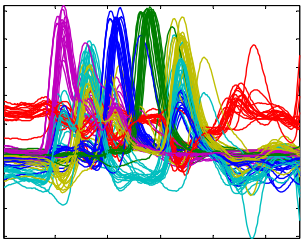
PARAFAC2_6unc
Az bar. %99.986
CC %81

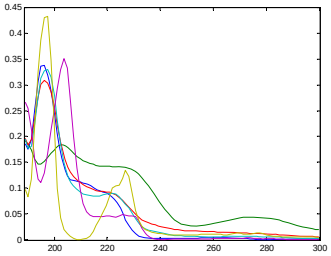

1. modua: eluzio profila

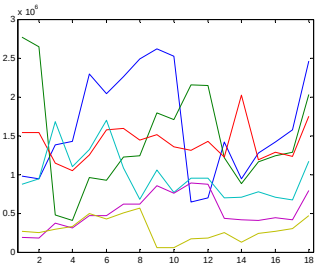

2. modua: espektro profila


3. modua: kontzentrazio profila

PARAFAC2_6nn
Az bar. %99.979
CC %51


1. modua: eluzio profila


2. modua: espektro profila


3. modua: kontzentrazio profila

6.9 Irudia. *PARAFAC2 modelo ezberdinen 3 moduen irudikapenak azaldutako bariantza eta CC-rekin."unc" eta "nn" kodeek mugatzaile gabe eta ez-negatibotasunarekin egindako modeloak adierazten dituzte, hurrenez hurren. Aldiz, 5 eta 6 modeloaren osagai kopuruak dira.*

5 osagai eta mugatzailerik gabeko modeloan (PARAFAC2_5unc) %99.973 azaltzen da bariantza eta nukleo-egonkortasunak nahiko ona dirudi (%95). Baina lortutako grafikoak begiratuz gero, bi puntu nabarmendu daitezke. Hasteko lehenengo moduan lortutako eluzio profilak. Gailur bakoitza analito ezberdin bati badagokio, osagai bakoitzak gailur bakar bat definitu beharko luke eta kasu honetan, 3. osagaiak (gorria) 2. eta 5. gailurrak (M eta TB) deskribatzen dituela ikusten da. Grafiko horretan bertan, 4. osagaiak (urdin argia) oinarrizko lerroa deskribatzen duela dirudi, eta 3. moduko irudikapena begiratuz gero, osagai horren kontzentrazioaren aldakuntza txikia dela dirudi lagin ezberdinetan zehar. Datu horrek, agian beste osagai baten beharra dagoela adierazten du. Azkenik, modelo honetan deigarria da 3. moduko grafikoan ageri diren kontzentrazio erlatiboaren balio negatiboak eta ez-negatibotasunaren erabileraren beharra adierazi dezake.

Beraz, beste modelo bat eraiki zen (PARAFAC2_5nn) 5 osagai erabiliz eta 2 eta 3 moduetan ez-negatibotasuna aplikatuz (esan bezala PARAFAC2-an ezin da mugatzailerik ezarri lehen moduan). Azaldutako bariantza eta nukleo-egonkortasuna %99.974 eta %<0 izan ziren, hurrenez hurren. Hots, modeloaren aldakortasuna ondo deskribatzen zuela bazirudien arren, osagai kopurua ez dela batere egokia esaten digu modeloak. 6.9. irudiko grafikoak begiratuz gero, bi fenomeno azpimarratu daitezke. Alde batetik 5. osagaiak (magenta) 2 eta 5 gailurrak (M eta TB) deskribatzen dituela ikusten da eta, bestetik, 2. osagaiak (berdea), berriz ere oinarrizko lerroa deskribatzen duela dirudien arren, kontzentrazio erlatiboak begiratuz gero, lehen osagaiaren (urdina) joera bera duela ikus daiteke.

Modeloari osagai bat gehiago gehituz eta mugatzailerik ez (PARAFAC2_6unc), azaldutako bariantza %99.986-koa izan zen eta nukleo egonkortasuna %81. Datu hauek nahikoa onak baziren ere, grafikoetan kontzentrazio negatiboen arazoa errepikatzen zen. Nahiz eta kasu honetan analito gailur bakoitza osagai ezberdin batek deskribatu, lortutako kontzentrazio profila guztiz desegokia izan zen.

Azkenik, 6 osagai eta ez-negatibotasunarekin eraiki zen modeloa (PARAFAC2_6nn). Azaldutako bariantza ona izan arren (%99.979) nukleo-egonkortasuna eskasa izan zen. (%51). Gainera, osagaiek lehenengo moduan deskribatutako joera guztiz nahasia izan zen: 1. osagaiak (urdina), 3 eta 5. gailurrak

deskribatzen zituen, 4. osagaiak (urdin argia), aldiz, 1., 2. eta 5. gailurrak. Eta azkenik, 6. osagaiak 2., 3. eta 5. gailurrak.

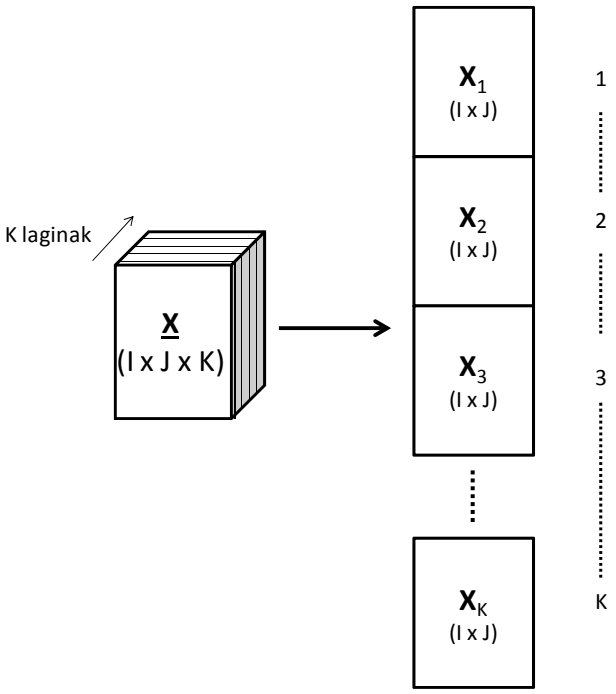
Azaldutako modelo bat bera ere ez zen guztiz ona izan, eta osagai kopuru gehiagorekin ere modeloak eraiki baziren ere, ez zen modelo onik lortu. Oro har ikusitako arazoa ondorengoa izan zen: lehenengo moduan, osagai bakoitzak ez zuen gailur bakar bat deskribatzen. Kasu gehienetan M eta TB nahasten ziren, baina bestelako nahasmenak ere ageri ziren. Bigarren moduak, nahikoa ondo deskribatzen zituen analito bakoitzaren espektro ultramoreak, nahiz eta kasuren baten ia bi espektro ultramore berdinak lortu (PARAFAC2_5nn modeloko lehen bi osagaiak esaterako (urdina eta berdea)). Azkenik, laginen kontzentrazio erlatiboak deskribatzen zituen grafikoetan balio negatiboak ageri ziren mugatzailerik gabeko modeloetan, baino ez-negatibotasuna aplikatutako modeloen grafikoetan ikusten zen joera ere nahiko nahasgarria zen.

Lortutako kontzentrazio erlatibo horiek, erauzketarako jarritako kontzentrazioekin korrelazionatu daitezke, kalibrazio-zuzen bat lortzeko eta modu horretan kuantifikazioa gauzatuz. PARAFAC2-ekin lortutako modeloetatik bat bera ere ez da ona izan. Hala eta guztiz ere, hurrengo atal batean modelo mota ezberdinen arteko konparazioa egin da baldintza berdinekin lortutako beste modeloak alderatuz.

6.3.2.2 Multivariate Curve Resolution (MCR)

MCR ere datu kromatografikoak maneiatzeko oso erabilia izan da [Tauler, 1995; Mas *et al.*, 2011; Salvatore *et al.*, 2013; Boeris *et al.*, 2014]. Halere, MCR-ren desabantaila nagusia azpimarratu behar da, izan ere, duen askatasun errotazionalagatik eta iterazio metodo bat izanik, iterazioaren hasiera puntuaren arabera ebazpen matematikoa ezberdina izan liteke. Horren ondorioz, modeloak errepikatuz gero emaitza bera ez lortzea gerta liteke; hots, soluzio ez-bakarra lortu daiteke. Arazo hau ekidin daiteke, behar bezalako mugatzaileak jarriz gero eta era horretan esanahi kimikoa duen egiazko soluzio bakar batera irits daiteke [Abdollahi eta Tauler, 2011].

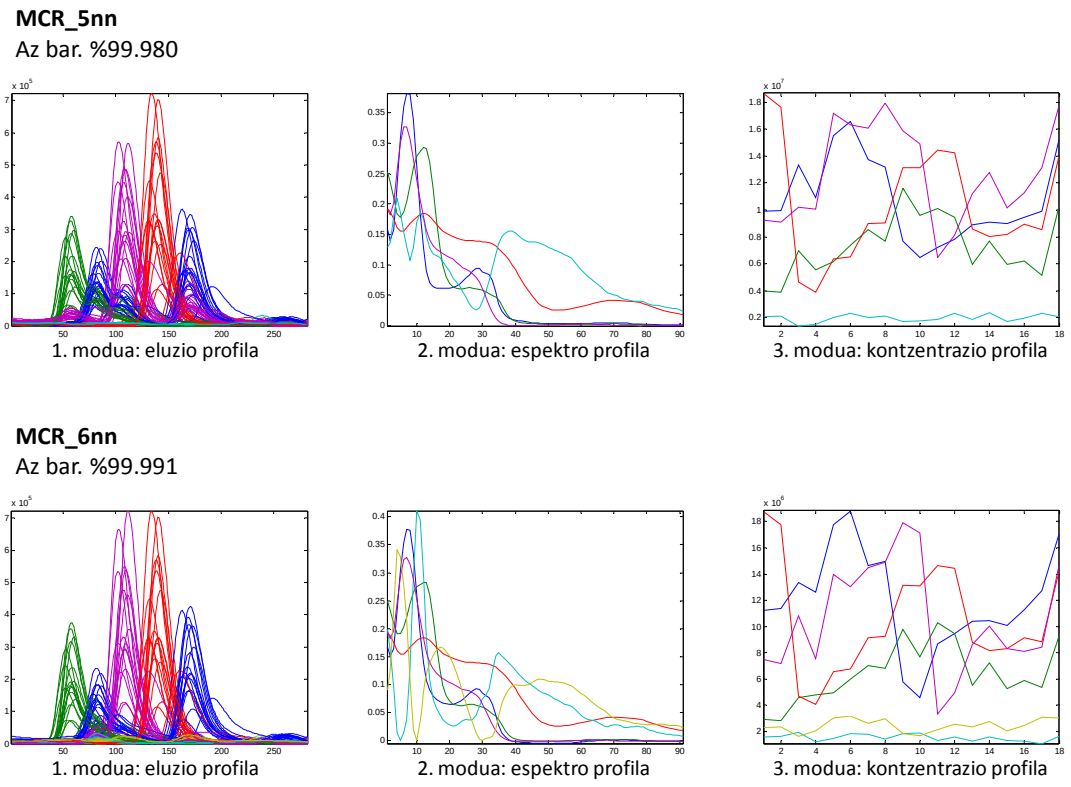
MCR modeloak eraikitzeko, hiru dimentsioko matrizea bi dimentsioko matrize bihurtu behar da. MCR datu trilinearrak maneiatzeko gai bada ere matrizea modu konkretu batean antolatu behar da: laginei dagokien kromatograma bakoitza, zutabe-moduan jarri behar da (ikus 6.10. irudia). Deskonposaketa honen ondorioz, MCR gauzatzeko ez da beharrezkoa lerrokatze tratamendurik, izan ere, modeloak kromatograma jarrai bat bezala jokatzen baitu.



6.10. Irudia. *Hiru dimentsioko datu matrizearen deskonposaketa bi dimentsioko matrizea lortzeko MCR burutzeko beharrezkoa den zutabe-norabidean.*

Beraz, soluzio bakarrak eta egiazkoak lortzeko mugatzaileak beharrezkoak dira MCR-n. 6.11. irudian ikus daitekeen bezala, ez-negatibotasuna erabili zen hiru profiletan. Lehenengo lerroko modeloan (MCR_5nn) PARAFAC2-an ikus zitekeen arazo bera ikus daiteke: modeloaren osagai batek (1. osagaia, urdina) 2. eta 5. gailurrak deskribatzen ditu (M eta TB). Azaldutako bariantza oso ona den arren, modeloak ez ditu datuak behar bezala deskribatzen. 4. osagaiak oinarrizko lerroa deskribatzen duela dirudi, eta agian osagai bat gehiagoren beharra nabari daiteke.

Beste osagai bat gehiago ere gehituta (MCR_6nn), ia modelo bera ikusten zen, lehen osagaiak (urdina) berriz ere M eta TB deskribatzen zituen, eta 4. eta 6. osagaiak oinarrizko lerroa. Azaldutako bariantza kasu honetan ere oso ona da (%99.991).

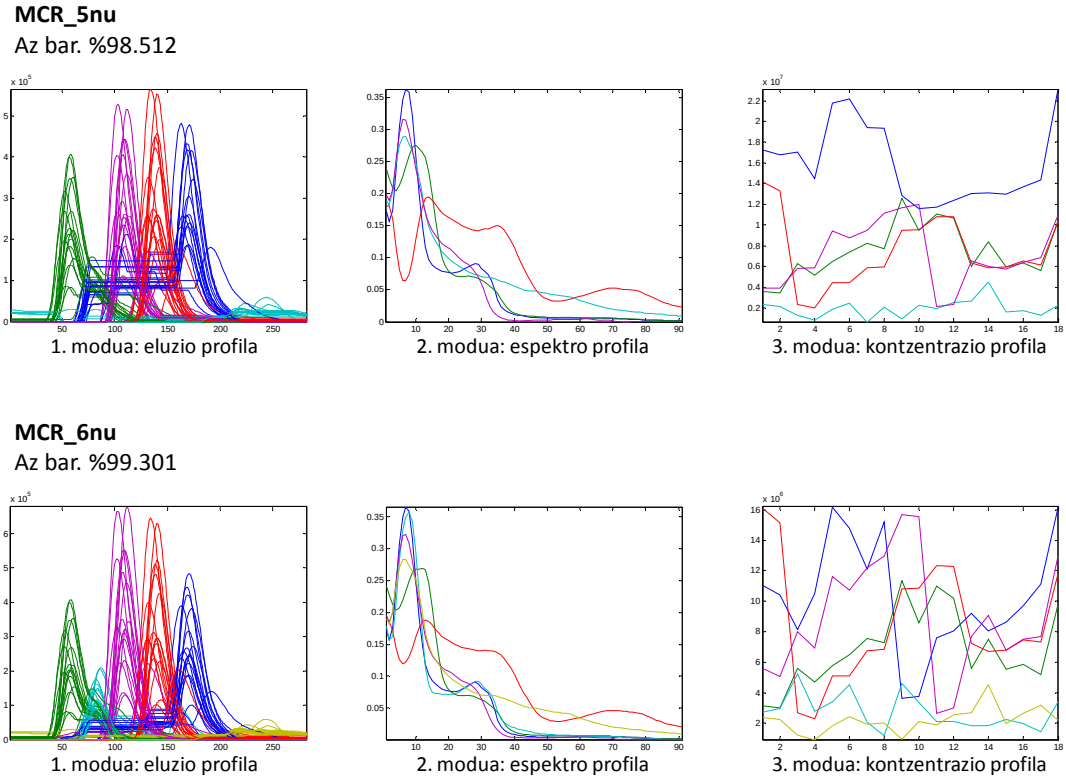


6.11 Irudia. *MCR modelo ezberdinen 3 moduen irudikapenak azaldutako bariantzarekin. Ez-negatibotasuna aplikatu da 5 eta 6 osagaiko modeloak eraikitzeko.*

Oraingoan, unimodalitatea aplikatu daiteke eluzio-profilean, PARAFAC2an ez bezala. Beraz, 6.12. irudian 5 eta 6 osagaiekin egindako modeloak ikus daitezke

(MCR_5un eta MCR_6un). Modelo hauek, profil guztietan dute ez-negatibotasun mugatzailea, eta horrez gain, eluzio-profilean unimodalitatea baita ere.

Unimodalitateak, ordea, ez du laguntzen. Berriz ere, M eta TB osagai bakar batekin modelatzen saiatzen denez, joera arraroak ikusten dira, nahiz eta berriz ere, azaldutako bariantza ona izan: %98.512 MCR_5nu kasuan eta %99.301 MCR_6nu kasuan.



6.12 Irudia. *MCR modelo ezberdinen 3 moduen irudikapenak azaldutako bariantzarekin. Ez-negatibotasuna aplikatu da 5 eta 6 osagaiko modeloak eraikitzeko. Eluzio-profilean unimodalitatea ere aplikatu da.*

Oro har, PARAFAC2 eta MCR modelo batzuk egin ostean, zera ondorioztatu daiteke: modelo matematikoek antzekotasun handiak aurkitzen dituzte bigarren eta bosgarren espektro profilen artean (M eta TB). Beraien benetako espektroak ikusita, korrelazio handia nabari daiteke. Hau da, modelo hauentzat espektro profiletan lau profil ezberdin daude, eta ez bost, analito gailur kopurua bezala. Horrek matematikoki

hein-urritasuna dagoela esan nahi du, izan ere bost espektro profiletatik bi ez dira linealki independenteak.

6.3.2.3 Parallel Factor Analysis with Linear Dependencies (PARALIND)

PARALIND-ek hein-urritasunak aintzat hartzen ditu. Kasu honetan, hein-urritasuna, arestian aipatutako espektro profilean aurkitzen da. Eluzio eta lagin kontzentrazioen profilean bost osagaien beharra agerikoa den bitartean, espektro profilean lau dira linealki independenteak diren osagaiak. PARALIND-ek modu batean beste bi moduetan ezberdina den osagai kopurua jartzea baimentzen du. Beraz espektro profilaren osagai kopurua lau izango da eta besteena bost.

PARALIND egin ahal izateko datuak lerrokatzea beharrezkoa da, ez baitu PARAFAC2-ak eskaintzen duen eluzio-denbora malgutasunik, ezta MCR-ren kromatograma jarraiaren izaerarik. Beraz, aurretik COSHIFT eta COW bidez lerrokatu ziren datuak.

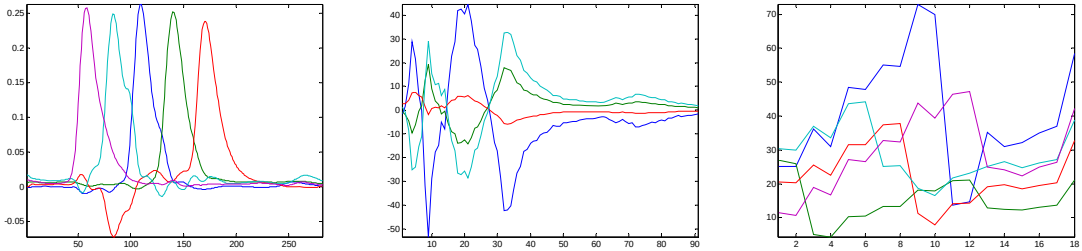
6.13. irudian ikus daitezke PARALIND modelo ezberdinak: mugatzailerik gabeko modeloa (PARALIND_4/5unc), ez-negatibotasuna modu guztietan duen modeloa (PARALIND_4/5nn) eta ez-negatibotasuna eta unimodalitatea (eluzio-profilean bakarrik) duen modeloa (PARALIND_4/5nu). Kasu guztietan osagai kopurua 5 da eluzio eta lagin kontzentrazio profilentzat, eta 4 espektro profilarentzat.

Hiru modeloetan lortutako azaldutako bariantzaren ehunekoak altuak dira: %99.484, %99.480 eta %99.443, mugatzailerik gabeko, ez-negatibotasuna eta ez-negatibotasuna + unimodalitatea erabilitako modeloetan, hurrenez hurren.

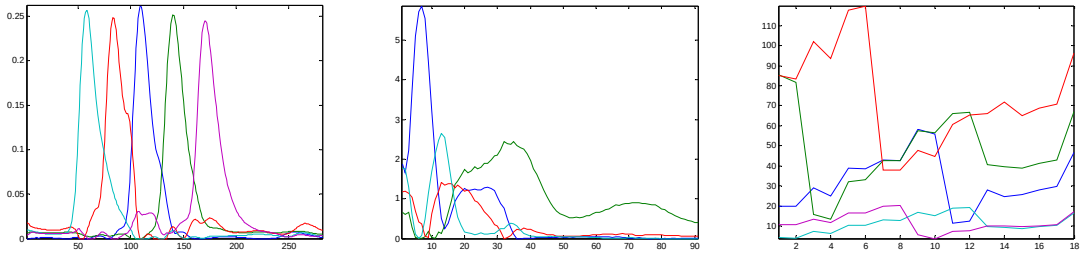
Mugatzailerik erabili ez den modeloan (PARALIND_4/5unc), gailur negatibo bat ikus daiteke bigarren gailurraren lekuan. Horrez gain, espektroen profiletan ere balio negatiboak ageri dira. Balio negatiboak zentzu handirik ez dute, baina PARAFAC2 eta MCR-rekin lortutako modeloekin gertatzen ez zena ikus daiteke: osagai bakoitzak gailur bakar bat deskribatzen du.

Ez-negatibotasuna aplikatzeak modeloari zentzu gehiago emango dionez, hurrengo modeloa profil guztietan ez-negatibotasuna aplikatuz egin zen (PARALIND_4/5nn). Modeloak itxura egokia du eluzio-denborei dagokionez, baina osagaiek deskribatzen duten gailur nagusiaz gain, beste gailurtxo batzuk ere ikus daitezke. Beraz, azkenik eluzio-profilean unimodalitatea ere aplikatzea erabaki zen (PARALIND_4/5nu). Azken modelo honek ere itxura egokia du, aurrekoaren oso antzekoa, aipatutako gailur txiki horiek gabe.

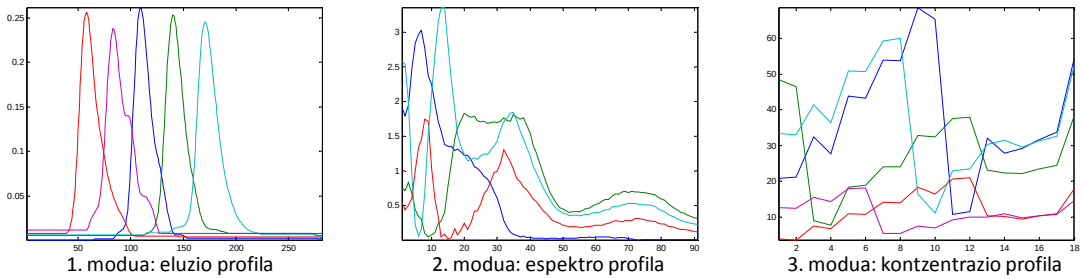
PARALIND_4/5unc
Az bar. %99.484



PARALIND_4/5nn
Az bar. %99.480



PARALIND_4/5nu
Az bar. %99.443



6.13 Irudia. *PARALIND modelo ezberdinen 3 moduen irudikapenak azaldutako bariantzarekin. 5 osagaiekin eraikitako modeloak, lehenengoa mugatzailerik gabe eraikia, bigarrena ez-negatibotasuna aplikatuz hiru moduetan, eta, azkena, ez-negatibotasuna modu guztietan eta eluzio profilean unimodalitatea.*

Halere, modeloen egokitasuna ikusteko, beharrezkoa da lortutako kontzentrazio profil horiek, benetako kontzentrazioekin erlazionatzea eta modeloek duten iragartzeko gaitasuna frogatzea. Behin hori jakinda, eta aipatutako datuak eta grafikoak interpretatuta, datuak ongien deskribatuko dituen modeloa hauta daiteke.

6.3.3 Modeloen konparazio kuantitatiboa

Modeloen itxura esanguratsua bada ere, analisi kuantitatiboak modeloaren egokitasunaren inguruan informazio asko eman dezake. Horretarako algoritmo ezberdinekin eraikitako zenbait modelo hautatu dira eta hoberena hautatzeko konparatu dira.

Analisi kuantitatiboa egiteko, laginen kontzentrazio profilean lortutako kontzentrazio erlatiboak analisirako jarri diren ezarritako kontzentrazioekin erlazionatu dira (6.1. taula) eta kalibrazio-sortekin kalibrazio zuzenak eraiki dira. 6.2. taulan, analito bakoitzari dagozkion kalibrazio tarteak, korrelazio koefizienteak eta detekzio-mugak ikus daitezke. Detekzio-muga hauek, kalibrazioan lortutako maldaren desbideratzearekin kalkulatuak izan dira, aurreko atalburuetan azaldu den bezala, ondoko formulak erabiliz:

$$s_{y/x} = \sqrt{\frac{\sum(\hat{y}_i - y_{i,ref})^2}{I-2}} \qquad (6.10)$$

$$LOD = \frac{3 \cdot s_{y/x}}{b} \qquad (6.11)$$

Analito bakoitzari dagozkion datuez gain, modeloari buruzko datuak ere ageri dira 6.2. taulan. Hala nola, modeloak eraikitzeko MATLAB softwareak behar izan duen konbergentzia denbora eta azaldutako bariantza.

Konparazio honetarako, algoritmo bakoitzarekin lortutako modelorik hoberenak aztertu dira aukera ezberdinen artean: 5 osagai eta ez-negatibotasuna duen PARAFAC2 modeloa, 5 osagai eta ez-negatibotasuna duen MCR modeloa eta 4 eta 5 osagai dituzten ez-negatibotasuna eta unimodalitatea dituzten bi PARALIND modeloak.

6.2. Taula. *Kalibrazio eta balidazio estandarren kontzentrazioak (µg L^{-1}).*

		TT	M	FS	FQ	TB
kalibrazio tartea (µg L^{-1})		5.5-33.0	5.5-33.0	7.0-42.0	5.4-32.40	8.4-50.4
Konbergentzia denbora	**PARAFAC2** [(a)]	8 h				
	MCR [(a)]	3 min				
	PARALIND [(a)]	10 min (+ lerrokatzea)				
	PARALIND [(b)]	13 min (+lerrokatzea)				
Azaldutako bariantza (%)	**PARAFAC2** [(a)]	99.974				
	MCR [(a)]	99.980				
	PARALIND [(a)]	99.480				
	PARALIND [(b)]	99.443				
R^2	**PARAFAC2** [(a)]	0.8742	0.1347	0.9690	0.9937	0.9167
	MCR [(a)]	0.8419	0.2085	0.7703	0.9847	0.7997
	PARALIND [(a)]	0.9920	0.9830	0.9805	0.9908	0.9830
	PARALIND [(b)]	0.9933	0.9883	0.9921	0.9904	0.9864
LOD (µg L^{-1})	**PARAFAC2** [(a)]	11.7*	78.2*	7.0*	2.4	14.2*
	MCR [(a)]	13.4*	60.2*	21.5*	3.8	23.6*
	PARALIND [(a)]	2.8	4.1	5.5	2.9	6.2
	PARALIND [(b)]	2.5	3.4	3.5	3.0	5.5

(a): ez-negatibotasun mugatzailea modu guztietan (PARAFAC2 modeloko lehen moduan ezik).

(b): ez-negatibotasun mugatzailea modu guztietan eta unimodalitatea ere bai eluzio profilaren moduan.

**: Detekzio-muga kalibrazio-tarteko kontzentrazio txikiena baino handiagoa.*

Lehengo datu deigarria konbergentzia denbora litzateke. MCR eta PARALIND metodoak minutu gutxiren barruan konbergentzia lortzen dute, baina PARAFAC2-arekin lortutako modeloak orduak behar izan zituzten. Halere, kontuan izan behar da PARALIND kasuan lerrokatzeak ere bere denbora behar izaten duela.

Modelo denek, azaldutako bariantzaren arabera, datuen joera oso ondo modelatzen dutela dirudi (>%99.443). Baina beste datuak begiratuz gero, ezberdintasun handiak nabari daitezke analitoaren arabera. Erregresio koefizienteei dagokienez, FQ analitoan hautatutako lau modeloekin emaitza onak lortzen badira ere (0.9847-0.9937), ezberdintasun handia dago beste analito batzuekin. M eta TB-ren kasuetan aurreikusi zitezkeen datu txarrak PARAFAC2 eta MCR algoritmoekin, grafikoki ondo ebatziak ez zeudela nabarmena zelako. Horregatik, batez ere M kasuan lortu dira korrelazio balio baxuak (0.1347 eta 0.2085) eta kalibrazio tartetik kanpo dauden detekzio-muga altuak (78.2 eta 60.2 µg L^{-1}). M PARALIND bidez ebatzitako erregresio

balioak 0.9830 eta 0.9883 izan ziren. Beste analitoei dagokienez, joera berdina da: bi PARALIND modeloekin lortutako emaitzak hobeak dira PARAFAC2 eta MCR-rekin lortutakoak baino. PARALIND-ekin lortutako korrelazio koefiziente balio guztiak 0.9805etik gorakoak izan ziren. PARAFAC2 eta MCR-rekin lortutakoak, aldiz, 0.7703 eta 0.9167 artekoak M (datu askoz okerragoak) eta FQ kanpoan utzita (PARALIND-ekin lortutako datuekin konparagarria).

Detekzio-mugak aztertzerakoan ere joera bera ikusten da: FQ kasuan lau modeloekin lortutako datuak antzerakoak dira (2.4-3.8 μg L^{-1}), M-ren datuak aldiz oso ezberdinak PARALIND (3.4 eta 4.1 μg L^{-1}) eta beste bi modeloak konparatuz (60.2 eta 78.2 μg L^{-1}), eta beste analitoen kasuan, balio okerragoak lortzen dira PARAFAC2 eta MCR modeloekin (11.7 eta 23.6 μg L^{-1} artean TT, FS eta TB-rentzat) PARALIND-ekin baino (2.5 eta 6.2 μg L^{-1} artean, TT, FS eta TB-rentzat). Nabarmendu daiteke FQ analitoan izan ezik, beste kasuetan PARAFAC2 eta MCR-rekin lortutako detekzio-muga balioak, kalibrazio-tarteko kontzentrazio txikiena baino balio altuagoak direla, eta beraz balio hauek baztergarriak direla.

Modeloen erroreak RMSEP (*Root Mean Square Error of Prediction*) eta RE (*Relative Error*) bidez kalkulatu dira kalibrazio eta balidazio sortentzako. Modeloak iragarritako balioak hartzen dira kontuan kalibrazio eta balidazio lagin multzoak kontuan hartuz. Betiere, aintzakotzat hartu behar da, kalibrazio datuak eta euren parametroak (esaterako R^2 eta LOD) kalibrazio-multzoarekin lortu direla, eta balidazio datuak kalibrazioan sartuta ez dauden kanpoko laginekin lortutako datuak direla. Datu hauek denak 6.3 eta 6.4 tauletan ikus daitezke. Ondoko ekuazioen bidez kalkulatu dira RMSEP eta RE:

$$RMSEP = \sqrt{\frac{\sum(\hat{c}_i - c_i)^2}{n}} \qquad (6.12)$$

$$RE\ (\%) = 100 \cdot \sqrt{\frac{\sum(\hat{c}_i - c_i)}{\sum c_i}} \qquad (6.13)$$

Non $\hat{c}_i$ modeloak iragarritako balioa den, c_i erreferentziazko kontzentrazio balioa eta *n* erabilitako lagin kopurua.

Errore hauek, aurreikusi litekeen bezala hobeagoak dira kalibrazio-multzoan, balidazio-multzoan baino, kalibrazio-multzoko laginak erabili baitira kalibrazio-zuzena eraikitzeko. Kalibrazio-multzoan ikus daiteke M ezik, gainerakoen erroreak onak direla

lau modeloetan (%1.1-7.0), nahiz eta PARALIND-en erroreak zertxobait txikiagoak izan (%1.2-2.0), guztien errore erlatiboa %10 azpitik daude. Halere, kalibrazio-laginetatik kanpo dauden balidazio-laginak iragartzeko gaitasuna ikusiz gero erroreak PARAFAC2 eta MCR kasuan handiagoak direla ikus daiteke (%4.8-10.6) PARALIND kasuan baino (%2.1-2.8).

PARAFAC2 eta MCR modeloetan orain arte ikusitakoarekin bat eginda, M-ren portaera kaskarra ikusi daiteke, kalibrazio nahiz balidazio-laginetan erroreak handiak dira: %35.7 eta %27.4 kalibrazio-multzoan eta %37.8 eta %24.2 balidazio-multzoan PARAFAC2 eta MCR modeloentzat hurrenez hurren.

Horrez gain, modeloaren berreskuratze ehunekoa aztertu da, modeloak duen iragartzeko gaitasuna ikusteko. 6.3 eta 6.4 tauletan ikus daitekeen bezala, berreskuratze ehunekoak oso onak dira PARALIND kasuetako kalibrazio-multzoan (%99.0-102.2) eta okerragoak PARAFAC2 eta MCR kasuetan (%95.2-101.7). Baina M-ren kasuan balio guztiz baztergarriak lortu ziren PARAFAC2 eta MCR modeloetan, %157.2 eta %33.3.

Balidazio-multzoan era joera antzekoa da: PARALIND modeloekin lortutako balioak hobeagoak dira (%94.7-103.2), PARAFAC2 eta MCR-rekin lortutakoak baino (%81.4-104.6). Berriz ere, M-rentzat lortu dira balio txarrenak, %37.2 eta 68.8 artekoak.

Laburbilduz, datu guztiek erakusten dute PARALIND-ek emaitza hobeak ematen dituela PARAFAC2 eta MCR modeloak baina batez ere M modelatzeko orduan, grafikoki aurreikusi den bezala. Bi PARALIND modeloen artean ez dago alde esanguratsurik, unimodalitatea erabili den modeloan emaitza zertxobait hobeak lortu dira, baina bi modeloak dira onak eta erabilgarriak.

PARALIND erabiltzearen alde txarra, behar duen lerrokatzea izango litzateke. Izan ere, kasu batzuetan denbora asko eska dezake eta konplikatua izan daiteke.

6.3. Taula. *Kalibrazio-multzoaren berreskuratzeak eta erroreak.*

			TT	M	FS	FQ	TB
Kalibrazio-multzoa	**$RMSEP_{kal}$**	**PARAFAC2** [(a)]	3.6	23.8	2.1	0.7	4.3
		MCR [(a)]	4.1	18.3	6.5	1.1	7.2
		PARALIND [(a)]	0.8	1.2	1.7	0.9	1.9
		PARALIND [(b)]	0.8	1.0	1.1	0.9	1.7
	RE_{kal} (%)	**PARAFAC2** [(a)]	5.3	35.7	2.5	1.1	4.2
		MCR [(a)]	6.1	27.4	7.7	1.8	7.0
		PARALIND [(a)]	1.3	1.9	2.0	1.4	1.9
		PARALIND [(b)]	1.2	1.5	1.3	1.4	1.7
	Batazbesteko kalibrazio berreskuratzeak (%)	**PARAFAC2** [(a)]	95.2 ± 22.9	157.2 ± 329.2	101.7 ± 14.0	100.4 ± 5.0	98.0 ± 16.3
		MCR [(a)]	96.6 ± 27.8	33.3 ± 238.8	97.3 ± 29.5	100.8 ± 9.2	100.1 ± 26.7
		PARALIND [(a)]	99.9 ± 5.1	102.2 ± 13.4	101.0 ± 7.4	99.0 ± 9.2	101.0 ± 14.1
		PARALIND [(b)]	99.8 ± 4.6	101.7 ± 9.8	101.1 ± 6.8	99.0 ± 9.2	100.1 ± 13.0

(a): ez-negatibotasun mugatzailea modu guztietan (PARAFAC2 modeloko lehen moduan ezik).

(b): ez-negatibotasun mugatzailea modu guztietan eta unimodalitatea ere bai eluzio profilaren moduan.

6.4. Taula. *Balidazio-multzoaren berreskuratzeak eta erroreak.*

			TT	M	FS	FQ	TB
Balidazio-multzoa	**$RMSEP_{bal}$**	**PARAFAC2** [(a)]	3.6	17.0	3.5	2.4	7.0
		MCR [(a)]	4.8	10.9	4.6	2.1	5.5
		PARALIND [(a)]	1.2	1.1	1.6	1.1	1.5
		PARALIND [(b)]	1.0	1.2	1.4	1.1	1.4
	RE_{bal} (%)	**PARAFAC2** [(a)]	8.0	37.8	6.2	5.4	10.2
		MCR [(a)]	10.6	24.2	8.0	4.8	8.0
		PARALIND [(a)]	2.8	2.5	2.8	2.4	2.1
		PARALIND [(b)]	2.2	2.6	2.5	2.4	2.1
	Batazbesteko balidazio berreskuratzeak (%)	**PARAFAC2** [(a)]	84.6 ± 14.8	37.2 ± 79.3	94.6 ± 16.9	89.2 ± 9.9	81.4 ± 22.0
		MCR [(a)]	85.2 ± 25.9	68.8 ± 49.9	104.6 ± 19.8	88.2 ± 4.3	88.4 ± 16.7
		PARALIND [(a)]	96.2 ± 6.7	103.1 ± 6.3	96.0 ± 7.2	94.7 ± 3.8	97.2 ± 5.1
		PARALIND [(b)]	97.1 ± 5.8	103.2 ± 6.4	96.7 ± 6.5	94.7 ± 3.8	97.6 ± 5.0

(a): ez-negatibotasun mugatzailea modu guztietan (PARAFAC2 modeloko lehen moduan ezik).

(b): ez-negatibotasun mugatzailea modu guztietan eta unimodalitatea ere bai eluzio profilaren moduan.

6.4 Ondorioak

Atalburu honetan PARALIND-en gaitasuna azpimarratu nahi izan da hein-urritasuna dagoenean. Kasu honetan espektro ultramoreak duten antzekotasunetik dator PARAFAC2-ak eta MCR-k modelatzeko gai ez diren hein-urritasuna.

Algoritmo bakoitzarekin zenbait modelo proposatu dira, eta beraien ezaugarri batzuk ikusita, hoberenak hautatu dira konparazio kuantitatibo bat egiteko. Grafikoki ikus daitekeena azpimarratu da datu kuantitatiboekin ere: miklobutanila eta tebukonazolaren antzekotasun espektralagatik, PARAFAC2 eta MCR ez ziren gai izan analito hauek ongi modelatzeko. Aldiz, PARALIND-ek gain hartzen duen hein-urritasunagatik eta modu espektralean osagai bat gutxiago proposatuz, lorturiko datuak analito guztiak ondo modelatzeko gaitasuna erakutsi dute. Kimikoki esanguratsuagoak dituen profilak lortu dira PARALIND kasuetan eta horrek informazio zehatzagoa eskaintzen du.

PARAFAC2 eta MCR-k tetrakonazola, flusilazola eta fluquinkonazola modelatzeko gai direla erakutsi dute, beraien arteko aldea espektrala nabarmenagoa baita. Hala eta guztiz ere PARALIND-ekin lortutako balioak hobeagoak izan dira kasu batzuetan.

PARALIND-en desabantaila datuak lerrokatu beharra litzateke, PARAFAC2 eta MCR eraikitzeko beharrezkoa ez den bitartean. Lerrokatzea kasu batzuetan konplikatua suerta baliteke ere, emaitzak ikusita merezi du denbora bat horretan ematea. Are gehiago PARAFAC2-ak behar dituen denborak ikusita, lerrokatzeak ematen duena baino denbora gehiago behar baitu modeloak berak.

PARALIND-ekin lortutako datuak kontuan hartuta emaitza hobeak eman dituela ondoriozta daiteke. Lortutako erroreak konparatuz gero PARALIND kasukoak izan dira baxuenak eta baita berreskuratze-ehuneko hoberenak ere. Oro har, PARALIND modeloekin lortutako detekzio-mugak ere hobeak izan dira kasu denetan, flukinkonazol kasuan ezik. Azken kasu honetan, balio antzekoak lortu dira PARALIND, PARAFAC2 eta MCR erabiliz. Erregresio koefizienteak (R^2) hobeak izan dira PARALIND-ekin ere.

6.5 Erreferentziak

Abdollahi, H. eta Tauler, R.; *Uniqueness and rotation ambiguities in Multivariate Curve Resolution methods.* **2011.** Chemometrics and Intelligent Laboratory Systems, 108(2), 100-111.

Amigo, J.M., Skov, T. eta Bro, R.; *ChroMATHography: Solving Chromatographic Issues with Mathematical Models and Intuitive Graphics.* **2010.** Chemical Reviews, (110), 4582-4605.

Amigo, J.M., Skov, T., Bro, R., Coello, J. eta Maspoch, S.; *Solving GC-MS problems with PARAFAC2.* **2008.** Trends in Analytical Chemistry, 27(8), 714-725.

Bahram, M. eta Bro, R.; *A novel strategy for solving matrix effect in three-way data using parallel profiles with linear dependencies.* **2007.** Analytica Chimica Acta, 584(2), 397-402.

Boeris, V., Arancibia, J.A. eta Olivieri, A.C.; *Determination of five pesticides in juice, fruit and vegetable samples by means of liquid chromatography combined with multivariate curve resolution.* **2014.** Analytica Chimica Acta, 814(0), 23-30.

Bro, R., *PARAFAC. Tutorial and applications.* **1997.** Chemometrics and Intelligent Laboratory Systems, 38(2), 149-171.

Bro, R., *Review on Multiway Analysis in Chemistry—2000–2005.* **2006.** Critical Reviews in Analytical Chemistry, 36(3-4), 279-293.

Bro, R., Andersson, C. eta Kiers, H.A.L.; *PARAFAC2: Part II. Modeling chromatographic data with retention time shifts.* **1999.** Journal of Chemometrics, 13(3-4), 295-309.

Bro, R., Harshman, R. eta Sidiropoulos, N.D., PARALIND. Modelling multi-way data with linearly dependent loadings. Eskuragarri http://www.models.life.ku.dk/algorithms helbidean. Azken aldiz [7/2014]-ean ikusia.

Bro, R., Harshman, R.A., Sidiropoulos, N.D. eta Lundy, M.E.; *Modeling multi-way data with linearly dependent loadings.* **2009.** Journal of Chemometrics, 23(7-8), 324-340.

Bro, R. eta Smilde, A.K.; *Principal component analysis.* **2014.** Analytical Methods, 6, 2812-2831.

de Juan, A., Rutan, S.C., Tauler, R. eta Massart, D.L.; *Comparison between the direct trilinear decomposition and the multivariate curve resolution-alternating least squares methods for the resolution of three-way data sets.* **1998.** Chemometrics and Intelligent Laboratory Systems, 40(1), 19-32.

de Juan, A. eta Tauler, R.; *Chemometrics applied to unravel multicomponent processes and mixtures: Revisiting latest trends in multivariate resolution.* **2003.** Analytica Chimica Acta, 500(1–2), 195-210.

de Juan, A. eta Tauler, R.; *Multivariate curve resolution (MCR) from 2000: progress in concepts and applications.* **2006.** Critical Reviews in Analytical Chemistry, 36(3-4), 163-176.

de Juan, A. eta Tauler, R.; *Factor analysis of hyphenated chromatographic data: Exploration, resolution and quantification of multicomponent systems.* **2007.** Journal of Chromatography A, 1158(1-2), 184-195.

Engel, J., Gerretzen, J., Szymańska, E., Jansen, J.J., Downey, G., Blanchet, L. eta Buydens, L.M.C.; *Breaking with trends in pre-processing?* **2013.** Trends in Analytical Chemistry, 50, 96-106.

Goicoechea, H.C., Culzoni, M.J., García, M.D.G. eta Galera, M.M.; *Chemometric strategies for enhancing the chromatographic methodologies with second-order data analysis of compounds when peaks are overlapped.* **2011.** Talanta, 83(4), 1098-1107.

Johnsen, L.G., Amigo, J.M., Skov, T. eta Bro, R.; *Automated resolution of overlapping peaks in chromatographic data.* **2014.** Journal of Chemometrics, 28(2), 71-82.

Kamstrup-Nielsen, M.H., Johnsen, L.G. eta Bro, R.; *Core consistency diagnostic in PARAFAC2.* **2013.** Journal of Chemometrics, 27(5), 99-105.

Kumar, N., Bansal, A., Sarma, G.S. eta Rawal, R.K.; *Chemometrics tools used in analytical chemistry: An overview.* **2014.** Talanta, 123, 186-199.

Maeder, M., Evolving factor analysis for the resolution of overlapping chromatographic peaks. **1987.** *Analytical Chemistry,* (3), 527.

Maeder, M. eta de Juan, A., **2009.** 2.16 - Two-Way Data Analysis: Evolving Factor Analysis. In: S.D. Brown, R. Tauler and B. Walczak, eds, *Comprehensive Chemometrics.* Elsevier, 261-274. Oxford.

Manne, R. eta Grande, B.; *Resolution of two-way data from hyphenated chromatography by means of elementary matrix transformations.* **2000.** Chemometrics and Intelligent Laboratory Systems, 50(1), 35-46.

Marini, F., D'Aloise, A., Bucci, R., Buiarelli, F., Magrì, A.L. eta Magrì, A.D.; *Fast analysis of 4 phenolic acids in olive oil by HPLC-DAD and chemometrics.* **2011.** Chemometrics and Intelligent Laboratory Systems, 106(1), 142-149.

Mas, S., Tauler, R. eta de Juan, A.; *Chromatographic and spectroscopic data fusion analysis for interpretation of photodegradation processes.* **2011.** Journal of Chromatography A, 1218(51), 9260-9268.

Nielsen, N.V., Carstensen, J.M. eta Smedsgaard, J.; *Aligning of single and multiple wavelength chromatographic profiles for chemometric data analysis using correlation optimised warping.* **1998.** Journal of Chromatography A, 805(1–2), 17-35.

Roma, T., *Multivariate curve resolution applied to second order data.* **1995.** Chemometrics and Intelligent Laboratory Systems, 30(1), 133-146.

Salvatore, E., Cocchi, M., Marchetti, A., Marini, F. eta de Juan, A.; *Determination of phenolic compounds and authentication of PDO Lambrusco wines by HPLC-DAD and chemometric techniques.* **2013.** Analytica Chimica Acta, 761, 34-45.

Skov, T. eta Bro, R.; *Solving fundamental problems in chromatographic analysis.* **2008.** Analytical and Bioanalytical Chemistry, 390(1), 281-285.

Skov, T., Van den Berg, F., Tomasi, G. eta Bro, R.; *Automated alignment of chromatographic data.* **2006.** Journal of Chemometrics, 20(11-12), 484-497.

Smilde, A., Bro, R. eta Geladi, P.; *Multi-way analysis. Applications in the chemical sciences.* **2004.** John Wiley & Sons Ltd. West Sussex (England).

Tauler, R., *Multivariate curve resolution applied to second order data.* **1995.** Chemometrics and Intelligent Laboratory Systems, 30(1), 133-146.

Tauler, R., de Juan, A. eta Jaumot, J., Multivariate Curve Resolution Homepage. Webpage of the MCR-ALS method with programs, tutorials and datasets. Eskuragarri http://www.mcrals.info/ helbidean. Azken aldiz [7/2014]-ean ikusia.

Tomasi, G., Van den Berg, F. eta Andersson, C.; *Correlation optimized warping and dynamic time warping as preprocessing methods for chromatographic data.* **2004.** Journal of Chemometrics, 18(5), 231-241.

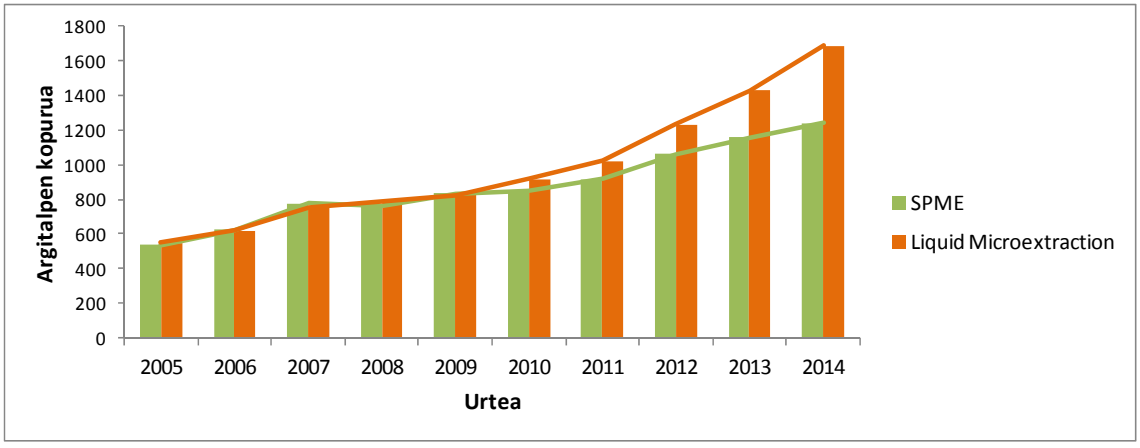
Van den Berg, F., Tomasi, G. eta Viereck, N., **2005.** Warping: investigation of NMR preprocessin and correction. In: S.B. Engelsen, P.S. Belton and H.J. Jakobsen, eds, *Magnetic resonance in food science: The multivariate challenge.* Royal Society of Chemistry, 131-138. Cambridge (UK).

Van Mispelaar, V.G., Tas, A.C., Smilde, A.K., Schoenmakers, P.J. eta van Asten, A.C.; *Quantitative analysis of target components by comprehensive two-dimensional gas chromatography.* **2003.** Journal of Chromatography A, 1019(1–2), 15-29.

7. Eztabaida orokorra

7. Eztabaida orokorra

Lan honetan zehar atal esperimentala batez ere SPME eta USAEME mikroerauzketa tekniketan oinarritu da. Mikroerauzketa teknikak analitoak aurrekontzentratzea eta beraien jatorrizko matrizetik isolatzea ahalbidetzen dute. Azken urteotan, mikroerauzketa tekniken erabileraren gorakada nabarmena ikusi da. SPME teknikaren hastapenak 90 hamarkadaren hasieran kokatzen badira ere [Arthur eta Pawliszyn, 1990], eduki ditzakeen mugak gainditzeko lehen likido mikroerauzketa teknikak garatzen hasi ziren 90 hamarkadaren erdialdean [Jeannot eta Cantwell, 1996]. Halere, likido mikroerauzketa teknika ezberdinen garapena eta erabileraren gorakada 2000 urtetik aurrera eman zen [Psillakis eta Kalogerakis, 2003; Lambropoulou eta Albanis, 2007; Pinto *et al.*, 2010; Sarafraz-Yazdi eta Amiri, 2010]. 7.1 irudian azken hamarkadan argitaratutako artikulu kopurua ikus daiteke. Azken urteetan likido mikroerauzketa tekniken inguruko artikulu kopuruak goraka jarraitzen duen bitartean SPME artikulu kopuruaren gorakada, aldiz, ez da likido mikroerauzketa teknikak bezain nabarmena.



7.1. Irudia. *Azken hamarkadan SPME eta likido mikroerauzketa teknikek izandako goraka (datuen iturria Science Direct bilatzailea).*

Atal honetan, lan zehar ikusitako teknikak konparatu dira. Alde batetik, tesi honetan landu diren tekniken berrikuspena egin da. Metodo bakoitzaren ezaugarri

analitikoak eta beste alde praktiko batzuk errepasatuko dira. Bestetik, tesi honekin lortutako emaitzak beste ikerlan batzuek lortutako ezaugarri analitikoekin alderatu dira.

7.1 *Garatutako mikroerauzketa metodoen ezaugarrien berrikuspena*

Hasteko, tesi honetako metodoak konparatu dira, SPME eta USAEME. Erauzketa etapak bakarrik konparatu dira, erauzketa materialak eta emaitza batzuk konparatu dira, baina baita teknikak berak dituen zailtasun/erraztasunak eta teknika maneiatzerakoan ikusitako ezaugarri praktiko batzuk (ikus 7.1, 7.2 eta 7.3 taulak).

Triazolen determinaziorako garatutako hiru metodoen ezaugarri analitikoak 7.1 taulan erakusten dira. Errepikakortasunak, RSD ehunekotan emanak, konparagarriak dira hiru prozeduretan. Emaitzarik hoberenak USAEME prozedurarekin lortu dira (%1.9 – 10.6) Baina tarte linealari dagokionez, deigarria den ezaugarri bat ikus daiteke. Izan ere tebukonazola SPME-GC/ECD bidez determinatzen denean, sentsibilitate urria erakusten du, eta horregatik bere linealtasun tartea (190 – 2560 µg L^{-1}) eta detekzio-muga (162 µg L^{-1}) bereziki altuak dira. Analito hau kanpoan utzita, ordea, USAEME kasuan detekzio-mugak zertxobait altuagoak direla esan daiteke. Halere, tarte lineala USAEME kasuan askoz zabalagoa dela azpimarratzekoa da. Izan ere, SPME kasuan zuntzen asetzea gerta daiteke puntu batetik aurrera, eta bere linealtasuna galtzen da. Lortutako berreskuratze ehunekoak hiru kasuetan antzekoak izan dira, naiz eta USAEME kasuko balioak zertxobait baxuagoak izan SPME kasuan baino. Hori gertatzearen arrazoia USAEME kasuan tanta birformatzeko arazoa izan daiteke. Izan ere, matrize konplexuen kasuan, zentrifugatu ondoren tanta formatzea zaila izan daiteke, eta gertatu ohi den erauztaile galerak, berreskuratze ehuneko baxuagoak eragin ditzake.

7.1.Taula. *Triazolen determinaziorako lan honetan garatutako metodoen ezaugarri analitikoak.*

Metodoa	**Matrizea**	**Analitoak**	**Linealtasuna ($\mu g\ L^{-1}$)**	**LOD[a] ($\mu g\ L^{-1}$)**	**RSD[b] (%)**	**EF[c]**	**Berreskuratzea (%)**	**Erreferentzia**
SPME-GC/ECD	Mahats-sagar zukuak	D-M-TT-TB	0.4-2560	0.2-162	3.3-18.0	-	93.6-112.1	[Bordagaray *et al.*, 2011]
SPME-HPLC/DAD	Ura, fruitu laginak	D-M-FQ-FS-TB-TT	5.5-50.4	1.5-5.9	2.8-13.1	-	94.5-123.4	[Bordagaray *et al.*, 2013]
USAEME-SFO-HPLC/DAD	Ura, mahats-sagar laginak	D-M-FQ-FS-TB-TT	20-890	10.9-17.2	1.9-10.6	226-255	82-112	[Bordagaray *et al.*, 2014]

SPME-GC/ECD: Fase solidozko mikroerauzketa elektroi harrapaketa detektagailua duen gas kromatografiarekin.
SPME-HPLC/DAD: Fase solidozko mikroerauzketa diodo lerrokatuen detektagailua duen bereizmen handiko likido kromatografiarekin.
USAEME-HPLC/DAD: Ultrasoinuz lagundutako emultsifikazio mikroerauzketa diodo lerrokatuen detektagailua duen bereizmen handiko likido kromatografiarekin.
Analitoak: D: dinikonazola; FS: flusilazola; FQ: flukinkonazola; M: miklobutanila; TB: tebukonazola; TT: tetrakonazola.
[a] LOD: Detekzio-muga; kalibrazio zuzenean oinarritua.
[b] RSD: Desbideratze estandar erlatiboa.
[c] EF: Aberastasun-faktorea.

Azken atalburua metodo kimiometrikoen bidezko gailur gainezarrien ebazpenean oinarritzen da. SPME eta HPLC/DAD-rekin garatutako metodoan bost gailurren gainezarpen partziala ikusi zen. Banaketa hobeago bat ezin izan zen lortu, SPME erabiltzeak dituen disolbatzaile mota mugatuagatik eta beraz, metodo kimiometrikoen erabilerak gailur gainezarrietan izan zezakeen eragina ikusi nahi izan zen. Izan ere, metodo kimiometrikoek horrelako kasuetan gailurren ebazpenean eta kuantifikazioan lagundu baitezakete.

Aldagai bakarreko eta PARALIND bidezko aldagai anitzeko kalibrazioen ezaugarri analitiko batzuk ikus daitezke 7.2 taulan. PARALIND-ek emandako emaitzak zertxobait hobeak badira ere, aldea ez da oso nabarmena, emaitzak antzekoak dira bi kasuetan.

7.2.Taula. *Aldagai bakarreko kalibrazioa eta PARALIND bidezko aldagai anitzeko kalibrazioaren ezaugarri batzuen konparazioa.*

Ezaugarria	**Aldagai bakarreko kalibrazioa (221nm)**	**PARALIND kalibrazioa**
RSD (%)	2.8 – 13.1	4.6 - 13.0
Berreskuratzea (%)	94.5 - 123.4	94.7 – 103.2
LOD (µg L^{-1})	1.5 - 5.9	2.5 – 5.5

Halere, PARALIND kasuarekin ikusitakoak bere garrantzia du. Izan ere, bigarren mailako datuak ebazterakoan gehienbat PARAFAC2 eta MCR erabili ohi dira. Baina analitoen espektro profilengatik metodo kimiometriko horiekin ez da emaitza onik lortu. Batez ere miklobutanil eta tebukonazol analitoen ebazpenean. Beraien espektroak antzekotasun handia dutenez, hein-urritasun arazoak ageri dira eta PARAFAC2 eta MCR-k arazo horri aurre egiteko gaitasun urria erakutsi dute kasu honetan. PARALIND-ek, ordea, hein-urritasuna bere gain hartzen du eta beraz, espektro antzekotasun handia dagoen kasuetan ere, ebazteko gaitasun egokia erakutsi du.

Tekniken alde praktikoaren inguruko ezaugarri batzuk bildu dira 7.3 taulan. Denborari dagokionez, erauzketak askoz azkarrago burutu ziren USAEME kasuan (18 min) SPME kasuan baino (45 eta 90 min). Izan ere, USAEME-k ahalbidetzen duen dispertsioaren ondorioz, kontaktu-azalera asko handitzen da eta masa-transferentzia askoz azkarrago egiten da. Horregatik denbora ia 5 aldiz gutxiagokoa izan zen USAEME erabiltzerakoan HPLC determinazioak konparatuz gero.

Metodoa aukeratzeko orduan kontuan hartu daitekeen beste puntu bat automatizatzeko aukera litzateke. Izan ere, lagin kopuru oso handia denean, eskuzko lana oso nekagarria eta luzea izan daiteke. Lan hauetan automatizaziorik egin ez bada ere, eskala handiagoko analisietan ezaugarri garrantzitsua da. SPME automatizatzeko zenbait gailu eskuragarri daude merkatuan eta beraz, behar bakoitzera egokitzen den sistema ezarri daiteke. USAEME-k, aldiz, eskuzko lan gehiagoren beharra eskatzen du. Pauso gehiago dituenez, bere automatizazioa konplexua izan daiteke. Halere, bestelako LPME teknikak automatizatzeko saiakerak egiten hasiak dira [Lee eta Lee, 2011] eta urte batzuen buruan gauzatu daiteke.

Lana eskuz eginez gero, ordea, USAEME tanta maneiatzea askoz errazagoa da SPME baino. Betiere, aldizka zenbait analisi oker egin daitezke, eta tanta erabiliz gero, tanta berri bat erabiltzeak ez du inolako arazorik ekartzen. SPME-rekin zailagoa izan liteke: SPME zuntzak oso sentikorrak dira, eta kontu handiz erabili behar dira apurtu nahi ez badira. Ahalik eta kontu handienarekin erabilita ere, zuntzak osorik edo partzialki puskatzea erraz gerta liteke, eta horrek eragin zuzena izango du analisian. Zuntz berri bat beharko litzateke, horrek dakartzan ondorioekin; alde batetik, zuntz bakoitzaren prezio garestia, eta bestetik, zuntz ezberdinez egindako analisiek (*batch-to batch reproducibility*) errepikakortasunean eragin dezakete.

USAEME erabiliz gero, une oro tanta berri bat erabiltzen denez, ez dago horrelako arazorik. Gainera, USAEME-k ez du egokitze etaparik behar, ezta garbiketarik ere SPME zuntzek behar duten bezala. SPME kasuan, analisia kaltetuko duten aurrez eginiko erauzketen arrastoak ala gatz kristalizazio arazoak ager daitezke. Horregatik beharrezkoa da garbiketa bat egitea erauzketa bakoitzaren ondoren.

SPME-k interfase baten beharra du HPLC-ra akoplatzerako orduan eta USAEME-k aldiz ez du gailu berezirik behar, xiringa batekin ohiko HPLC injektore batean sartu baitaiteke.

7.3.Taula *Lan honetan garatutako mikroerauzketa metodoen alderdi praktiko batzuen konparazio taula.*

Ezaugarriak	**SPME**		**USAEME-SFO**
Determinazioa	**GC/ECD**	**HPLC/DAD**	**HPLC/DAD**
Erauzketa denbora (min)	45	90	18
Automatizatzeko aukera	Bai		Ez
Zailtasun maila	Altua		Ertaina
Analisi arteko ezberdintasunak	Zuntzen arteko ezberdintasunak		Ez (aldiro tanta berri bat erabili da)
Garbiketa etapa	Bai		Ez
Egokitzea	Bai		Ez
Interfaseen beharra	Ator berezia	Bai	Ez
Material erauztailea	PDMS/DVB		Undekanola
Kostua (€)	398.50 (3 zuntzeko kaxa)		39.80 (100 g botila/120.5mL)
Pakete edo botilako erauzketa kopurua	150 (~50 zuntz bakoitzeko)	180 (~60 zuntz bakoitzeko)	~2300 (botilako)
Erauzketa kostua (€)*	2.66	2.21	0.02

* Erauzketa kostuak bakarrik hartu dira kontuan, determinazio kromatografikoaren kostuak ez.

Azkenik, SPME teknika erabiltzerakoan PDMS/DVB zuntzak erabili ziren. Hiru unitateko zuntz kaxa bakoitzaren salneurria 398.50 €-koa izan zen (azken aldiz 2015ko urtarrilean ikusia), beraz, zuntz bakoitzak ia 133 €-ko kostua duela esan daiteke. Garatutako metodoan, zuntzak likidotan murgilduta erabili direla aintzat hartuz, zuntzek 60 erauzketa inguru iraun dute HPLC kasuan eta 50 inguru GC kasuan, beraz,

erauzketa bakoitzaren prezioa 2.21€ eta 2.66 € bitartekoa dela esan daiteke. Bestalde, USAEME teknika erabiltzerakoan, undekanola erabili zen. Botila bakoitzak 39.80 € balio zuen (100 g-ko botila = 120.5 mL), eta metodoan erauzketa bakoitzeko 50 µL erabiltzen zirela kontuan hartuz, botila bakoitzarekin 2000 analisi baina gehiago egin daitezke. Beraz, erauzketa bakoitzeko kostua erauztaileari dagokionez, 0.02 € ingurukoa litzateke, SPME-ren prezioarekin konparatuz 100 aldiz txikiagoa da.

7.2 *SPME eta USAEME metodoekin lortutako ezaugarri analitikoen konparazioa argitaratutako beste lan batzuekin*

Oso erabiliak izan dira teknika hauek pestizida ezberdinen determinaziorako, ondorengo tauletan (7.4, 7.5 eta 7.6) matrize ezberdinetan triazolen determinazioetarako proposatutako metodo ezberdinak adierazten dira. 7.4 taulan ur laginetan triazolen determinaziorako garatu diren beste lanen ezaugarriak ikus daitezke; 7.5 taulan fruitu matrizeetan determinatutako metodoak ageri dira eta azkenik, 7.6 tauletan triazolak determinatu diren beste metodoak bestelako matrize likido konplexuagotan. Bibliografian lortutako metodo horietatik triazol zehatzen datuak jaso dira tauletan, lan honetan landu diren analitoenak hain zuzen ere. Lan horiek beste analito batzuk determinatzen badituzte ere, ez dira taula horietan adieraziko.

Ikus daitekeen bezala batez ere azken 5 urteotan metodo ugari garatu dira triazolen determinaziorako, eta azkeneko urte hauetako metodoetan batez ere likido mikroerauzketan oinarritutako teknika ezberdinak nabarmendu daitezke. Analitoak determinatzeko oro har, GC eta HPLC erabili dira, mota guztietako detektagailuak erabiliz: MS, DAD, UV, FID, ECD, NPD, besteak beste. Detektagailu hauek, sentsibilitate oso ezberdinak izan ditzakete analitoekiko eta horrek eragin zuzena izango du ezaugarri analitikoekin. Halere, badirudi, HPLC determinazioa sarriago erabili dela analito hauen analisirako GC baino.

Detekzio-muga era ezberdinetan kalkula daiteke, lan honetan kalibrazio zuzena erabili da, eta seinale/soinu (S/N) erlazioa hiru hautatu da. Beste lan gehienek ere S/N=3 erabili dute, baina kasu askotan ez dute adierazten. HPLC erabili duten

metodoetan detekzio-mugei dagokienez, hiru ikerketa lirateke azpimarratu beharrekoak [Tang *et al.*, 2010; Wang *et al.*, 2011a; Wang *et al.*, 2012], lortutako balioak oso baxuak baitira (0.005-0.1 μg L^{-1} bitartekoak) (ikus 7.1 eta 7.4 taulak). GC erabili den kasuetan baliorik onenak DLLME-GC/NPD erabiliz lortu ziren [Farajzadeh *et al.*, 2014]. Gainerako balioak lan honetan garatutako metodoekin lortutako balioen antzekoak edo okerragoak dira.

Linealtasun tartea hautatzerakoan detektagailua naiz mikroerauzketa teknikak dute garrantzia. Oro har, likido mikroerauzketen kasuan tarte zabalagoak lor daitezke, erauztailearen bolumenak zerikusi handia baitu. SPME kasuan zuntzaren bolumena mugatua da, baina LPME kasuetan mikrolitro bolumenak tartea handitzea baimendu dezake.

Desbideratze estandar erlatiboak (% RSD) ia kasu guztietan %10 baino txikiagoak dira, SPME-rekin lortutako desbideratze estandarrak, ordea, kasu batzuetan balio hori gaindi dezake. Esaterako DI-SPME-GC/ToF/MS erabiltzerakoan lortutako balioak %4 eta %20 bitartekoak izan ziren [Souza-Silva *et al.*, 2013]. Lehendik ere, lan honetan garatutako metodoetan ikusi da SPME-z determinatutako metodoetan errepikakortasuna likido mikroerauzketan oinarritutako errepikakortasunean baino balio okerragoak lortzen zirela. Baina esan daiteke antzeko balioak lortu direla lan honetan eta beste lanetan.

Berreskuratze-saioetan lortutako emaitzetan ere tarte zabalak ikus daitezke. Ehunekorik baxuena %45ean lortzen da, behi-esnean egindako analisietan [Farajzadeh *et al.*, 2011b]. Matrizea konplexua izanik, berreskuratzeak okerragoak izan ohi dira (ikus 7.6 taula). Lan hori kanpoan utzita, berreskuratze baxuena %70-ean aurki dezakegu [Souza-Silva *et al.*, 2013], altuena berriz %120-koa da [Celeiro *et al.*, 2014]. Balio hauek fruitu matrizeetan lortu dira, baina ur matrizeetako berreskuratzeak bakarrik aintzakotzat hartuz, balioak %74 [Farajzadeh *et al.*, 2012] eta %110 [Wang *et al.*, 2011a] bitartekoak dira. 7.1. taulan ikus daitekeen moduan, lan honetan lortutako balioak %82 eta %123.4 bitartekoak dira. Kontuan izanik, berreskuratze hauek fruitu laginetan egin direla, bibliografian lortutako balioekin konparagarriak dira.

Azkenik, aberastasun-faktorea konparatu da likido mikroerauzketetan oinarritutako tekniketan. Tesi honetan garatu den USAEME metodoaz lortutako balioak 226-255 bitartekoak dira. Oro har, DLLME laguntzen duten beste metodoekin konparatuz gero (SBSE-DLLME, SEV-DLLME, AALLME esaterako) balio zertxobait

txikiagoak lortu dira tesi honetan, baina maila berdinekoak kontsidera daitezke. Batez ere deigarria dena DLLME-GC/NPD bidez lortutako balioak dira [Farajzadeh *et al.*, 2014]: 1943 eta 1988 bitartekoak.

Oro har, tesi honetan gauzatu diren metodoen emaitzak bibliografian ageri diren emaitzekin konparagarriak dira. Ezaugarri analitiko gehienak maila bertsukoak dira, salbuespenak salbuespen. Detekzio-mugak salbuespen batzuk dituen arren, lan honetako balioak maila bertsukoak dira. Errepikakortasun balioak (RSD ehunekoek adierazia) hobexeagoak dira likido mikroerauzketetan solido faseko mikroerauzketetan baino. Berreskuratze ehunekoak matrize motaren araberakoak dira, matrize sinpleetan (ura) emaitza hobeagoak lortzen dira, matrize konplexuagoetan baino (behi-esnea).

Ondorioz, lan honetan garatutako SPME eta USAEME metodoen emaitzak antzekoak direla esan daiteke, baina USAEME-k abantaila garrantzitsu batzuk eskaintzen ditu SPME-ren aurrean: maneiatzeko errazagoa da eta bere kostu baxuagoak aukera interesgarria egiten du mikroerauzketa teknika ezberdinen artean.

7.4.Taula. *Triazolen determinaziorako ur laginetan garatutako beste metodoen ezaugarri analitikoak.*

Metodoa	**Matrizea**	**Analitoak**	**Linealtasuna ($\mu g\ L^{-1}$)**	**LOD[a] ($\mu g\ L^{-1}$)**	**RSD[b] (%)**	**EF[c]**	**Berreskuratzea (%)**	**Erreferentzia**
IL-DLLME-HPLC/DAD	Ura	TB	122-6830	3.9*	2.5	-	93	[Ravelo-Pérez *et al.*, 2009]
CPE-HPLC/UV	Ura	D-TB	0.05-20	0.02-0.03*	4.2-5.3	> 60	93-95	[Tang *et al.*, 2010]
DLLME-SFO-HPLC/DAD	Ura	M-TB	0.5-200	0.08-0.1*	4.3-5.7	190-289	86-110	[Wang *et al.*, 2011a]
DLLME-HPLC/UV	Ura	TB	2.0-100	1.2	2.8-5.3	82.8	90.6-105.3	[Ye *et al.*, 2012]
GBMNE-HPLC/UV	Ura	M-TB	0.05-50	0.005-0.01	4.2-6.6	-	86.0-100.8	[Wang *et al.*, 2012]
DLLME-GC/FID/MS	Ura	D-TB	2-5000	0.5-2.0	2-12	306-380	74-99	[Farajzadeh *et al.*, 2012]
DLLME-HPLC/DAD	Ura	TB	30-1500	19.8-25.4*	0.9-6.5	-	87.8-103.7	[Luo *et al.*, 2013]
ALLME-GC/FID	Ura	D-TB	2-3500	0.4-0.7	3-4	456-504	93-105	[Farajzadeh *et al.*, 2013]

OHARRA: Metodoen laburdurak laburdura zerrendan daude azalduak.

[a] LOD: Detekzio-muga; *S/N= 3-an oinarritua.

[b] RSD: Desbideratze estandar erlatiboa.

[c] EF: Aberastasun-faktorea.

7.5.Taula. *Triazolen determinaziorako fruitu matrizeetan garatutako beste metodoen ezaugarri analitikoak.*

Metodoa	**Matrizea**	**Analitoak**	**Linealtasuna ($\mu g\ L^{-1}$)**	**LOD[a] ($\mu g\ L^{-1}$)**	**RSD[b] (%)**	**EF[c]**	**Berreskuratzea (%)**	**Erreferentzia**
SBSE-DLLME-GC/FID	Ura Zukuak	D-TB	10-50000	1.3-1.6*	-	690-710	72-108	[Farajzadeh *et al.*, 2010]
SEV-DLLME-GC/FID	Ura Zukuak	D-TB	0.5-2000	0.14*	3.4	482 (TB)	86-103	[Farajzadeh *et al.*, 2011a]
HF-LPME-GC/MS	Ura Mahats zukua	D	1-5000	0.4	9.0	173.9	83.1-118.9	[Sarafraz-Yazdi *et al.*, 2012]
DI-SPME-GC/ToF/MS	Mahatsa Marrubiak	M-TB-FS-D	0.25-1000	0.025-5**	4-20	-	70-120	[Souza-Silva *et al.*, 2013]
AALLME-GC/FID	Ura, luzokerra, tomatea, mahats zukua	D-TB	2-750	0.56-0.59*	3-6	713-782	100-109	[Farajzadeh eta Khoshmaram, 2013]
UAE + PLE-GC/TQ/MS	Mahatsa	TB	2-1000	0.44-22.8*	5.8-9.6	-	91-120	[Celeiro *et al.*, 2014]
USAEMME-GC/FID	Udare, sagar eta mahats zukuak	M-TB	5-500	1.77-2.26*	3.9-7.7	183-209	91.6-105	[Li *et al.*, 2014]

OHARRA: Metodoen laburdurak laburdura zerrendan daude azalduak.

[a] LOD: Detekzio-muga; *S/N= 3-an oinarritua,** LOQ: Kuantifikazio-muga.

[b] RSD: Desbideratze estandar erlatiboa.

[c] EF: Aberastasun-faktorea.

7.6.Taula. *Triazolen determinaziorako beste matrize likido konplexuagotan garatutako metodoen ezaugarri analitikoak.*

Metodoa	**Matrizea**	**Analitoak**	**Linealtasuna ($\mu g\ L^{-1}$)**	**LOD[a] ($\mu g\ L^{-1}$)**	**RSD[b] (%)**	**EF[c]**	**Berreskuratzea (%)**	**Erreferentzia**
IL-DLLME-HPLC/DAD	Ardoa	D	50-2000	9.7*	5.2-9.2	-	74.6-83.5	[Wang *et al.*, 2011b]
DLLME-GC-FID/MS	Behi-esnea	TB	50-80000	11*	3.6-6.0	156-380	45-96	[Farajzadeh *et al.*, 2011b]
IL-DLLME-HPLC/DAD	Arratoi odola	M-TB-D	6-500	4-6**	3.0-6.5	178-197	88.9-98.5	[Li *et al.*, 2013]
DLLME-GC/NPD	Eztia	D-TB	0.1-45	0.03-0.045*	4-6	1943-1988	97-99	[Farajzadeh *et al.*, 2014]

OHARRA: Metodoen laburdurak laburdura zerrendan daude azalduak.

[a] LOD: Detekzio-muga; *S/N= 3-an oinarritua,** LOQ: Kuantifikazio-muga.

[b] RSD: Desbideratze estandar erlatiboa.

[c] EF: Aberastasun-faktorea.

7.3 Erreferentziak

Arthur, C.L. eta Pawliszyn, J.; *Solid Phase Microextraction with Thermal Desorption Using Fused Silica Optical Fibers.* **1990.** Analytical Chemistry, 62, 2145-2148.

Bordagaray, A., García-Arrona, R. eta Millán, E.; *Optimization of Solid-Phase Microextraction Procedure Coupled to GC-ECD for Triazole Fungicides Determination in Juice Samples.* **2011.** Food Analytical Methods, 4(3), 293-299.

Bordagaray, A., García-Arrona, R. eta Millán, E.; *Development and application of a screening method for triazole fungicide determination in liquid and fruit samples using solid-phase microextraction and HPLC-DAD.* **2013.** Analytical Methods, 5(10), 2565-2571.

Bordagaray, A., García-Arrona, R. eta Millán, E.; *Determination of Triazole Fungicides in Liquid Samples Using Ultrasound-Assisted Emulsification Microextraction with Solidification of Floating Organic Droplet Followed by Hihg-Performance Liquid Chromatography.* **2014.** Food Analytical Methods, 7(6), 1195-1203.

Celeiro, M., Llompart, M., Lamas, J.P., Lores, M., Garcia-Jares, C. eta Dagnac, T.; *Determination of fungicides in white grape bagasse by pressurized liquid extraction and gas chromatography tandem mass spectrometry.* **2014.** Journal of Chromatography A, 1343, 18-25.

Farajzadeh, M.A., Bahram, M., Jafary, F. eta Bamorowat, M.; *Combination of Extraction by Silylated Vessel-Dispersive Liquid–Liquid Microextraction as a High-Enrichment Factor Technique: Optimization and Application in Preconcentration of Some Triazole Pesticides from Aqueous Samples Followed by GC-FID Determination.* **2011a.** Chromatographia, 73(3), 393-401.

Farajzadeh, M.A., Djozan, D. eta Khorram, P.; *Development of a new dispersive liquid–liquid microextraction method in a narrow-bore tube for preconcentration of triazole pesticides from aqueous samples.* **2012.** Analytica Chimica Acta, 713, 70-78.

Farajzadeh, M.A., Djozan, D., Mogaddam, M.R.A. eta Bamorowat, M.; *Extraction and preconcentration technique for triazole pesticides from cow milk using dispersive liquid-liquid microextraction followed by GC-FID and GC-MS determinations.* **2011b.** Journal of Separation Science, 34(11), 1309-1316.

Farajzadeh, M.A., Djozan, D., Nouri, N., Bamorowat, M. eta Shalamzari, M.S.; *Coupling stir bar sorptive extraction-dispersive liquid liquid microextraction for preconcentration of triazole pesticides from aqueous samples followed by GC-FID and GC-MS determinations.* **2010.** Journal of Separation Science, 33(12), 1816-1828.

Farajzadeh, M.A. eta Khoshmaram, L.; *Air-assisted liquid–liquid microextraction-gas chromatography-flame ionisation detection: A fast and simple method for the assessment of triazole pesticides residues in surface water, cucumber, tomato and grape juices samples.* **2013.** Food Chemistry, 141(3), 1881-1887.

Farajzadeh, M.A., Mogaddam, M.R.A. eta Aghdam, A.A.; *Comparison of air-agitated liquid–liquid microextraction technique and conventional dispersive liquid–liquid micro-extraction for determination of triazole pesticides in aqueous samples by gas chromatography with flame ionization detection.* **2013.** Journal of Chromatography A, 1300, 70-78.

Farajzadeh, M.A., Mogaddam, M.R.A. eta Ghorbanpour, H.; *Development of a new microextraction method based on elevated temperature dispersive liquid–liquid microextraction for determination of triazole pesticides residues in honey by gas chromatography-nitrogen phosphorus detection.* **2014.** Journal of Chromatography A, 1347, 8-16.

Jeannot, M.A. eta Cantwell, F.F.; *Solvent Microextraction into a Single Drop.* **1996.** Analytical Chemistry, 68(13), 2236-2240.

Lambropoulou, D.A. eta Albanis, T.A.; *Methods of sample preparation for determination of pesticide residues in food matrices by chromatography–mass spectrometry-based techniques: a review.* **2007.** Analytical and Bioanalytical Chemistry, 389(6), 1663-1683.

Lee, J. eta Lee, H.K.; *Fully Automated Dynamic In-Syringe Liquid-Phase Microextraction and On-Column Derivatization of Carbamate Pesticides with Gas Chromatography/Mass Spectrometric Analysis.* **2011.** Analytical Chemistry, 83, 6856-6861.

Li, Y., Yang, X., Zhang, J., Li, M., Zhao, X., Yuan, K., Li, X., Lu, R., Zhou, W. eta Gao, H.; *Ultrasound-assisted emulsification magnetic microextraction: a fast and green method for the determination of triazole fungicides in fruit juice.* **2014.** Analytical Methods, 6, 8328-8336.

Li, Y., Zhang, J., Peng, B., Li, S., Gao, H. eta Zhou, W.; *Determination of triazole pesticides in rat blood by the combination of ultrasound-enhanced temperature-controlled ionic liquid dispersive liquid-liquid microextraction coupled to high-performance liquid chromatography.* **2013.** Analytical Methods, 5(9), 2241-2248.

Luo, M., Liu, D., Zhou, Z. eta Wang, P.; *A New Chiral Residue Analysis Method for Triazole Fungicides in Water Using Dispersive Liquid-Liquid Microextraction (DLLME).* **2013.** Chirality, 25(9), 567-574.

Pinto, M.I., Sontag, G., Bernardino, R.J. eta Noronha, J.P.; *Pesticides in water and the performance of the liquid-phase microextraction based techniques. A review.* **2010.** Microchemical Journal, 96(2), 225-237.

Psillakis, E. eta Kalogerakis, N.; *Developments in liquid-phase microextraction.* **2003.** Trends in Analytical Chemistry, 22(9), 565-574.

Ravelo-Pérez, L.M., Hernández-Borges, J., Asensio-Ramos, M. eta Rodríguez-Delgado, M.A.; *Ionic liquid based dispersive liquid–liquid microextraction for the extraction of pesticides from bananas.* **2009.** Journal of Chromatography A, 1216(43), 7336-7345.

Sarafraz-Yazdi, A. eta Amiri, A.; *Liquid-phase microextraction.* **2010.** Trends in Analytical Chemistry, 29(1), 1-14.

Sarafraz-Yazdi, A., Assadi, H. eta Ibrahim, W.A.W.; *Determination of Triazole Fungicides Using Hollow Fiber Liquid Phase Microextraction Prior to Gas Chromatography–Mass Spectrometry Analysis.* **2012.** Industrial & Engineering Chemistry Research, 51(7), 3101-3107.

Souza-Silva, E.A., Lopez-Avila, V. eta Pawliszyn, J.; *Fast and robust direct immersion solid phase microextraction coupled with gas chromatography–time-of-flight mass spectrometry method employing a matrix compatible fiber for determination of triazole fungicides in fruits.* **2013.** Journal of Chromatography A, 1313, 139-146.

Tang, T., Qian, K., Shi, T., Wang, F., Li, J. eta Cao, Y.; *Determination of triazole fungicides in environmental water samples by high performance liquid chromatography with cloud point extraction using polyethylene glycol 600 monooleate.* **2010.** Analytica Chimica Acta, 680(1-2), 26-31.

Wang, C., Wu, Q., Wu, C. eta Wang, Z.; *Application of dispersion–solidification liquid–liquid microextraction for the determination of triazole fungicides in environmental water samples by high-performance liquid chromatography.* **2011a.** Journal of Hazardous Materials, 185(1), 71-76.

Wang, S., Ren, L., Xu, Y. eta Liu, F.; *Application of ultrasound-assisted ionic liquid dispersive liquid-phase microextraction followed high-performance liquid chromatography for the determination of fungicides in red wine.* **2011b.** Michrochimica Acta, 173, 453-457.

Wang, W., Ma, X., Wu, Q., Wang, C., Zang, X. eta Wang, Z.; *The use of graphene-based magnetic nanoparticles as adsorbent for the extraction of triazole fungicides from environmental water.* **2012.** Journal of Separation Science, 35(17), 2266-2272.

Ye, C., Liu, Q., Wang, Z. eta Fan, J.; *Dispersive liquid-liquid microextraction combined with high-performance liquid chromatography-ultraviolet detection for the determination of three triazole derivatives in environmental water samples.* **2012.** International Journal of Environmental Analytical Chemistry, 92(10), 1176-1186.

8. Ondorioak

8. Ondorioak

Jarritako helburuekin eta tesi honetan lortutako emaitzekin ondorengoa ondoriozta daiteke:

1) <u>Triazolen determinaziorako mikroerauzketa prozeduren garapena</u>

Fase solidoko mikroerauzketa eta fase likidoko mikroerauzketa tekniketan oinarritutako prozedurak erabili eta garatu ziren lan honetan. Teknika hauek analitoak isolatu eta prekontzentratzen dituzte matrize mota ezberdinetatik. Murgiltze zuzena erabili zen SPME kasuan eta ultrasonikazioaren erabilera aukeratu zen LPME teknika ezberdinen artean tanta organikoa dispertsatzeko zuen gaitasunagatik, izan ere, dispertsio horrek masa transferentzia abiadura handiagotzen baitu.

SPME-GC/ECD prozeduran (3. ATALBURUA) aztertutako lau analitotik hiruentzat (tetrakonazola, miklobutanil eta dinikonazola) emaitza egokiak lortu ziren. Tebukonazola, ordea, ez zen metodoarekin oso sentikorra. Halere, prozedurak sinpletasuna eta lagin tratamendu urrien erabilera eskaintzen zuen. Kimika berdearen ikuspuntutik babestutako metodoa dela esan daiteke, disolbatzaile organikorik ez baita erabiltzen prozesu osoan.

Bolumen handiko analitoekin erlazionatutako arazoei aurre egiteko SPME-HPLC/DAD prozedurarekin (4. ATALBURUA) eman zitzaion jarraipena lanari. Analitoak oso lurrunkorrak ez zirenez, HPLC/DAD determinazioa GC/ECD baino egokiagoa zela iritzi zen. Bi analito gehiago gehitu ziren lanaren zati honetan (flusilazola eta flukinkonazola). Ezaugarri analitikoei dagokienez, emaitza onak lortu ziren, nahiz eta gailur kromatografikoen bereizmen osoa ez lortu.

Baina SPME-ren desabantaila batzuk nabarmenak ziren, hala nola, kostua eta zuntzen hauskortasuna, batez ere murgiltze zuzena erabiltzen zenean. Hutsune hauek betetzeko likido faseko mikroerauzketa tekniken

erabilera proposatu zen, zehazki ultrasoinuz lagundutako mikroerauzketa. USAEME-k erauzketa azkarrak bermatzen zituen, gainazal azalera handiak transferentzia abiadura handiagotzen baitzuen.

USAEME-SFO-HPLC/DAD prozedura (5. ATALBURUA) garatu zen beraz. Lortutako emaitzak onak izan ziren analito guztientzat, batez ere errepikakortasunari zegozkion datuak. Analisi bakoitza erauztaile tanta berri batez hasten zenez, hondakin arrastorik ez zegoen, ezta zuntzaren zahartzeak zekartzan arazorik ere. Gainera USAEME-rekin lan egitea errazagoa eta erosoagoa izan zen SPME-rekin baino.

2) GC eta HPLC kromatografia teknika ezberdinen erabilera zabala

Gas kromatografia (GC) eta bereizmen handiko likido kromatografia (HPLC) analitoen banaketa eta determinaziorako ohiko teknikak dira. Lan honetan HPLC-ko banaketa baldintzatzen zuten zenbait parametro aztertu ziren, adibidez, eluitzailearen konposizio eta fluxua, disolbatzaile gradienteak etab. Banaketa baldintza hoberenak lortu ziren aztertutako parametroekin. Betiere kontuan izanda SPME-k disolbatzaile organiko batzuen erabilera muga zezakeela.

GC-rekin elektroi harrapaketa detektagailua (ECD) eta HPLC–rekin diodo lerrokatuen detektagailua (DAD) erabili ziren. ECD-k emaitza onak eman zituen triazol pestizidentzat, beren atomo halogenatuagatik. Bestalde DAD-k hiru dimentsioko datu matrizeak eskaintzen zituen, eta beraz, kuantifikaziorako uhin-luzera bakar bat baino gehiago hautatzeko aukera eskaintzen zuen, UV espektroa erabili zitekeen analitoen identifikaziorako eta bigarren mailako abantaila erabili zen azterketa kimiometrikoak egiteko gainezarpen arazoak dauden kasuetan 6. ATALBURUAN bezala.

Aipatutako detektagailuez hornitutako instrumentu horiek ohiko laborategientzat eskuragarria den zentzuzko kostua dute. Horregatik, beraien erabilera aplikazio ezberdinetan ikus daiteke errutinako analisi moduan.

Geroago, GC/MS eta LC/MS bezalako instrumentu sentikorragoak ere erabil daitezke berrespen metodo gisara.

3) Metodo kimiometrikoen erabilera

- **Diseinu esperimentala**

Mikroerauzketa prozesuak eragiten dituzten aldagai esperimental asko aurki daitezke. Hori horrela, aldagai esanguratsuen aukeraketa eta balio optimoen bilaketa eginkizun garrantzitsua izan zen. Diseinu esperimentalak erabilerak bi eginkizunak bete zituen, aldagai esanguratsuak aurkitu eta baita baldintza esperimental hoberenak ere. Miaketarako diseinu faktorial zatikatuak erabili ziren (3 eta 4 ATALBURUAK) eta diseinu konposatu zentralak optimizaziorako (3, 4 eta 5 ATALBURUAK). Horrez gain, desiragarritasun erantzun azalerak aztertu ziren aukeratutako aldagaien erantzun global moduan.

SPME prozeduretan, miaketa diseinuak aplikatu ondoren aldagai kualitatiboak eta esanguratsuak ez ziren aldagai kuantitatiboen baliorik mesedegarrienak finkatu ziren. Erauzketa denbora eta tenperaturak optimizaziorako aldagaitzat hartu ziren eta SPME-HPLC/DAD kasuan gatz gehikuntza ere diseinuan gehitu zen. Bi metodoetan desiragarritasun funtzio globala aplikatu ondoren 60^{o}C-ko erauzketa tenperatura eta denbora luzeak aukeratu ziren (45 min SPME-GC/ECD metodoan eta 90 min SPME-HPLC/DAD prozeduran). Gehitutako NaCl kopurua 180 g L^{-1} balioan kokatu zen, balio optimotik gertu. Izan ere, gehiegizko gatz kantitateak zuntzaren estaldura kaltetu zezakeen.

USAEME-SFO-HPLC/DAD prozeduran erauzketa tenperatura eta denbora eta NaCl gehikuntza aztertu ziren optimizazio diseinuan. Emaitzak aztertu ondoren, baldintza esperimental hoberenak 30^{o}C, 18 min eta 250 g L^{-1} NaCl gehikuntzan finkatu ziren.

- **Aldagai anitzeko analisia**

Metodologia honek kromatografian gerta daitezkeen gainezarpen arazoak konpon ditzake. SPME-HPLC/DAD prozeduran gertatzen den bezala, tetrakonazol, miklobutanil, flusilazol, flukinkonazol eta tebukonazol analitoen bereizmena ez zen izan guztiz osoa. Horretarako, PARAFAC2, MCR eta PARALIND algoritmoak erabili ziren eta beraiekin lortutako emaitzak konparatu ziren (6. ATALBURUA). PARAFAC2 eta MCR bigarren mailako datuak ebazteko gehien erabilitako algoritmoak dira, baina kasu honetan ez zuten emaitza onik eman. PARALIND-ek hein-urritasun arazoak bere gain hartzen ditu eta gainezarritako gailurren arazoa konpontzeko gaitasuna erakutsi zuen azaldutako kasuan ikusi bezala. PARALIND-ekin eraikitako modeloek emaitza kuantitatibo onak eman zituzten, PARAFAC2 eta MCR-k porrot egin zuten bitartean, batez ere miklobutanil eta tebukonazol analitoei dagozkien gailurretan, non beraien espektroek antzekotasun handia erakusten zuten.

4) Tekniken aplikazioa fruitu eta likido laginetan

Garatutako metodoak sagar eta mahats laginetan aplikatu ziren. SPME-GC/ECD prozeduran (3. ATALBURUA) fruitu zukuak erabili ziren eta triazol pestizida arrasto batzuk aurkitu ziren. SPME-HPLC/DAD metodoa (4. ATALBURUA) fruitu eta likido lagin ezberdinetan aplikatu zen. Mahats eta sagar arbolei pestizidak zituzten produktu komertzialekin tratamendua eman zitzaien aurretik eta lagin ezberdinak jaso ziren hurrengo bi asteetan zehar. Horrez gain, euri laginak ere jaso ziren. Azterketa horrek erakutsitakoaren arabera, produktu hauek azaleko tratamenduak ziren, izan ere pestizida arrastoak fruituen azalean eta euri laginetan bakarrik aurkitu baitziren. USAEME-SFO-HPLC/DAD (5. ATALBURUA) metodoan fruitu zuku ezberdinak erabili ziren berreskuratze-frogetan. USAEME prozedurak bere egokitasuna erakutsi zuen fruitu zukuak aztertzerakoan, baina edari alkoholdunekin ez zuen portaera ona eduki.

Laburtuz, mikroerauzketa teknikek triazol pestiziden determinaziorako gaitasun ona erakutsi dute fruitu eta likido laginetan. Kimiometriak eskaintzen dituen erremintak, prozedura horietan emaitzak hobetzea eragin dezakete. Mikroerauzketa teknika ezberdinen artean, zailtasun eta kostua kontuan hartuz, USAEME-k abantaila praktiko gehiago eskaintzen ditu, nahiz eta matrize konplexu batzuetan hain egokia ez dela ikusi.

ARGITALPENAK

ARTIKULU ZIENTIFIKOAK

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Optimization of solid-phase microextraction procedure coupled to GC-ECD for triazole fungicides determination in juice samples.* **2011**. Food Analytical Methods, 4,293–299.

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Development and application of a screening method for triazole fungicide determination in liquid and fruit samples using solid-phase microextraction and HPLC-DAD.* **2013**. Analytical Methods, 5, 2565-2571.

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Determination of triazole fungicides in liquid samples using ultrasound-assisted emulsification microextraction with solidification of floating organic droplet followed by high-performance liquid chromatography.* **2014**. Food Analytical Methods, 7,1195–1203.

Bordagaray, Ane, Amigo, José Manuel, *Modelling highly co-eluted peaks of analytes with high spectral similarity*. **(TrAC, Trends in Analytical Chemistry aldizkarian onartua)**

KONGRESU ETA BILTZARRETAKO EKARPENAK

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Experimental design application for triazole fungicides determination in food liquid samples by solid-phase microextraction and GC/ECD*. VII Colloquium Chemiometricum Mediterraneum, Granada (Espainia), **2010ko ekainaren 21-24**.

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Triazole fungicides determination using Solid- Phase Microextraction (SPME)*. Cross Border Doctorials, Angelu (Frantzia), **2011ko urriaren 17-21**.

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda, Ostra, Miren; *Multi-way chemometric methods for the determination of triazole fungicides in environmental samples by SPME-HPLC-DAD*. XIII. Chemometrics in Analytical Chemistry, Budapest (Hungaria), **2012ko ekainaren 25-29**.

Bordagaray, Ane, García-Arrona, Rosa, Millán, Esmeralda; *Determination of triazole compounds in liquid samples using ultrasound-assisted liquid phase microextraction with solidification of floating droplet (USALPME-SFO) followed by HPLC-DAD*, XVII. Euroanalysis, Varsovia (Polonia), **2013ko abuztuaren 25-29**.