Determination of food colorants in food matrices by microemulsion electrokinetic capillary chromatography

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Abstract

Color additives are widely used in food industry to confer a desirable appearance to edible products. Some of the most used colorants (Tartrazine (E102), Sunset Yellow (E110), Red Allure (E129) and Blue Brilliant (E133)) are determined in this study by microemulsion electrokinetic capillary chromatography (MEECK). The method has been validated regarding linearity, RSD %, LOD and LOQ and recoveries in all analyzed samples. Regression coefficients were higher than 0.9981 for all linear ranges, intra and inter-day precisions were less than 7.01 % and 8.55 % respectively and recoveries were between 90 and 100% in almost all the cases. LODs and LOQs were ranged from 0.24 to 1.21 mg L^{-1} and from 0.80 to 4.03 mg L^{-1} respectively. Proposed method is considered suitable for the determination of colorants in food analysis in order to confirm their correct usage regulated by EU.

Keywords: MEECK; colorants; food; method development

1. Introduction

Food additives are defined by the *Codex Alimentarius* as “any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing,

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packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods." (World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO),). The aim of the addition of colorants to the food relies on modifying sensory properties and conservative properties as well as on making the comestibles more attractive to the consumers.

The use of additives has been increased since nowadays more elaborated products are consumed by the society. Those elaborated products need to be attractive for the consumers, thus the use of substances improving conservation, texture, smell, flavor and appearance is a common practice in the food industry (Martins, Roriz, Morales, Barros, & Ferreira, 2016). Color additives, according to Amchova *et al.* (Amchova, Kotolova, & Ruda-Kucerova, 2015), are used for the (i) the compensation of color loss due to exposure to light, air, temperature and storage conditions; (ii) enhancement of natural colors to make the food more attractive; (iii) provision of color to colorless foodstuff or (iv) to allow identification by consumers of products on sight, especially drugs.

Food additives are classified by the European Union (EU) in four groups (Commission regulation (EU) Nº 1129/2011, 12.11.2011): additives in general (group I), food colors authorized in *quantum satis* (group II), food colors with combined maximum limit (group III) and polyols (group IV). Apart from the doses defined by EU (Commission regulation (EU) Nº 1129/2011, 12.11.2011) which states the maximum allowed quantities that can be used in food industry, acceptable daily intake (ADI) is also defined for each additive, which is the amount that can be ingested on a daily basis without appreciable health risk. This ADI value is calculated regarding the No Observed Adverse Effect Level (NOAEL), which is corrected with a factor (usually 100) to extrapolate the differences between animals and humans as well as the interindividual variability (Lu, 1988).

Among others, the most used colorants are tartrazine (E102), sunset yellow (E110), red allure (E129) and blue brilliant (E133). Regarding EU classification, these substances are included in group III, which means that maximum limits are established. Those maximum limits are classified depending on the food categories. For ice-creams, candies, flavored drinks and other food supplements the sum of the group III additives should not exceed a limit that goes from

20 to 500 mg L^{-1} or mg kg^{-1} depending on the food category, and in most cases each colorant cannot be more than 50 mg L^{-1} or mg kg^{-1} (Commission regulation (EU) Nº 1129/2011, 12.11.2011). On the other hand, ADI values are 7.5 mg kg^{-1} per body weight (b.w.) for E102, 2.5 mg kg^{-1} b.w. for E110, 7 mg kg^{-1} b.w. for E129 and 12.5 mg kg^{-1} b.w. for E133 (World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), 2015).

Some of these additives have not shown prejudicial effects and their use is limited to an amount called *quantum satis* (which means, "add as much of this ingredient as is needed to achieve the desired result, but not more"). Other additives have shown effects in humans, being kids especially vulnerable. Firstly, because children are the group of people that consume most candies, especially rich in artificial colorants; secondly because ADI doses can be easily reached by children due to their lower weight and finally because the harmful effects are more pronounced in underage population. A study shows that some colorant mixtures (including E102, E110 and E129) have important effects on children behavior increasing the level of hyperactivity (McCann *et al.*, 2007).

Several methods have been developed to determine azo dyes. Some of them include extraction steps using solid-phase extraction (SPE) (Bonan, Fedrizzi, Menotta, & Elisabetta, 2013; Soylak, Unsal, & Tuzen, 2011); liquid-liquid extraction (LLE) (Yoshioka & Ichihashi, 2008; Zou, He, Yasen, & Li, 2013) including eco-friendly procedures without using organic solvents (Khanavi et al., 2012) and other approaches such us ultrasound-assisted extraction (UAE) (Shen, Zhang, Prinyawiwatkul, & Xu, 2014) or microwave-assisted extraction (Sun, Sun, Li, Zhang, & Ynag, 2013). Probably the most used technique for determination is HPLC coupled with UV, DAD or MS detectors (González, Gallego, & Valcárcel, 2003; Minioti, Sakellariou, & Thomaidis, 2007; Yoshioka & Ichihashi, 2008; Harp, Miranda-Bermudez, Baron, & Richard, 2012; Bonan et al., 2013; Wu et al., 2013; Zou et al., 2013; Shen et al., 2014). Spectrophotometric determinations can be also performed but matrix interferences must be resolved. This can be performed by isolating analytes before the measurements (e.g. with SPE and LLE procedures) or by using multivariate approaches with the obtained spectra (Dinç, Baydan, Kanbur, & Onur, 2002; Khani, Ghasemi, Shemirani, & Rahmanian, 2015; Lachenmeier & Kessler, 2008; López-de-Alba, Wróbel-Kaczmarczyk, Wróbel, López-Martínez, & Hernández, 1996).

Capillary electrophoresis (CE) has been used in the last years for the determination of colorants in many different matrices such as alcoholic beverages (Prado, Boas, Bronze, & Godoy, 2006), ice-cream (Del Giovine & Piccioli Bocca, 2003), milk beverages (Huang, Shih, & Chen, 2002) or other foodstuff (Pérez-Urquiza & Beltrán, 2000). CE is a separation technique based on the mobility of charged and uncharged molecules under an applied voltage. Apart from the applied voltage, a buffered background electrolyte (BGE) is used to lead analytes to detector, placed in the end of a capillary column.

When a surfactant such as sodium dodecyl sulphate (SDS) is added to the BGE a microemulsion pseudostationary phase is form and then the separation mechanism is due to the different partition of solutes into a micellar pseudophase. This is called microemulsion micellar electrokinetic capillary chromatography (MEECK) (Landers, 1994). Surfactant molecules form micelles that provide ionic and hydrophobic interaction sites and therefore the separation between uncharged and charged solutes is improved due to differences in migration behaviours. Electrophoretic mobility for uncharged molecules is negligible and separation in MEECK is only due to the partitioning of molecules between micelles and electrolyte solution (Ryan, Donegan, Power, & Altria, 2010). Therefore, neutral molecules can often be used as a marker for electroosmotic flow (EOF) velocity. Sometimes the use of additives can improve the separation. Some studies report that adding cyclodextrines (CD) allow analytes to interact with the interior of CD and the micelles enhancing resolution (Altria & McLean, 1998; Ryan, Altria, McEvoy, Donegan, & Power, 2013). Even enantiomeric separation can be carried out using CDs like MEECK additives (Abushoffa, Burjanadze, Blaschke, Crommen, & Chankvetadze, 2002; Ryan et al., 2013). β-CD and SDS can be both used in the buffer as pseudostationary phases; β-CD forms a complex with analytes whereas SDS form micelles with analytes in the nucleus. The complexation with β-CD and the partition of analytes into SDS micelles has effectively demonstrated to improve separation (Li, Chen, Liao, & Liu, 2006).

The aim of this work is to develop and validate a MEECK method able to separate and quantify a group of colorants (E102, E110, E129 and E133) used in different drinks, food colorants, candies and drug samples. The effect of different concentrations of buffer, surfactant and β-cyclodextrine were studied in order to obtain an efficient electrophoretic separation. The

developed method was applied to different colored foodstuff in order to check the compliance with the current regulations.

2. Experimental

2.1. Colorant standards

4-methyl-3-penten-2-one (Sigma-Aldrich) was used as the neutral marker to control the operation conditions. Tartrazine (E102, 87%), Sunset Yellow (E110, 87%), Red Allure (E129, 85%) and Blue Brilliant (E133, 85%) were acquired in Roha Epsa S.L. (Valencia, Spain). 1 g L^{-1} stock solutions were prepared and stored at 4ºC at dark. Under these conditions, stock solutions are kept stable for at least one month. Working solutions were daily prepared and filtered .

2.1. Background electrolyte solution for CE

A background electrolyte (BGE) for CE was daily prepared. The BGE was prepared using a disodium tetraborate (Borax) (Sigma-Aldrich, Madrid) solution adjusted to pH 10 with NaOH 0.1 M, sodium dodecyl sulphate (SDS) (Panreac, Barcelona) and β-cyclodextrine (β-CD) (Sigma-Aldrich, Madrid). BGE composition for the optimum separation of analytes had 12.5 mM Borax, 6 mM SDS and 5 mM β-CD. Once the BGE was prepared it was filtered with a 0.45 μm micropore filter before placed in the CE.

2.2. Equipment

All CE experiments were carried out using a 7100 Capillary Electrophoresis from Agilent Technologies (Morges, Switzerland) and the used software was 3D-CE 7100 System. The dimensions of the used capillary for separation were an inner diameter of 50 μm and a length of 40 cm with a high sensitivity light path (Agilent).

A conditioning step of the capillary was performed both at the beginning and at the end of the working day consisting on a cleaning with NaOH 1M for 600 s following by a rinsing with water for another 600 s. Air was also passed through the column to dry it afterwards. A short

cleaning step was set up before every analysis consisting in the use of NaOH 0.1 M for 150s, water for 120s and BGE for 120s. Longer cleaning steps were also carried out every 5-6 analysis.

Then the samples were injected for 10 s (50 mbar) and a voltage of 25 kV was used. The electropherogram was completed in 15 minutes taking into account the full procedure (cleaning plus the analysis time). The temperature was kept constant during all the analysis time at 25 ºC.

Although a 3D electropherogram (from 190 to 600 nm) was obtained for each analysis, the selected wavelengths for the determination of analytes were fixed at 210, 410, 430, 485 and 500 nm, corresponding to maximum absorbance of the colorants.

2.3. Samples

Different samples were acquired in local stores. Samples included liquid jellies (4, coded as CU, CG, CH and CN), ice-pops (2, coded as FU and FN), non-alcoholic beverages (1, coded as R), isotonic drinks (2, coded as P and IG) food liquid dyes (3, coded as VU, VH and VG) and solid dyes for paella (3, coded as Du, H, K). A children drug was also included in the study (coded as D). 16 samples were analyzed in total.

Sample pretreatment was kept as simple as possible. Dilutions were made when necessary and all the samples were filtered with a 0.45 μm micropore filter before analysis. Some samples were also centrifuged before filtration for 10 minutes (10000 rpm) and the supernatant was only analyzed.

3. Results and discussion

3.1. Separation optimization

The first step to develop a CE procedure is to get a good separation. For this aim, different levels of experimental parameters were tried as it can be seen in Table 1. Several compositions and concentrations for BGE components were tried to find the optimum conditions.

Different buffers were tried including borax and phosphate buffers adjusted at pH 10 and 11 respectively. Phosphate buffer produced a splitting for one analyte peak at different concentrations, so it was discarded. Increasing concentration of borax results in increasing migration times, but longer migration times did not resolve the strong overlapping of E110 and E129.

Consequently, SDS was added to try to separate the analytes, but the use of only SDS was not enough to separate all of them. E133 and E102 were separated from E110 and E129 easily, but it was difficult to resolve the latter due to their similar chemical structure. Therefore, the addition of β-CD was necessary. By increasing the concentration of β-CD, the separation of E110 and E129 was finally achieved. Moreover, the migration times slightly decreased with the addition of β-CD.

Temperature has also influence in the separation. Even minor changes can affect the separation, so it should be kept constant during the analysis. A slight change from 25 to 30 °C reduces analysis time in more than a minute but does not improve the separation.

Voltage is a major parameter to consider. Increasing voltage reduces considerably the migration times but the overlapping can be more pronounced. In the lowest applied voltage (10 kV), migration times were longer than 15 minutes.

After considering different variables, the best separation was obtained using a voltage of 25 kV, 25 °C, and the next composition for BGE: 12.5 mM Borax, 6 mM SDS and 5 mM β-CD. Under these conditions, a good separation for all the colorants was achieved as it can be shown in the electropherogram (Figure 1). The total time for each analysis was less than 9 minutes.

3.2. Area normalization

Capillaries used for CE have a light path at the final section of the capillary. This window is where the analytes are detected by a DAD. Since the detection is performed into the capillary, the different mobility of the analytes along the separation plays a major role in the quantification. Ideally, the peak area should be only proportional to the analyte concentration, but differences in the migration velocity of the solutes also influence on the area. When the analyte moves faster,

184 both the migration time and the residence time in the detector are shorter, and the measured area
185 will be lessened. Therefore, the area (A) of each analyte can be corrected or normalized (A_{norm})
186 with the migration time ($t_{migration}$) using the following equation (Altria, 1993):

187 $$A_{norm} = \frac{A}{t_{migration}}$$

188 For the studied analytes of this work, the relative standard deviation (RSD %) in the
189 migration times ranged from 1.4 % (for E102) to 17.9 % (for E110). The area normalization
190 improved significantly the calibration linearity as well as the reproducibility, especially in the
191 sample analysis.

192 ## 3.3. Method validation

193 Figures of merit of the procedure were calculated in order to validate the method. Two
194 concentration ranges were considered for calibration due to different colorant contents in the
195 analyzed samples. Results are shown in Table 2. Calibration curves with values over 0.99 were
196 obtained for each analyte in both ranges.

197 Intra and inter-day precision was checked with replicate measurements in two different
198 levels, one corresponding to a value in the low range (L1 ~15 mg L^{-1}) and one to the high range
199 (L4 ~150 mg L^{-1}). Nine replicas were performed to calculate the relative standard deviation (RSD
200 %). For intra-day data, nine measurements were done in the same day, whereas for inter-day
201 data the measurements were performed in three different days during two weeks. As it can be
202 seen in Table 2 all RSD values are lower than 10 % but in most cases are below 5 %.

203 Limits of detection and quantification (LOD and LOQ) are calculated according to
204 Konieczka (Konieczka & Namieśnik, 2009). In the proposed method, a low concentration (c_{min})
205 close to an expected LOD is measured several times (n = 10) and the standard deviation is
206 calculated. This standard deviation is multiplied by a factor to calculate both the LOD and the
207 LOQ, which are three and ten respectively. Then the conditions established by the Konieczka
208 method are checked:

209 $$10 \cdot LOD > c_{min}$$

$$LOD < c_{min}$$

If the above conditions are not fulfilled, another concentration (c_{min}) has to be tried to calculate again the standard deviation. The procedure is repeated until the Konieczka method conditions are fulfilled.

A previous study of colorants in different foodstuffs by CE (Pérez-Urquiza & Beltrán, 2000) obtained LODs from 1.0 to 1.7 mg L^{-1}, slightly higher than the ones obtained in this work. Nevertheless, similar regression coefficients and relative standard deviations have been obtained in both cases. Prado *et al.* (Prado et al., 2006) obtained similar LODs to the ones obtained in this work (0.6 to 2.5 mg L^{-1}),

3.4. Sample analysis

16 different samples were analyzed with the developed method. Samples were chosen according to their color and label information about the presence of the colorants of interest. Nevertheless, some labels were confusing, especially in liquid jellies and ice-pops, because they said “it might include some of the listed colorants”. This could mean that some of the colorants are used in some specific sweets but not in all the candies of the container. All the samples were analyzed three times and Table 3 shows the results obtained in terms of mean and standard deviation (sd).

Generally, expected colorants were found at different concentrations in the analyzed samples (according to its color). As exception, E129 was not found in some samples (VG, IG and CG) where it was expected. This could be because their labels include the possible presence of other colorant with same color (red).

Samples with higher concentrations of colorants were the food dyes, both liquids and solids. Moreover, all the food colorants exceeded drastically the allowed limit (50 mg L^{-1} or mg kg^{-1}) by the EU (Commission regulation (EU) Nº 1129/2011, 12.11.2011). Nevertheless, it should be considered that those colorants are not directly edible; their use is aimed to cooking. Therefore, the safety in the use of these products will rely on consumer’s responsibility.

Apart from the mentioned dyes, there are two other samples (FN an D) which exceed the maximum allowed limit by the EU, being the concentration of E102 79.1 mg L^{-1} in FN and the concentration of E110 99 mg L^{-1} in D. Nevertheless, the regulation is specifically for foods and not for drugs, where the ingested amount should be considerably less than for any foodstuff.

Two colorants (E102 and E129) were found in the non-alcoholic drink, but the sum of them did not exceed the permitted limit.

Besides the EU regulation, consumers should be responsible for the consumption of these kinds of analyzed products. A regular consume of these products can be prejudicial for health, especially for children, as ADI can be exceeded easily taking into account the found concentrations of additives. ADI values for these colorants ranging from 2.5 to 12.5 mg/kg b.w. that means that the amount that can be ingested by a kid of 20 kg is between 50 and 140 mg per day. For example, the individual package amount of the sample named as H was about 1.4 g with the E102 composition of 21.7%. This means that if the whole package is used in a dish cooked for four people, each of person is consuming 75.9 mg of E102, which exceeds the ADI level of E102 for a kid with a weight below 30 kg.

To check the accuracy of the method recovery studies were carried out in real samples at two concentration levels, N1 and N2. Because in most of the cases the producers provided no reference value, recovered concentration should be the original presented in the sample plus the added one (N1 or N2 in each case).

In the case of the drug sample, a reference was provided by the technical datasheet (100 mg L^{-1} of E110), so the found concentration was also compared to this. All the recovery values are included in Table 4.

In most cases, obtained recoveries were from 90 to 110 %, but in some cases, the recovery was below 90 % or above 110 %. Especially low values were obtained with CN (74.6 %) and FU (64.4 %). In those samples, the filtration was difficult even after the centrifugation. The filter obstructed easily even with very little volumes and probably some analyte remained in the filter.

Recovery values from 85.0% to 109.1 % for the same colorants in milk were obtained in a previous study (Huang et al., 2002). SPE extraction procedure before EC determination to eliminate matrix interferences was used in this case.

4. Conclusions

A fast and reliable method has been developed for the determination of four different colorants (E102, E110, E129 and E129) in different food products. Obtained method validation parameters show the reliability of the developed MEECK method to determine those analytes in very short times (each analysis by EC was performed in less than 9 minutes) as well as good linearity in a very wide range of concentrations. Both good precision and accuracy were obtained in almost all the cases and LOD and LOQ values are consistent with the ones obtained by other authors for this kind of analytes. Furthermore, they are low enough accordingly to the amount of colorants found in the analyzed samples.

Sixteen different samples have been analyzed including food dyes, isotonic and non-alcoholic drinks, ice-pops, liquid jellies and drugs. Two of the samples analyzed exceed the limit established by EU and the analyzed samples used for cooking contain a very high amount of colorant content, so they must be carefully used to not exceed the allowed limit in the prepared food. Nevertheless, it seems that under a responsible consumption of the analyzed samples the ADI level is hardly exceeded. This assumption, however, can be not reliable for children, not only because ADI restriction is lower for them (less body weight) but also due to children consume probably more candies than adults.

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Table 1. Figures of merit obtained for the developed method including both calibration ranges (low and high). L1 and L4 correspond to concentration levels established at 15 and 150 mg L^{-1} for E133, 16 and 160 mg L^{-1} for E110, 11 and 110 mg L^{-1} for E129 and 11 and 110 mg L^{-1} for E102.

Additive		Calibration			Concentration range (mg L^{-1})		RSD %			
		R^2	**Intercept**	**slope**	**Min**	**Max**	**Intra-day (n=9) L1 /L4**	**Inter-day (n=9) L1/L4**	**LOD (mg L^{-1})**	**LOQ (mg L^{-1})**
E102	*Low range*	0.9988	-0.3498	0.6688	2.29	22.89	4.15 (L1)	3.52 (L1)	0.68	2.26
	High range	0.9995	2.7342	0.6444	22.89	915.61	3.64 (L4)	3.94 (L4)		
E110	*Low range*	0.9981	-0.0147	0.7224	3.28	32.84	4.88 (L1)	5.47 (L1)	0.24	0.80
	High range	0.9987	11.7100	0.6924	32.84	1313.62	5.39 (L4)	5.41 (L4)		
E129	*Low range*	0.9995	-0.2304	0.7650	2.17	21.69	4.99 (L1)	8.55 (L1)	0.24	0.80
	High range	0.9998	2.1949	0.7075	21.69	867.77	7.01 (L4)	4.61 (L4)		
E133	*Low range*	0.9991	0.1075	0.3263	2.98	29.82	4.73 (L1)	7.77 (L1)	1.21	4.03
	High range	0.9992	3.8608	0.2815	29.82	1192.69	6.10 (L4)	7.21 (L4)		

Table 2. Found concentration levels in different samples in mg L^{-1}, except for solid samples (%). Each value was obtained from triplicate measurements and the standard deviation from the measurements is included in parenthesis.

		Concentration (sd) [n=3]			
Samples	**Code**	**E102**	**E110**	**E129**	**E133**
Food liquid colorants*	**VG**	-	-	-	-
	VH	6270 (135)	-	-	-
	VU	-	-	-	6137 (46)
Food solid colorants (for paella) **	**Du**	4.3 % (0.1)	-	-	-
	K	6.23 % (0.02)	-	-	-
	H	21.69 % (0.08)	-	-	-
Energy driknks *	**IG**	-	-	-	-
	P	-	-	-	3.5 (0.1)
Non alcoholic drinks*	**R**	7.0 (0.2)	-	2.5 (0.1)	-
Ice-pops*	**FU**	-	-	-	14.3 (0.1)
	FN	79.1 (0.7)	-	-	-
Liquid jellies*	**CU**	-	-	-	33.4 (0.4)
	CG	-	-	-	-
	CH	15.6 (0.2)	-	-	-
	CN	12.20 (0.06)	-	-	-
Drugs*	**D**	-	99 (3)	-	-

* Concentrations in mg L^{-1}

** Concentrations expressed in %

Table 3. Recovery studies performed in the samples. Addition levels N1 and were 15 and 30 mg L^{-1} for E133, 16 and 32 mg L^{-1} for E110; 11 and 22 mg L^{-1} for E129 and 15 and 30 mg L^{-1} for E102. Those concentrations are in the final solution, after the necessary processing (including dilution, centrifugation and filtration) was made.

		Recovery (%)			
Samples	**Code**	**E102**	**E110**	**E129**	**E133**
Food liquid dyes	**VG_N1**			93.9	
	VG_N2			100.3	
	VH_N1	98.2			
	VH_N2	97.8			
	VH_N1				97.2
	VH_N2				95.1
Food solid dyes (for paella)	**Du_N1**	91.7			
	Du_N2	93.4			
	K_N1	87.0			
	K_N2	100.0			
	H_N1	97.3			
	H_N2	96.9			
Energy drinks	**IG_N1**			91.2	
	IG_N2			94.0	
	P_N1				101.2
	P_N2				98.4
Non alcoholic drinks	**R_N1**	105.6		107.7	
	R_N2	112.2		117.3	
Ice-pops	**FU_N1**				87.4
	FU_N2				64.4
	FN_N1	97.8		92.2	
	FN_N2	99.1		90.8	
Liquid jellies	**CU_N1**				94.2
	CU_N2				100.6
	CG_N1			109.3	
	CG_N2			106.0	
	CH_N1	95.3			
	CH_N2	101.3			
	CN				
	CN_N1	83.2		74.6	
	CN_N2	87.7		77.4	
Drugs	**D**		98.9		
	D_N1		112.3		
	D_N2		119.1		

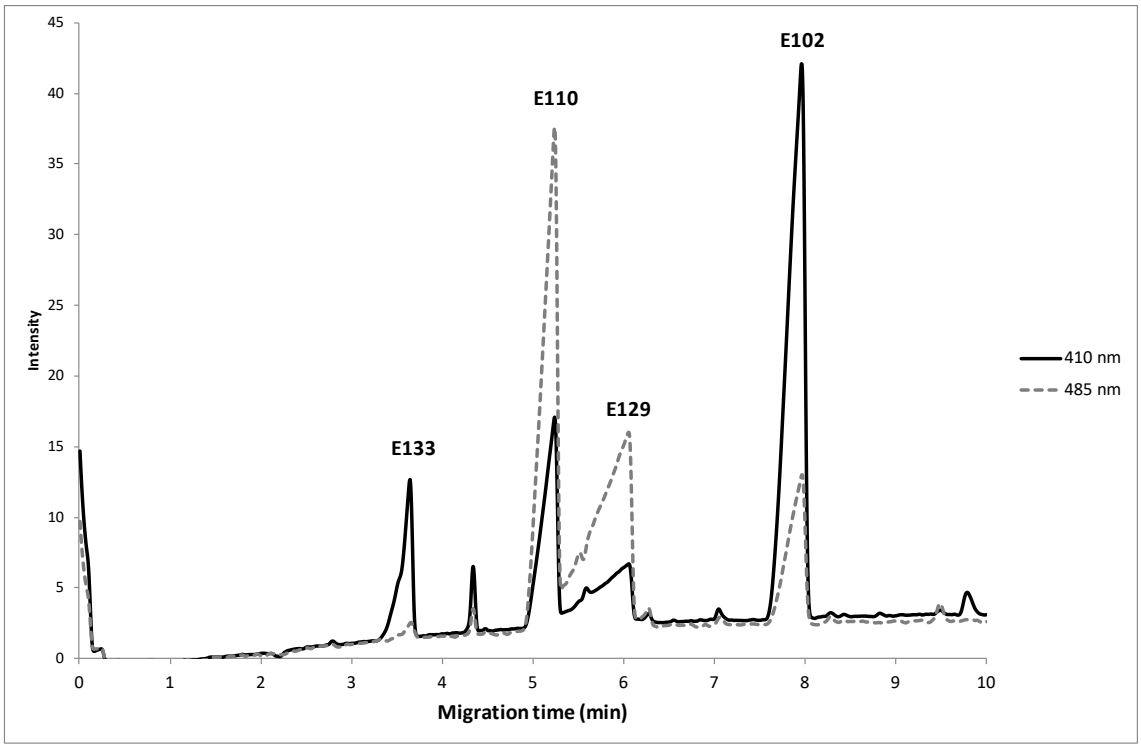

Figure 1. Electropherogram obtained under optimal conditions (BGE composition of 12.5 mM Borax, 6 mM SDS and 5 mM β-CD, 25kV and 25 ºC) and at concentrations of 112 mg L^{-1} for E133, 115 mg L^{-1} for E110, 114 mg L^{-1} for E129 and 118 mg L^{-1} for E102. Continuous line shows the electropherogram obtained at 410 nm and dashed line shows the same electropherogram at 485 nm.