HELTZE DENBORAK ZALDI HARAGIAN DUEN ERAGINA: IKUSPEGI INSTRUMENTALA ETA SENTSORIALA

EFECTO DEL TIEMPO DE MADURACIÓN SOBRE LA CALIDAD DE LA CARNE DE CABALLO: ENFOQUE INSTRUMENTAL Y SENSORIAL

EFFECT OF AGEING TIME ON HORSE MEAT QUALITY: INSTRUMENTAL AND SENSORY APPROACH

Doktorego-tesia |Tesis Doctoral | Ph.D. Thesis

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2022

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Universidad del País Vasco

Euskal Herriko Unibertsitatea

Heltze denborak zaldi haragian duen eragina: ikuspegi instrumentala eta sentsoriala

Efecto del tiempo de maduración sobre la calidad de la carne de caballo: enfoque instrumental y sensorial

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Vitoria-Gasteiz, July 2022

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ACKNOWLEDGEMENTS FOR THE FINANCIAL SUPPORT

Lorea Rivera Beldarrain fully acknowledges the doctoral fellowship provided by the Department of Economic Development and Infrastructures of the Basque Government.

This research has been funded by Lactiker research group (Basque Government IT944-16).

ACKNOWLEDGEMENTS TO THE EDITORIALS

Authors thank the editorials for granting permission to reuse the published articles in this Ph.D. Thesis. The published versions can be accessed at the following links:

Insausti *et al*. (2021) *Animal Frontiers*, 11, 47-54. (**Appendix I**)

https://doi.org/10.1093/af/vfab003

Beldarrain et al. (2018) *J. Proteomics*, 183, 25-33. (**Appendix II**)

https://doi.org/10.1016/j.jprot.2018.05.005

Beldarrain *et al*. (2021) *Animals*, 11, 1421. (**Appendix III**)

https://doi.org/10.3390/ani11051421

Beldarrain et al. (2022) *Meat Sci*., 184, 108692. (**Appendix IV**)

https://doi.org/10.1016/j.meatsci.2021.108692

Beldarrain *et al*. (2022) *Meat Sci*., 188, 108804. (**Appendix V**)

https://doi.org/10.1016/j.meatsci.2022.108804

Beldarrain *et al*. (2020) *Food Res. Int.,* 129, 108871. (**Appendix VI**)

https://doi.org/10.1016/j.foodres.2019.108871

“One cannot think well, love well, sleep well, if one has not dined well.”

Virginia Woolf, A Room of One's own

Lehenik eta behin, Eusko Jaurlaritzako Ekonomiaren Garapen eta Azpiegitura Saila eskertu nahiko nuke, nire aurre-doktoretzarako beka finantziatu izanagatik.

Nire tesi-zuzendariei ere eskerrak eman nahi dizkiet. Esker bereziak Noeliari, emaitza onenak lortzeko atsedenik gabe borrokatzera irakasteagatik, eta azken hilabetetan tesi hau zuzentzeko egindako lan sakonagatik. Gracias también a Miguel, por hacerme sentir como en casa en el IATA y por compartir siempre mis triunfos y fracasos con un buen consejo y unas palabras serenas. Sin vuestro apoyo esto no hubiera sido posible, mil gracias. A Claudia, mi compañera de laboratorio, congresos, lloros (bastantes) y risas (muchas). Gracias por ponerle un toque de sinceridad a cada etapa del camino, crecer juntas ha sido un auténtico placer.

Benetan eskertzen dut proiektu hau arlo askotako profesionalekin partekatu izana, izugarri ikasi dudalako imajinaezinak iruditzen zitzaizkidan gauzei buruz. Urteetan zehar laguntza handia izan dut eta oso presente daukat. Una mención muy especial a Lara, porque me enseñó a tomarme con serenidad los imprevistos profesionales y personales, a cortar carne como una diosa y a reír cuando te sobrepasan los problemas sin que puedas hacer nada al respecto. Eres para mí un ejemplo como científica y como persona.

Eskerrak Iñaki Etaiori kontsumitzaile froga aurrera eramaten laguntzeagatik, plazer bat izan da hain ezezaguna nuen eremu batean lan egitea, eta eskerrak ere Luisjari, beti egon delako aholkuak emateko prest. Eskerrak orokorrean Lactiker ikerketa taldeko lankideei: Xabiri bidea zabaltzeagatik eta ostean nigandik hurbil egon direnei (Maider, Ana, Garazi…), beti izan dugulako barrerako tartetxo bat.

Lactikerretik kanpo ere laguntza handia izan dut. Eskerrak eman nahi dizkiot Kizkitzari, UPNA-n hain harrera ona egiteagatik eta texturometroa erabiltzen erakusteko denbora hartzeagatik. Gracias a Quique, por tu trabajo con el masas y por tu buen humor, y a la gente del laboratorio 102-104 del IATA, en especial a Javi, Laura, Nerea y Alejandro por acogerme con tanto cariño. I am especially grateful to Dr. Ingrid Miller (Vetmeduni, Vienna), for her hospitality and continuous support as a visiting researcher in her lab. I am really thankful for the large amount of tips and knowledge in proteomics that I learnt in that brief but fruitful time. Your professionalism and commitment with science are inspiring; I

hope that we have the opportunity to collaborate again in the near-future. Vienan ere ezagutu nuen Ana, *m'emporto al cor les teves ganes de donar guerra*! I would also like to thank John Kramer (Guelph, Canada) for his advices.

Azken urte hauek nahiko gogorrak izan dira eta askotan ez da erreza izan nirekin denbora konpartitzea, baina hala ere beti izan dut inguruan jendea, aurrera jarraitzeko indarra ematen. Argi daukat zuek gabe ez nintzela honaino iritsiko, ez behintzat indarrekin eta umore onean. Eskerrak etxean jasan nautenei, Alazne eta Mikel, Nahiari elkar zaintzeari inoiz ez uzteagatik eta Ostebiri deskonektatzeko plan iturri agortezina izateagatik. Deustuko tropari juerga eta maitasun guztiagatik eta Saniri beti etxea izateagatik. En esta última etapa, a Pam y a Selva por las risas y el terraceo, *y per descomptat, Gràcies Xavier per tot el suport i afecte aquests últims anys*.

Y por último, gracias de corazón a mi familia. A Estibaliz, Marisol y Aitor, por apoyarme y creer en mí (quizá hasta demasiado), pero sobre todo por enseñarme a ser fuerte y demostrarme que no hay mala situación que no se pueda superar. A aita, Sabino y Izaskun porque, aunque ya no estén, los siento muy cerca.

Guztioi, mila esker bihotzez.

LABURPENA

Doktorego-tesi hau Euskal Herriko Unibertsitateko (UPV/EHU) Lactiker Ikerketa Taldearen - animalia-jatorriko elikagaien kalitatea eta segurtasuna da honen jardueraren ardatza - eta Instituto de Agroquímica y Tecnología de los Alimentos, Consejo Superior de Investigaciones Científicas-eko Haragiaren eta Haragi produktuen Biokimika Ikerketa Taldearen arteko elkarlanean egin da.

Doktorego-tesi honen helburu nagusia heltze denborak zaldi haragiaren kalitate atributuetan duen eragina ikertzea izan da, metodo instrumental eta sentsorialak erabiliz. Orokorrean, zaldi haragiaren kalitatea gutxiago ikertu da beste espezie batzuenarekin alderatuz, zaldi haragiaren ekoizpen praktikak eta kalitatea erlazionatzen dituen ikerketa falta nabarmena dago. Beraz, lan horretatik sortutako informazioak zaldi haragiaren kalitatean eragina duten *post-mortem* faktoreak hobeto ulertzea ahalbidetuko du.

Lehenik eta behin, Espainia iparraldeko zaldi haragiaren ekoizpen sistemei buruzko berrikuspen bat idatzi zen, eta horrek zaldi haragi ekoizpenak dituen hainbat abantaila ulertzen lagundu zuen. Berrikuspenetik ondorioztatu zen zaldi ekoizpen estentsiboak

kalitate handiko haragia ematen duela, eta ingurumenarekin lotutako hainbat abantaila dituela (adibidez, bioaniztasuna eta paisaia mosaikoa hobetzea; baso suteen prebentzioa; eta hausnarkariekin alderatuz, negutegi efektudun gasen isurketa gutxitzea), baita landa garapenarekin eta garapen sozialarekin lotutako beste batzuk ere (tradizioak eta arriskuan dauden arrazak kontserbatzea, landa enplegua eta landa ekonomia sustatzea). Horrela, zaldi haragia alternatiba egokia izan daitekeela ondorioztatu zen, baina gaur egun dagoen informazio faltari aurre egiteko haragi mota honen kalitatean eragin dezaketen *ante-* eta *post-mortem* faktoreak gehiago ikertzea falta da (**I. Publikazioa**).

Tesi honen arreta *post-mortem* faktoreetan zentratu zen, eta zehazki, heltze denborak haragi kalitatearen atributu garrantzitsuenetan duen eraginean. Zaldi haragiaren kalitatean eragina izan dezaketen faktore guztietatik heltze prozesua aukeratu zen, zaldi haragian egindako aurreko ikerketak oso mugatuak direlako eta beste espezie batzuetatik eratorritako okelan duen eragina ezaguna delako. Jakina da heltze denborak haragiaren kalitatean garrantzi handia duela prozesuan zehar gertatzen diren aldaketa biokimiko eta fisiologikoen ondorioz. Batez ere, samurtasuna hobetzen da eta hori da kontsumitzaileak haragian kontuan hartzen duen ezaugarri organoleptiko garrantzitsuenetako bat. Heltze prozesuak haragiaren beste atributu batzuei ere eragiten die; hala nola, kolorea, zaporea edo ura eusteko ahalmenari.

Kontuan izanda heltze prozesuaren efektuak espezie, arraza eta muskulu bakoitzaren araberakoak direla, heltze denboraren eragina gaur egun asko kontsumitzen den zaldi arraza batean ikertzearen garrantzia azpimarratu zen. Zentzu horretan, kontsumitzaileak kalitate hobe batekin erlazionatuko duen heltze denbora optimo bat ezartzeak, heltze prozesu luzeagoekin beharrezkoak diren inbertsioak saihestuko lituzke. Erronka horri aurre egiteko Hispano-Bretón zaldi arrazatik eratorritako solomoa ikertu zen; arraza hau Espainia iparraldean kokatzen da, ondare genetikoaren zati garrantzitsua da eta haren kontserbazioa giltzarria da mendiko ekosistemak mantentzeko.

Hasteko, konposizio azterketaren arabera, ekoizpen erdi estentsiboan hazitako Hispano-Bretón zaldietatik eratorritako haragiaren hezetasun, proteina eta errauts edukierak (% 75,3, % 20,4 eta % 1,03, hurrenez hurren) antzeko adinarekin hildako beste zaldi arraza

batzuenaren antzekoak izan ziren. Mioglobina edukia (3,47 mg/ g haragi) adin antzeko behi haragiarena baino handiagoa eta n-3 gantz azido poliasegabeen edukia (gantz azido totalen % 1,53) hausnarkarietan deskribatuta dagoena baino handiagoa izan ziren. Gantz azido poliasegabeen pilaketa zaldien gantz azido horiek xurgatzeko eta ehunetan pilatzeko daukaten gaitasunarekin lotuta dago eta prozesu hau hidrogenazio mikrobianoa gertatu aurretik ematen da. Guzti hori kontutan hartuta, zaldi haragiaren kontsumoa herrialde gehienetan nahiko ezezaguna bada ere, okela gorri alternatibotzat har daiteke, batik bat, haren konposizio nutrizionala eta inguruko ekosisteman eragiten dituen efektu positiboak kontutan hartzen badira (**I. eta III. Publikazioak**).

Heltze prozesuaren ikerketarako, zaldi solomo xerrak hutsean ontziratu eta 4 °C-an, ilunetan, 0, 7, 14 eta 21 egunez gorde ziren. Lehenik eta behin, heltze denboraren eragina haragiaren kalitate atributu garrantzitsuenetan neurtu zen metodo instrumentalak erabiliz. Zehazki, konposatu bolatilen konposizioa, pH-a, kolore instrumentala, mozketa indarra, ur galera kozinatzean eta sub-proteoma miofibrilarra neurtu ziren. Ondoren, metodo sentsorialak erabiliaz, kontsumitzaileen onarpena eta zaldi haragiaren deskribapen sentsoriala aztertu ziren.

Ehundurarekin erlazionatutako kalitate atributuetatik mozketa indarra eta kozinatzean ematen den ur galera aukeratu ziren. Lehen bi asteetan haragiaren samurtasuna hobetu zen (mozketa indarra txikiagotuz) eta kozinatzean gertatutako ur galerak lehen astean eman ziren. Ondoren, aste batetik aurrera, ur galera gehiagorik ez zen gertatu eta kontsumitzaileek haragia urtsua bezala deskribatu zuten, heldu gabeko haragiarekin gertatu ez bezala.

Ondoren, heldutako eta kozinatutako zaldi haragiaren usaina ikertu zen. Konposatu lurrunkorren azterketa egitean heltze prozesuan zehar hexadekanala eta 2- eta 3-metilbutanala bezalako zenbait aldehidoren pilaketa gertatu zen. Honek, ustez, zaldi haragiaren usainean eragina izan dezake konposatu horien inpaktu aromatikoa handia delako. Hamalau egun baino gehiagoko heltze denborak beharrezkoak izan ziren konposatu lurrunkorren profilean aldaketa esanguratsuak gertatzeko eta, ondorioz, zaldi haragiaren usainean eragiteko (**IV. Publikazioa**).

Bestalde, zaldi haragiaren sub-proteoma miofibrilarra aztertu zen. Haragiaren samurtzea muskulu proteinen *post-mortem* degradazioaren ondorio zuzena denez, degradazio honen mekanismoak argitzeak heltze estrategia eraginkorren garapenean lagundu dezake. Hortaz, isoelektrofokatze likidoa (OFFGEL) eta dimentsio bateko elektroforesia (1-DE) oinarri dituen metodologia garatu zen. Metodologia hau bi dimentsioko (2-DE) elektroforesi tradizionalaren alternatiba bezala proposatu zen. Horrek, zenbait abantaila eskaintzen ditu; adibidez, proteinak likido fasetik zuzenean berreskuratzea eta automatizazio maila handiagotzea (**II. Publikazioa**). Metodologiaren egokitasuna frogatu ondoren zaldi haragian aplikatu zen. Lortutako emaitzen arabera, proteina aldaketa gehienak lehen bi asteetan gertatu ziren, mozketa indarraren bidez ikusitako samurtasunaren hobetzearekin egoki erlazionatuta. Gainera, eta lehen aldiz, hainbat proteinen *post-mortem* ugaritasun aldaketa behatu zen zaldi haragiaren heltze prozesuan zehar. Zehazki, 1 miosinaren kate arina, miosinari lotutako C proteinaren pusketa, eta T eta I troponinak (eta beraien pusketak) modu esanguratsuan aldatu ziren eta horrek zaldi haragiaren samurtasunaren biomarkatzaile gisa erabiltzeko aukera zabaltzen du (**V. Publikazioa**).

Azkenik, kontsumitzaile ikerketa bat egin zen. Kontsumitzaileen heldu gabeko (0 egun) eta 7, 14 eta 21 egunez heldutako zaldi haragiaren inguruko pertzepzioa ebaluatu zen. Ikerketa hau Euskal Herriko Unibertsitateko Zentzumen Analisien Laborategian (LASEHU) egin zen 120 boluntariorekin. Kontsumitzaileek zaldi haragiari emandako onarpena, ahozkoa eta ikusizkoa, ebaluatu ziren, eta emaitzak 'Check-all-that-apply' (CATA) metodologiaren bidez lortutako deskribapen sentsorialarekin bateratu ziren. Aste bateko heltze prozesuak bai ahozko eta bai ikusizko zaldi haragiaren onarpena hobetu zituela ikusi zen. Bestalde, heltze denbora tarte luzeagoek ez zuten kontsumitzaileen onarpena hobetu. CATA analisi deskriptiboarekin emaitza interesgarriak lortu ziren, eta instrumentalki neurtutako atributuekin batera, zaldi haragiaren kalitatean izandako eragin agregatua hobeto ulertzen lagundu zuten. Ehundurari lotutako ezaugarriei dagokienez, kontsumitzaileek astebetez heldutako haragiaren samurtasun eta urtsutasun handiagoak deskribatu zituzten eta denbora luzeagoekin ez zen aldaketa gehiagorik nabaritu. Horrek zera esan nahi du; kontsumitzaileek ez zutela heltze prozesuaren bigarren astean zehar mozketa indarrak zehaztutako samurtasun handiagoa hautematen. Aitzitik, kontsumitzaileek egindako zaldi haragiaren usainaren deskripzioa bat zetorren neurri instrumentalek neurtutakoarekin; biek

adierazi zuten zaldi haragian bi astetik gorako heltze prozesua behar dela usain bereizgarri bat lortzeko. Itxurari dagokionez, bi aste baino denbora tarte luzeagoan heldutako haragiak ebaluazio negatiboa jaso zuten; haragi hondatuaren itxura, kolore marroixka eta ez uniformeagatik (**VI. Publikazioa**). Emaitza hauek bat datoz kolore instrumental neurketekin.

Oro har, Hispano-Bretón arrazako zaldi solomoaren heltze denbora optimoa 7 egunekoa dela ondorioztatu zen. Denbora tarte horretan kolorea eta samurtasuna hobetu ziren, baita kontsumitzaileen onarpen hobea lortu ere. Bi astetan zehar heldutako haragia gorriagoa eta samurragoa izan arren, kontsumitzaileen onarpen ebaluazioa ez zen hobetu eta, beraz, bi astetan heltzeko behar den inbertsioa ez dago arrazoituta, ez behintzat kontsumitzaileen ikuspuntutik. Gainera, 14 egun baino heltze denbora luzeagoak ez dira gomendagarriak haragiaren kolorean gertatzen den hondapenagatik eta, baita ere, usain bereizgarrien sorreragatik. Guzti hori ez da gainera kontsumitzaileen onarpen balorazio altuago batean bihurtzen. Lortutako emaitz guzti horiekin zaldi haragiaren heltze prozesuaren ezaugarritze zehatz bat lortu da; alde batetik, prozesua hobeto ulertzeko, eta bestalde, zaldi haragi industriari heltze denbora optimoa zein den adierazteko, kalitate homogeneo eta altuko zaldi okela lortze aldera.

RESUMEN

La presente Tesis Doctoral se ha realizado en colaboración entre el Grupo de Investigación multidisciplinar Lactiker de la Universidad del País Vasco (UPV/EHU) cuya actividad está centrada en la Calidad y Seguridad Alimentaria de los Alimentos de Origen Animal, y el Grupo de Investigación de Bioquímica de la Carne y Productos Cárnicos del Instituto de Agroquímica y Tecnología de Alimentos del Consejo Superior de Investigaciones Científicas, cuya actividad investigadora está centrada en el estudio de los mecanismos bioquímicos responsables de la transformación del músculo en carne.

El objetivo general de esta Tesis Doctoral ha sido investigar el efecto del tiempo de maduración sobre los parámetros de calidad de la carne de caballo, utilizando tanto metodologías instrumentales como sensoriales. En general, la calidad de la carne de caballo ha sido menos investigada que la de otras especies de abasto, y existe una notable falta de estudios que relacionen las prácticas de producción de éste tipo de carne con su calidad. Por ello, la información generada en este trabajo ayudaría a tener una mejor comprensión de los factores *post-mortem* que afectan a la calidad de la carne de caballo.

La primera parte de la Tesis Doctoral consistió en una revisión bibliográfica sobre las explotaciones de equino orientadas a la producción de carne del norte de España, basadas en sistemas extensivos de pastoreo, que ayudó a comprender las externalidades positivas de este tipo de producciones. De la revisión bibliográfica se concluyó que la producción caballar en régimen de pastoreo da lugar a una carne de alta calidad, a la vez que ofrece una serie de ventajas medioambientales (*p.e.*, mejora de la diversidad y del paisaje en mosaico, prevención de incendios y menores emisiones de gases de efecto invernadero comparando con los rumiantes) y beneficios asociados al desarrollo rural y social (*p.e.*, conservación de tradiciones y razas autóctonas, promoción del empleo rural y economía agrícola). Se concluyó por tanto que la carne de caballo podría convertirse en una carne roja alternativa, sin embargo, se detecta la necesidad de estudios adicionales que ayuden a superar la falta de información en relación al efecto de factores *ante*- y *post- mortem* sobre la calidad de la carne de caballo (**Publicación I**),

La atención de la presente Tesis Doctoral se centró en los factores *post-mortem,* particularmente en el efecto del tiempo de maduración, sobre los parámetros de calidad más relevantes. Entre todos los factores que pudieran influir en la calidad de la carne de caballo se escogió el proceso de maduración, debido a que éste ha sido poco estudiado este tipo de carne y teniendo en cuenta que su efecto en la calidad de la carne de otras especies es ampliamente conocido. De hecho, la maduración juega un papel imprescindible en la calidad de la carne, debido a las transformaciones bioquímicas y fisiológicas que tienen lugar durante el proceso. Esto es especialmente relevante para el incremento de la terneza, considerada una de las características organolépticas más importantes para el consumidor. A su vez, la maduración afecta a otros parámetros de calidad de la carne como el color, aroma o capacidad de retención de agua.

Teniendo en cuenta que la maduración influye de manera específica en diferentes especies, razas y músculos, se consideró relevante estudiar el efecto del tiempo de maduración en una raza de caballo altamente consumida en la actualidad. De este modo, el establecimiento de un tiempo óptimo de maduración que se traduzca en un incremento de la calidad percibido por el consumidor, evitaría inversiones innecesarias asociadas a tiempos de maduración más largos. Para abordar este reto, se eligió investigar en lomos de caballos de

la raza Hispano-Bretón. Se trata de una raza bien establecida en el norte de España, que es parte importante de la herencia genética, y cuya conservación es clave para mantener los ecosistemas de montaña.

El estudio inicial de la composición grosera puso de manifiesto que la carne proveniente de caballos de la raza Hispano-Bretón criados en condiciones semi-extensivas cuenta con un contenido medio de humedad, proteínas y cenizas (75,3 %, 20,4 % y 1,03 % respectivamente) comparable a los valores descritos en la literatura en carne de caballos de otras razas sacrificados a una edad similar. Además, se encontró un contenido más alto de mioglobina (3,47 mg/ g) y ácidos n-3 poliinsaturados (1.53 % de todos los ácidos grasos) que el habitualmente descrito en carne de rumiantes. Esto último tiene su raíz en la mayor capacidad de los caballos para absorber los ácidos poliinsaturados de la dieta, antes de que se sometan a hidrogenación microbiana. Todo esto confirmó que la carne de caballo tiene potencial para ser una carne roja alternativa, por su composición nutricional y por las externalidades positivas o servicios ecosistémicos (**Publicaciones I y III**)

Para el estudio de la maduración, los filetes de lomo de caballo fueron envasados al vacío y conservados a 4 °C en la oscuridad durante 0, 7, 14 y 21 días. En primer lugar, se estudió el efecto del tiempo de maduración sobre los parámetros de calidad más relevantes mediante métodos instrumentales. Más concretamente, se determinaron la composición de compuestos volátiles, pH, color instrumental, fuerza de corte, pérdidas por cocinado y sub-proteoma miofibrilar de la carne de caballo. En segundo lugar, se investigó el efecto de maduración sobre la preferencia y descripción sensorial de los consumidores, utilizando métodos sensoriales.

Con relación a la textura, los parámetros elegidos para su determinación instrumental fueron la fuerza de corte y las pérdidas por cocinado. El incremento en la terneza (disminución de la fuerza de corte) se observó durante las primeras dos semanas de maduración, mientras que las pérdidas por cocinado disminuyeron únicamente durante la primera semana de maduración, manteniéndose después constantes. Tras este periodo de una semana, la carne sería percibida como más jugosa por el consumidor.

El aroma de la carne de caballo cocinada fue estudiado a diferentes tiempos de maduración. Se observó que varios aldehídos como el hexadecanal y 2- y 3-metilbutanal incrementaban su abundancia durante el proceso de maduración. Este hecho podría afectar al olor de la carne, ya que se trata de compuestos con un impacto odorante considerable. En general, fueron necesarios más de 14 días de maduración para generar cambios significativos en el perfil de volátiles y, en consecuencia, en el aroma de la carne de caballo cocinada (**Publicación IV**). En cuanto al color, factor más importante a la hora de la compra de carne, es conocido que el consumidor preferentemente elige carnes luminosas de color rojo cereza, ya que relaciona estas características de color con un estado saludable del producto. En este sentido, se observó un incremento en el color rojo de la carne durante las primeras dos semanas de maduración, y después de este tiempo las propiedades de color comenzaron a deteriorarse. De hecho, la carne madurada durante 21 días se volvió de color pardo (**Publicación III**).

Por otra parte, se estudió el efecto del tiempo de maduración sobre el sub-proteoma miofibrilar de la carne de caballo. Puesto que la tenderización de la carne ocurre como consecuencia de la degradación *post-mortem* de las proteínas que componen las fibras musculares, descifrar los mecanismos de dicha degradación contribuiría a desarrollar estrategias eficientes de maduración. Para ello, se desarrolló una estrategia novedosa de análisis basada en el isoelectroenfoque en medio líquido (OFFGEL) combinado con la separación mediante electroforesis monodimensional el gel (1-DE). Esta metodología se propuso como alternativa a la clásica electroforesis bidimensional (2-DE), ofreciendo ventajas sobre ésta última tales como la recuperación directa de las proteínas de la fase líquida o un mayor grado de automatización (**Publicación II**).

Tras comprobar la eficacia de la técnica propuesta, ésta se aplicó al estudio de la carne de caballo. En general, los resultados demostraron que la mayor parte de cambios en abundancia de las proteínas tuvieron lugar durante las primeras dos semanas de maduración, coincidiendo con el aumento de la terneza observado mediante la determinación de la fuerza de corte. Además, por primera vez, se describió el cambio de abundancia de varias proteínas durante la maduración. Concretamente, se observó que abundancia de la cadena ligera de miosina 1, así como de un fragmento de la proteína C

fijadora de miosina 1 y de las troponinas T e I (junto a sus fragmentos) se alteraba con la maduración, abriendo así el camino para estudiar dichas proteínas como potenciales marcadores de terneza en carne de caballo (**Publicación V**).

Finalmente, se realizó un estudio de consumidores para evaluar la percepción de los consumidores sobre la carne de caballo sin madurar (0 días) y madurada durante 7, 14 y 21 días. Este estudio se llevó a cabo en el Laboratorio de Análisis Sensorial Euskal Herriko Unibertsitatea (LASEHU), y en él participaron 120 voluntarios. Los consumidores evaluaron la aceptabilidad en boca y visual de la carne, en combinación con el método "*Check-all-that-apply*"(CATA). Éste último se utilizó para obtener una descripción sensorial de la carne de caballo. Se observó que la aceptabilidad de la carne de caballo, tanto en boca como visual, incrementó después de la primera semana de maduración. Sin embargo, periodos más largos de maduración no se trasladaron en una mayor aceptabilidad. De hecho, la carne madurada durante más de 14 días fue rechazada por los consumidores.

El análisis descriptivo mediante CATA proporcionó unos interesantes resultados que en combinación con las medidas instrumentales contribuyeron a entender mejor el efecto del tiempo de maduración sobre la calidad de la carne. En cuanto a parámetros de textura, los consumidores describieron un aumento en la terneza y jugosidad de la carne madurada durante una semana, mientras que no se observó mayor aumento en la carne madurada durante más tiempo. Esto significaría que el aumento en la terneza determinado instrumentalmente (fuerza de corte) después de la primera semana de maduración, no sería percibida por el consumidor. Por el contrario, la descripción de los consumidores del aroma de la carne madurada coincidió con lo observado mediante medidas instrumentales, ya que ambas indicaban que eran necesarias más de dos semanas de maduración para alterar el aroma en la carne de caballo. En lo que respecta a su apariencia, la carne madurada durante más de dos semanas obtuvo una valoración negativa por parte de los consumidores, que la describieron como podrida, parda y sin uniformidad en el color (**Publicación VI**). Estos resultados coincidirían con los obtenidos mediante medidas instrumentales.

En conclusión, los resultados obtenidos en esta Tesis Doctoral apuntan a que el tiempo óptimo de maduración para carne del lomo de Hispano-Bretón sería de 7 días. Durante este

periodo, la terneza y el color mejoran, así como la aceptabilidad del consumidor. La segunda semana de maduración da lugar a una carne más roja y más tierna, pero ésta mejora no es suficiente para ser percibida por el consumidor. De esta manera, la inversión requerida para la segunda semana de maduración no estaría justificada, al menos desde el punto de vista del consumidor. Además, no se recomiendan tiempos de maduración más largos que 14 días, debido al deterioro de color (carne parda) y a la generación de un olor diferenciado que no se traslada en una mayor aceptabilidad por parte del consumidor. Estos resultados ofrecen una caracterización exhaustiva y una mejor comprensión del proceso de maduración en la carne de caballo, aportando suficiente conocimiento a la industria como para establecer un tiempo de maduración óptimo que de lugar a una carne de caballo homogénea y de alta calidad.

SUMMARY

The present Ph.D. Thesis has been conducted in collaboration between Lactiker Research Group at the University of the Basque Country (UPV/EHU), which performs multidisciplinary research in the field of Quality and Safety of Food from Animal Origin, and the research group of Biochemistry of Meat and Meat Products from the Institute of Agrochemistry and Food Technology at the Spanish Research Council (CSIC), which focuses on the study of the biochemical mechanisms responsible for the conversion of muscle to meat.

The main objective of this Ph.D. Thesis was to investigate the effect of ageing time on horse meat quality attributes, which have been measured using instrumental and sensory methodologies. Overall, the quality of horse meat has been less investigated in comparison to other livestock species, and there is a noticeable lack of studies referred to horse meat production practices and related horse meat quality investigations. Therefore, the information generated from this work would allow a better understanding of *post-mortem* factors affecting horse meat quality.

First of all, a literature review about pasture-based horse meat production systems in northern Spain was drafted, which helped to comprehend the associated positive externalities. From the review process, it was concluded that extensive equine breeding produces high-quality meat, while providing several advantages related to the environment (*i.e*., improvement of biodiversity and mosaic landscape, prevention of wildfires, and decrease of greenhouse gas emissions compared to ruminants) and several others related to rural and social development (*i.e*., conservation of traditions and endangered breeds, promotion of rural employment and farm economy). It was, thus, concluded that horse meat could be an alternative red meat although it is considered that further studies are needed to overcome the lack of information about the effect of *ante*- and *post*-mortem factors influencing its quality (**Publication I**).

The attention of this thesis was focused on *post-mortem* practices and specifically, on the effect of ageing time on meat quality attributes; on the most relevant quality parameters. From all the factors that could have an impact in horse meat quality, the ageing process was selected due to the very limited previous studies performed in horse meat and to its well-known effect on the quality of meat from other domestic species. Indeed, it is known that ageing plays a pivotal role in meat quality due to the biochemical and physiological changes occurring during the process. This is especially true for the improvement of tenderness, which is considered one of the most relevant organoleptic attributes considered by the consumer. Other quality attributes such as colour, flavour or water-holding capacity are also affected throughout the process.

Given the species-, breed- and muscle-specific nature of its effect, it was considered relevant to study the ageing time in a currently highly consumed horse breed. In this sense, the establishment of an optimum ageing time, with significant quality improvement detectable by the consumer, would avoid unnecessary investments associated with longer ageing periods. To address the proposed challenge, loin muscle from Hispano-Bretón horse breed was investigated. This breed is well-stablished in northern Spain; it is an important part of the genetic heritage and its conservation is key to maintain mountain ecosystems.

The initial gross composition study proved that meat from semi-extensively reared Hispano-Bretón horses contain mean moisture, protein and ash contents (75.3 %, 20.4 % and 1.03 %, respectively) which are comparable to values reported in meat from other horse breeds slaughtered at similar age. Additionally, a higher content of myoglobin (3.47 mg/ g) and n-3 polyunsaturated fatty acids (1.53 % of total fatty acids) than that usually described in meat from ruminant origin was found. The latter has its origin in the higher capacity of horses to absorb dietary polyunsaturated fatty acids before microbial hydrogenation happens. From this, it was confirmed that although its consumption is currently not popular in most countries, horse meat has the potential to be an alternative red meat taking into account its nutritional composition and associated positive externalities or ecosystem services (**Publication I & III**).

For the ageing study, horse loin steaks were vacuum packed and stored at 4 °C in the dark for 0, 7, 14 and 21 days. First, the effect of ageing time on most relevant meat quality attributes was studied using instrumental methods. More concretely, volatile compound composition, pH, instrumental colour, shear force, cook loss, and myofibrillar sub-proteome. Then, consumer preference and sensory description of horse meat were investigated using sensory methods.

First of all, in order to study texture quality attributes, shear force and cook loss were selected. The improvement of tenderness (decrease in shear force) was observed during the first two weeks of ageing, while cook loss decreased only during the first week of ageing and then maintained constant. After this time (one week) the consumer described meat as 'juicier' compared to the non-aged horse counterpart.

Then, aroma of cooked horse meat was studied at different ageing periods. It was found that several aldehydes such as hexadecanal, and 2- and 3-methylbutanal increased during the ageing process of horse meat, presumably affecting meat odour due to their considerable odorant impact. Overall, periods longer than 14 days were necessary to generate significant changes in the volatile profile, and, consequently, in the aroma of horse meat (**Publication IV**). In terms of meat colour, which is the most determinant factor during purchase, consumer is known to choose a bright cherry-red meat colour, considering it an indicator

of wholesomeness. An increase of redness was observed during the first two weeks of ageing, while colour properties started to deteriorate thereafter. Indeed, meat aged for 21 days shifted to brownish colour (**Publication III**).

Additionally, the effect of ageing time on the myofibrillar sub-proteome of horse meat was studied. Since meat tenderization happens as a direct consequence of *post-mortem* degradation of muscle fibre proteins, unravelling underlying mechanisms would contribute to develop efficient ageing strategies. A novel approach based on liquid isoelectric focusing (OFFGEL) followed by one-dimensional gel electrophoresis (1-DE) was developed. This methodology was proposed as an alternative to traditional two-dimensional electrophoresis (2-DE), offering several advantages as the direct recovery of proteins from the liquid phase and a higher degree of automation (**Publication II**). After testing the suitability of this approach, it was applied to the study of horse meat. Overall, results demonstrated that most protein changes happened during the first two weeks of ageing, in good agreement with increased tenderness confirmed by shear force measurements. In addition, and for the first time, the abundance of several proteins was reported to change in horse meat during *post-mortem* ageing. More concretely, myosin light chain 1, myosin binding protein C fragment and troponins T and I (and their fragments) were significantly changed during ageing, opening the possibility of using them as potential meat tenderness biomarkers (**Publication V**).

Finally, a consumer study was performed in order to evaluate the perception of the consumer about the non-aged (0 days) and aged horse meat (7, 14 and 21 days). This study was performed in the Sensory Laboratory of the University of the Basque Country (Vitoria-Gasteiz) with 120 volunteer participants. Consumers evaluated the in-month and visual acceptability in combination with Check-all-that-apply (CATA) methodology in order to get a sensory description of the horse samples. It was observed that one week of ageing improved both in-mouth and visual acceptability of horse meat, while longer ageing periods were not translated to higher consumer acceptability scores. In fact, the appearance of meat aged for more than 14 days was rejected. CATA descriptive analysis also provided interesting results that, in combination with instrumental measurements, contributed to a better understanding of the ageing effect on horse meat quality. For texture attributes, the

consumer described an increase in tenderness and juiciness of meat aged for one week, while no difference was observed thereafter. This meant that the increase in tenderness determined by shear force measurements after the first week of ageing was not perceived by the consumer. On the contrary, horse meat aroma description made by the consumer was in good agreement with that observed by instrumental measurements, and both indicated that more than two weeks of ageing are needed to create a differentiated horse meat aroma. Regarding the appearance, meat aged for more than two weeks resulted in a negative consumer visual evaluation and was described as spoilt, brownish and not uniform colour (**Publication VI**). These results were in good agreement with the observations made by instrumental colour measurements.

Overall, it was concluded that the optimum ageing time for Hispano-Bretón loin muscle could be established at 7 days. During this period, an improvement in colour and tenderness happened, as well as in consumer acceptability. The second week of ageing resulted in a redder and more tender meat, but this enhancement was not sufficient to be perceived by the consumer. Therefore, the investment required for the second week of ageing would not be justified, at least, from the consumer point of view. Moreover, ageing periods longer than 14 days would not be advisable due to meat colour deterioration (brownish meat) and to the generation of differentiated odour that are not translated into a higher consumer acceptability. These results provide a thorough characterization and a better understanding of the ageing process in horse meat giving the horse meat industry enough knowledge to establish an optimum ageing time in order to get an homogeneous and high quality horse meat.

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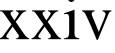

Fatty acid nomenclature

	Systematic name	Common name
12:0	Dodecanoic acid	Lauric acid
14:0	Tetradecanoic acid	Myristic acid
15:0	Pentadecanoic acid	
16:0	Hexadecanoic acid	Palmitic acid
17:0	Heptadecanoic acid	Margaric acid
18:0	Octadecanoic acid	Stearic acid
***i*-16:0**	14-methyl-pentadecanoic acid	Isopalmitic acid
9*c*-14:1	9*c*-tetradecenoic acid	Myristoleic acid
7*c*-16:1	7*c*-hexadecenoic acid	
9*c*-16:1	9*c*-hexadecenoic acid	Palmitoleic acid
9*c*-18:1	9*c*-octadecenoic acid	Oleic acid
11*c*-18:1	11*c*-octadecenoic acid	Asclepic acid
13*c*-18:1	13*c*-octadecenoic acid	
11*c*-19:1	11*c*-nonadecenoic acid	
9*t*-18:1	9*t*-octadecenoic acid	Elaidic acid
9*c*,11*t*-18:2	9*c*,11*t*-octadecadienoic acid	Rumenic acid
20:3n-9	5*c*,8*c*,11*c*-eicosatrienoic acid	
18:2n-6	9*c*,12*c*-octadecadienoic acid	Linoleic acid
20:2n-6	11*c*,14*c*-eicosadienoic acid	
20:3n-6	8*c*,11*c*,14*c*-eicosatrienoic acid	Dihomo-γ-linolenic acid
20:4n-6	5*c*,8*c*,11*c*,14*c*-eicosatetraenoic acid	Arachidonic acid (ARA)
22:4n-6	7*c*,10*c*,13*c*,16*c*-docosatetraenoic acid	Adrenic acid
22:5n-6	4*c*,7*c*,10*c*,13*c*,16*c*-docosapentaenoic acid	
18:3n-3	9*c*,12*c*,15*c*-octadecatrienoic acid	α-linolenic acid
20:3n-3	11*c*,14*c*,17*c*-eicosatrienoic acid	
20:5n-3	5*c*,8*c*,11*c*,14*c*,17*c*-eicosapentaenoic acid	EPA
22:5n-3	7*c*,10*c*,13*c*,16*c*,19*c*-docosapentaenoic acid	DPA
22:6n-3	4*c*,7*c*,10*c*,13*c*,16*c*,19*c*-docosahexaenoic acid	DHA

Protein nomenclature

PROTEIN ID	Full name
ALDOA	Fructose-bisphosphate aldolase A
ATP5F1C	ATP synthase subunit gamma mitochondrial
ATP5PO	ATP synthase subunit O, mitocondrial
CA3	Carbonic anhydrase 3
CAPZA2	F-actin-capping protein subunit alpha-2-like protein
CKM	Creatine kinase M type
CRYAB	Alpha-crystalin B chain
ENO3	Beta enolase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
LDHA	L-lactate dehydrogenase A chain
MDH2	Malate dehydrogenase mitochondrial
MYBPC1	Myosin-binding protein C1 slow type
MYL1	Myosin light chain 1/3 skeletal muscle
MYL3	Myosin light chain 3
MYLPF	Myosin regulatory light chain 2 skeletal muscle
PGAM2	Phosphoglycerate mutase 2
TNNI2	Troponin I fast skeletal muscle
TNNT3	Troponin T fast skeletal muscle
TPM2	Tropomyosin 2
TUBB4A	Tubulin alpha-4A chain
VDAC3	Voltage-dependent anion-selective channel protein 3

Abreviations

1-DE	One dimensional gel electrophoresis
2-DE	Two dimensional gel electrophoresis
A	Animal
a*	Redness
ACN	Acetonitrile
AHC	Agglomerative hierarchical clustering
AS	Animal sex
AT	Ageing time
ATP	Adenosine triphosphate
b*	Yellowness
BCFA	Branched chain fatty acid
C	Consumer
C*	Chroma
c	cis
CATA	Check-all-that-apply
CF	Citation frequency
CFU	Colony forming unit
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CLA	Conjugated linoleic acids
CS	Carcass side
CW	Carcass weight
d	Day
D	Slaughter day
DTT	Dithiothreitol
DMA	Dimethylacetals
DNPH	2,4-dinitrophenylhydrazine
ESI	Electrospray ionization
FA	Fatty acid
FAME	Fatty acid methyl ester
FID	Flame ionizing detector
FoA	Formic acid
GC	Gas chromatography
GLM	General linear model
GHG	Greenhouse gas
h	Hour

h*	Hue
HB	Hispano-Bretón
IHD	Italian Heavy draft
IMA	In-mouth acceptabilityy
IMe	Identification method
IPG	Inmobilized pH gradient
IS	Internal standard
L*	Lightness
LAB	Lactic acid bacteria
LASEHU	Laboratory of the University of the Basque Country
LC	Liquid chromatography
LTL	*Longissimus thoracis et lumborum*
LOD	Limit of detection
LRI	Linear Retention Index
Min	Minutes
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
OFFGEL	Liquid isoelectric focusing
OIR	Odour impact ratio
OT	Odour threshold
PCA	Principal component analysis
PLS-DA	Partial least squares discrimination analysis
pI	Isoelectric point
PUFA	Polyunsaturated fatty acid
PVC	Polyvinylchloride
S	Session
SC	Subcutaneous
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFA	Saturated fatty acid
SPME	Solid-phase microextraction
T	Tissue
t	trans
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
Tris	Tris-hydroxymethyl-aminomethane
VA	Visual acceptability
WBSF	Warner–Bratzler shear force
WHC	Water holding capacity

SECTION I

1. STATE OF THE ART

1.1 General framework

The global population is predicted to grow from 7.9 billion in 2022 to 9.7 by 2050 (1) and agricultural production is expected to scale up worldwide in response to this trend (2). According to the 2030 Agenda for Sustainable Development from the United Nations, in an increasing agricultural production scenario, the main challenges that need to be addressed are undernourishment, sustainable use of natural resources and response to climate change (3). In this regard, the Food and Agriculture Organization of the United Nations has concluded that, overall, alternative production systems that are less detrimental to the environment need to be developed (2). These challenges of the whole agricultural production system are also undoubtedly translated to livestock farming and, consequently, to the production of meat, an important component of human diet that supplies primarily proteins and essential macronutrients.

1.2 Horse meat production and related ecosystem services

Horse meat production, although less popular comparing to that from other species (4), constitutes an alternative that offers several positive externalities (**Figure 1.1**). The latter are usually related to the typical pasture-based production systems from equine farms located in mountain regions. With reference to environmental services, when appropriate grazing management is carried out (5), extensive equine breeding has proved to effectively prompt biodiversity. More concretely, the consumption of dominant plant species by horses promotes the coexistence of a wider variety of plants, and the concomitant diversity in animal species (6). In addition, horses feed selectively, maintaining grassland patches of good fodder quality and zones of taller grass which they avoid and where faeces are often concentrated ('latrine patches') (7). This contributes, with the creation of a heterogeneous mosaic of vegetation patches, to the recovery of the characteristic landscape mosaicism that has been disappearing mainly by the exodus and abandonment of traditional practices (8).

Changes in landscape have been exacerbated by drier and hotter conditions than in the last decades, as well as by the loss of open spaces caused by forest expansion and shrub encroachment. Ultimately, all these factors entail a risk for wildfires due to the accumulation of vegetation fuels (9,10). The big frame size and rapid digestion process of horses ensures the digestion of high amounts of biomass while grazing. This means that pasture-based equine breeding constitutes an effective tool for reducing vegetation fuels in critical areas of the landscape.

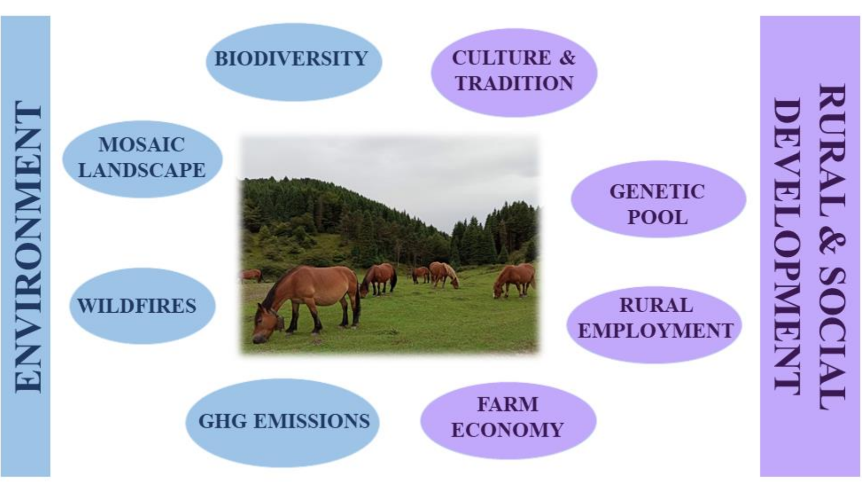

Figure 1.1 Advantages related to pasture-based horse meat production system. Environmental advantages have been represented in blue while advantages related to rural and social development have been represented in purple.

A further environmental advantage of horse meat production over that of big frame ruminants such has bovine is related to greenhouse gas (GHG) emissions. Overall, GHGs originated from livestock farming are on average up to 14.5 % of all the human-induced ones (11) and, therefore, there is a need to reduce these emissions in the race of managing global warming. Among the GHGs emitted from livestock, methane (CH_4) is an especially important target because it is a potent and a short-lived gas, thus, reducing its emissions would provide fast positive returns. It is in this context that horses may consist on a less stressful alternative for the environment. Indeed, the emission factor of methane (kg CH_4 per head) coming from enteric fermentation is, in general, much lower in horses compared to ruminants. This phenomenon has been attributed to the simple stomach of horses (monogastric), which follows an opposite strategy compared to ruminants. In horses, the

large digestive fermentative chambers are located in the hindgut, where a much higher reductive acidogenesis than in the rumen fermentation takes place, resulting in a lower methanogenesis (12). However, other authors state that still undefined reasons, other than rumen or hindgut, influence CH_4 production levels (13). Nevertheless, horse meat production is not generally mentioned in mitigation strategies designed for livestock farming, probably due to the general lack of popularity of the use of horses for meat production purposes.

Together with the environmental benefits, equine farms dedicated for meat production have the potential to contribute to the rural and social development. Farms maintain a primary activity that is part of the cultural heritage, which is being abandoned in most European mountain regions (14). In addition, local breeds are often used, many of which have been classified as endangered breeds and have been included in the list of Domestic Animal Diversity Information System hosted by the Food and Agriculture Organization of the United Nations (15). To this extent, equine farming supports the conservation of these breeds (12). Besides that, employment and economy could be enhanced by diversification of the production in rural areas, which means, for example, additional income to the regular beef cattle breeding. In essence, the promotion of horse meat production, commercialization and consumption in rural and urban areas could help mitigating depopulation.

The role of horses as grazers has also some disputed points, as is the case when horses share grasslands with other grazing species such as cattle or sheep. In those cases, having similar diet preferences, they would compete for quality forage. In addition, equids are less efficient in fibre digestion, so they need to consume more than ruminants to reach their nutritional requirements (12). However, considering all the aforementioned points, it seems clear that horse meat production systems have the potential to provide the market with an alternative food obtained with less environmental impact. This is also in good accordance with the demands of the modern consumer, increasingly concerned about the environmental impact of the products they purchase (16). Despite all this, there is a noticeable lack of studies and standardized practices related to *ante-mortem* (breed, feeding strategy, sex, slaughter age and weight) and *post-mortem* (meat ageing, packaging) practices related to horse meat production resulting in heterogeneous or non-consistent horse meat quality in

the market. It is, therefore, considered that additional investigations are needed, especially in relation to *post-mortem* practices, to move forward and prompt the horse meat industry towards the production of a high quality product with the ability to attract consumer trust (**Publication I,** Appendix I).

1.3 The ageing process and its applicability to horse meat

Meat ageing refers to the intervention in which meat is *post-mortem* chill-stored for a certain duration of time in order to develop desired quality attributes while preventing spoilage. During decades, ageing strategies have been refined and a whole research field has opened in order to investigate its impact on meat quality attributes.

1.3.1 Meat ageing

Traditionally, carcasses or primal cuts of meat were hung (tender stretch and Achilles methods) in a cold place for ageing, but the appearance of vacuum packaging allowed conducting the process under more controlled conditions. Currently, wet ageing (also named vacuum ageing) is the prevailing form of ageing in European countries and consists on placing primal or sub-primal cuts of meat in a vacuum package and storing them under a controlled temperature for a specific period. Throughout the process, a proper vacuum package and temperature control are necessary, while the main purpose is to achieve a higher degree of tenderness (softer meat) (17,18). This is the type of ageing covered in the present Ph.D. Thesis. Alternatively, dry ageing is another form of ageing in which carcasses, pieces or cuts are placed unpacked in specific coolers or chambers, under controlled temperature, relative humidity and airflow. The higher costs of specialized infrastructure together with product loss caused by shrinkage, moisture loss and trimming makes this form of ageing particularly expensive and directed mainly to specialised restaurants or gourmet markets. To this extent, it is usually practised with specific high quality beef sub-primals, such as rib and loin, and the main objective of dry ageing is the development of specific flavours together with tenderness (19,20).

1.3.2 Horse meat ageing: opportunities and current research

It is widely known that the effect of ageing on meat is species-specific. This means that the rate of ageing is different for different animal species; however, most of research with respect to meat ageing has been carried out in beef. Furthermore, differences in ageing effect among cattle breeds have been observed (21) and, thus, differences among horse breeds are also expected. In this regard, the ageing process should be studied in meat from at least those horse breeds primarily destined to human consumption. This would generate valuable information that would help the horse meat industry to understand and standardize *post-mortem* practices such as ageing.

To date, very limited studies have been performed regarding horse meat ageing. The evolution of several meat quality attributes during ageing periods of up to two weeks has been studied in meat from Galician Mountain (22), Hispano-Bretón (HB) x Galician Mountain crossbreeds (23), Italian Heavy draft (IHD) (24-26), Burguete (27), and Galician Mountain x Burguete horses (28). Additionally, the ageing period was extended up to 30 days in meat from Jeju crossbreeds (29). In general, due to the limited number of studies and numerous sources of variation, specific conditions to maximize the positive impact of ageing on horse meat are far from being understood and, therefore, further improved.

1.3.3 Major biochemical and physiological events taking place in *post-mortem* muscle

The ageing process plays a pivotal role in the quality attributes of meat because of *post-mortem* biochemical and physiological events occurring (represented in orange colour in **Figure 1.2**). The extent of these changes, considering the same time frame, is affected by several key factors. Among them, pre-slaughter factors involving animal and muscle characteristics or animal handling and post-slaughter factors related to ageing conditions are the most relevant ones (represented in green colour in **Figure 1.2**). These factors influence meat quality attributes (represented in blue colour in **Figure 1.2**) in different ways. In this regard, multiple factors and interactions need to be considered for the development of effective ageing strategies to improve meat quality. In the following sections, the principal biochemical mechanisms and physiological changes by which *post-mortem* ageing time affects the most relevant meat quality attributes will be addressed.

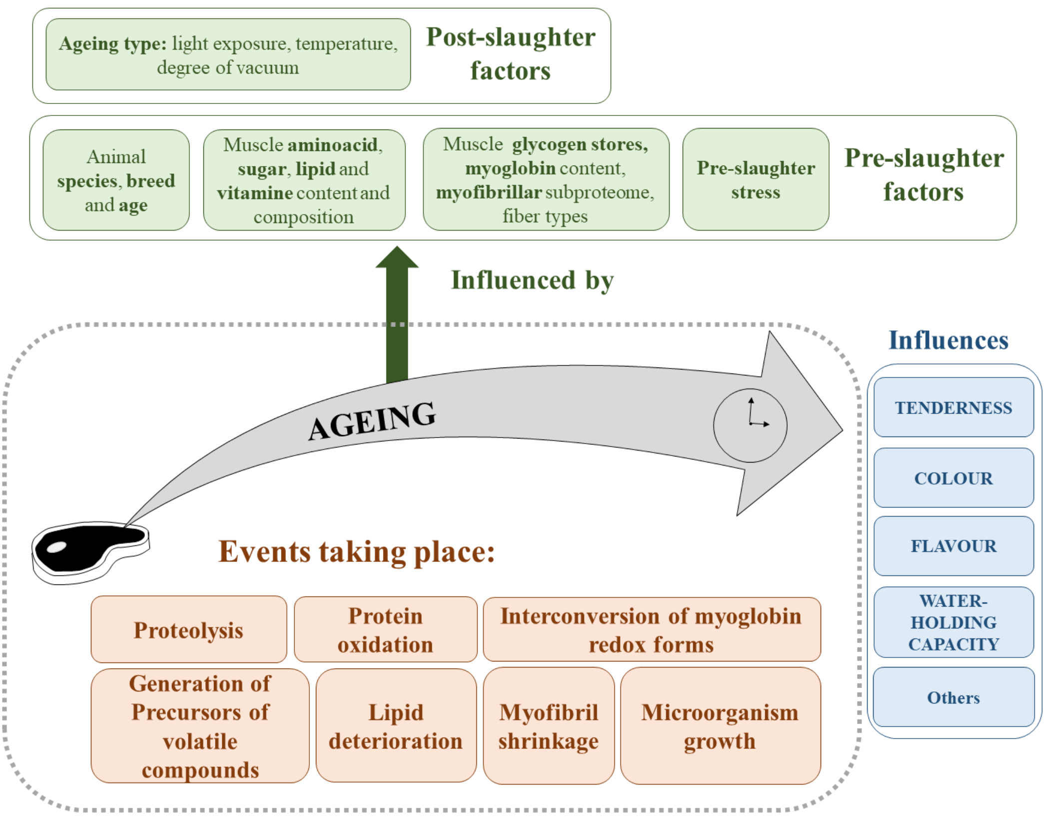

Figure 1.2 The most relevant biochemical and physiological events taking place in *post-mortem* muscle (orange), factors influencing the rate of the ageing process in *post-mortem* muscle (green) and meat quality attributes influenced by the ageing process (blue).

1.3.3.1 Conversion of muscle to meat and proteolysis

After animal slaughter, the blood flow ceases, oxygen supply is cut off and, consequently, energy sources are depleted. At this early *pre-rigor* phase, there are factors that trigger programmed cell death or apoptosis in muscle. Simultaneously, at this phase, the objective of the cell is to produce energy through the synthesis of mitochondrial adenosine triphosphate (ATP) in order to fight against apoptosis, try to preserve cellular functions and keep with the process of muscle contraction. In this context, initially, aerobic metabolism is maintained thanks to the presence of ATP, residual oxygen and phosphocreatine. Moreover, at this early *pre-rigor* stage, increased concentrations of several heat shock proteins have been described. More concretely, their synthesis is induced in response to cell damage, since these proteins preserve cellular proteins against denaturation and possible loss of function (30).

Once ATP and oxygen reserves are exhausted, metabolism is switched from aerobic to anaerobic, being glycolysis the main pathway for ATP supply and entering in a *rigor* phase. During anaerobic glycolysis, glucose is converted to pyruvate, leading to lactate production and resulting in the characteristic *post-mortem* pH drop until reaching the ultimate pH (24-hour pH or pH_{24h}). The glycolytic pathway ends when one or more glycolytic enzymes are deactivated due to the acidic pH; or when there is no more glucose available (depleted glycogen stores). At this point, no ATP is produced to assure the muscle contraction mechanism and meat reaches the *rigor mortis* phase; meats toughest state where the highest shear force values are recorded.

After reaching the *rigor mortis* state, the degradation of the highly organized muscle fibre takes place; a process named meat tenderization and characterized by a decrease in meat toughness or the mechanical resistance of meat (**Figure 1.3**) (30-32).

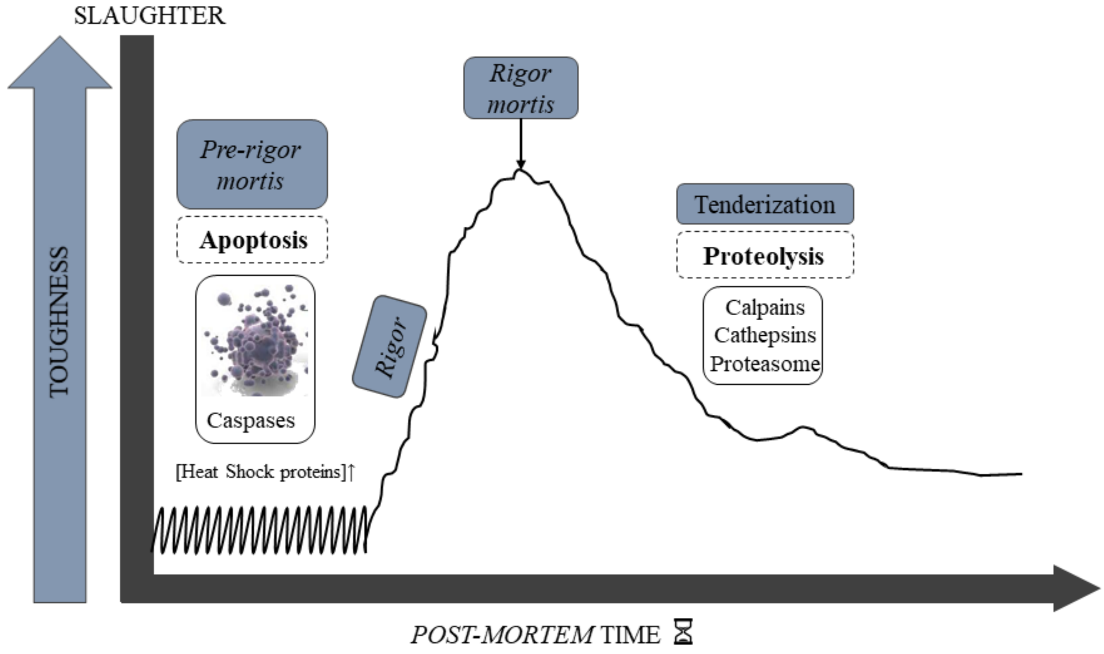

Figure 1.3 Graphical representation of the different phases in the conversion of muscle to meat. Adapted from Ouali *et al*. (31).

Muscle fibres, composed of myofibrils, have a well-known and defined structure, with a highly organized protein network in charge of maintaining muscle architecture and

contraction properties. Repeating contractile units, known as sarcomeres, are linearly arranged as follows: Z line, half I band, A band, half I band, and Z line. Longitudinally, the protein titin connects Z and M lines (**Figure 1.4**).

The structure of the sarcomere is composed by thin and thick filaments made of actin and myosin, respectively. These two proteins, together with nebulin, the troponin complex (T, C and I) and tropomyosin, are the proteins involved in the process of muscle contraction by the formation and dissociation of the actomyosin complex. During the ageing process, all these proteins are well-known targets for protein degradation due to the action of endogenous muscle proteases. A series of peptidase families (and their inhibitors) have been identified in skeletal muscle and proposed to play an important role in the softening of the myofibrillar structure. This is the case of calpains, cathepsins, proteasome, matrix metallopeptidases and caspases (33).

Calpains have been traditionally the most extensively studied protease family in relation to meat ageing. They constitute a large family of intracellular cysteine proteases that are expressed in skeletal muscle as μ-calpain, m-calpain and p94 (tissue-specific calpain 3). Associated with these calpains, the calpain-specific endogenous inhibitor, calpastatin, prevents their proteolytic activation and the expression of their catalytic activity. Experimental evidence has supported the contribution of calpains on meat tenderization and the positive relation of calpastatin with meat toughness (34). Additionally, cathepsins are a complex group of enzymes comprised of both exo- and endo-peptidases belonging to cysteine (cathepsins B, H, L and X), aspartic (cathepsins D and E) and serine (cathepsins G) peptidase families. Although their role in meat tenderization has been questioned due to their location in the lysosomes, evidences have also supported the contribution of cathepsins to meat tenderization (34). Matrix metallopeptidases, on the other hand, degrade collagen and other proteins in charge of the spatial organization of the collagen fibrils.

The proteasome is a multicatalytic protease complex that has also been suggested to be related to *post-mortem* structural changes of myofibrils. This complex is involved in the regulation of basic cellular pathways by degrading abnormal, denatured or damaged proteins in the cytosol and nucleus of cells (33). Its catalytic core, the 20S proteasome, is the one that has been suggested as responsible for *post-mortem* protein degradation,

supported by evidence found in bovine muscle. Moreover, the loss of activity of 20S proteasome caused by *post-mortem* muscle conditions was found to be lower compared to the activity of μ-calpain. Therefore, authors suggested that, in *post-mortem* muscle, the 20S proteasome would very likely have a higher proteolytic potential than μ-calpain (35).

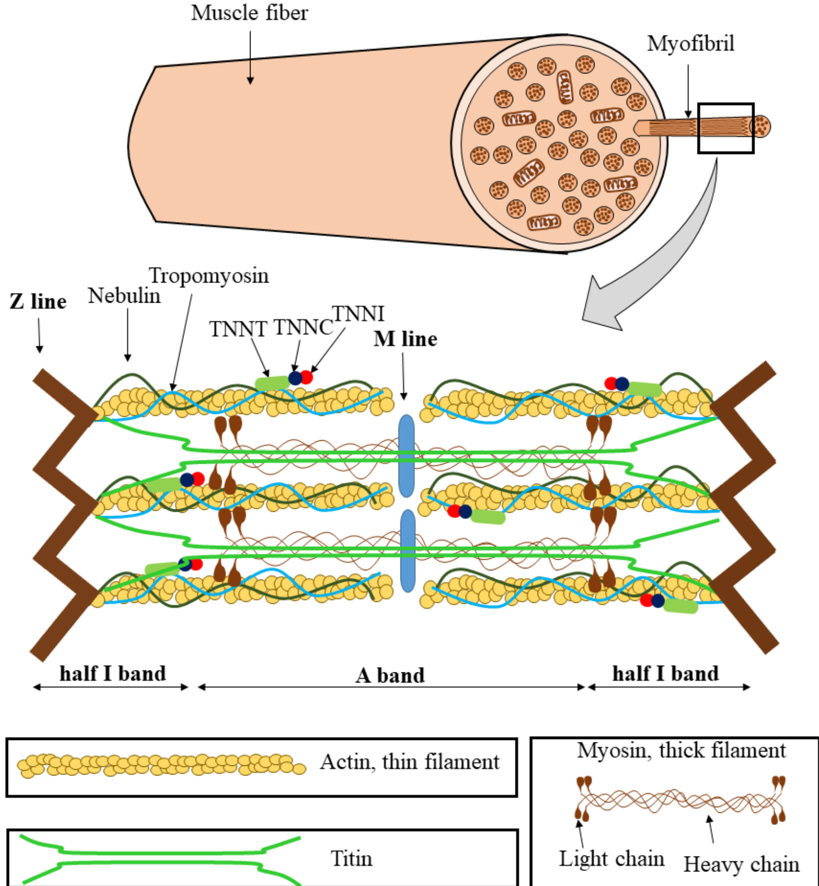

Figure 1.4 Simplified schematic representation of the myofibril and its structure, with the succession of basic structural and contractile units (Z-I-A-I-Z) known as sarcomeres. Principal proteins integrating the myofibrils have been localized.

TNNT, troponin T; TNNC, troponin C; TNNI, troponin I.

More recently, caspases have been introduced as a group of enzymes related to *post-mortem* muscle biochemistry, although these are reported to act during the first steps of muscle to meat conversion (**Figure 1.3**). Caspases are a family of cysteine dependent peptidases that are mainly synthesised as inactive zymogens (pro-caspases). Under stimuli, they initiate a

series of controlled reactions that ultimately lead to cell death by selectively cleaving C-terminal aspartic acid residues of peptides/proteins (36). More in detail, these peptidases are classified according to the point of entry to the cell death pathway as initiator (caspases 8, 9, 10 and 12) or executioner (caspases 3, 6 and 7) caspases. In *post-mortem* muscle, the most extensively investigated mechanism for cell death consists on caspase activation through an intrinsic pathway by means of internal stimuli (metabolic or hypoxic stress), favouring the release of cytochrome C from the mitochondria and the formation of caspase 9 apoptosome complex. The latter leads to the activation of executioner caspases 3 and 7 that will proceed with cell dismantling. The second mechanism involves an extrinsic activation pathway, in which caspases 8 and 10 are activated through extracellular receptors and subsequently activate executioner caspases 3 and 7. On the contrary, the activation of caspase 12 initiates the apoptotic cascade directly through the endoplasmic reticulum, while caspase 6 is still not as extensively studied as the rest and its role in cell death is still unclear (36-38).

In essence, proteolysis associated to the process of muscle to meat conversion would be a multi-enzymatic process.

1.3.3.2 Generation of precursors of volatile compounds

Odour or aroma is developed during cooking by the generation of odour active volatile compounds. These compounds result from different complex reactions such as Maillard reaction between amino acids and sugars, lipid oxidation, vitamin degradation and interaction between Maillard reaction products with lipid oxidation products, such as Strecker degradation (39,40). The contribution of volatile compounds to cooked meat aroma depends on the concentration and odour threshold (OT) of the odour active volatile compounds (41). In addition, intrinsic factors including animal species, breed, sex and muscle have been reported to influence the volatile profile of cooked meat (39).

During the ageing process, changes in the concentration of aroma precursors (amino acids, sugars, lipids and vitamins) take place in raw meat, mainly by bacterial action, lipid oxidation (section 1.3.3.4) and enzymatic reactions. As a result of occurring changes, the volatile profile of cooked meat can be modified.

1.3.3.3 Interconversion of myoglobin redox forms

Myoglobin is a sarcoplasmic protein primarily responsible for the colour of meat. Overall, the content of myoglobin varies primarily according to animal species, breed and/or muscle. This, together with its chemical forms (**Figure 1.5**), influences the basal colour of meat as well as *post-mortem* colour evolution. Myoglobin protein consists of a peptide chain (globin) and a pigment component (heme group), which is present in a hydrophobic pocket of the globin. The heme group, containing an iron atom with four connections to the heme ring, is bound to the globin (5th ligand) via a histidyl residue from the latter (93 position).

Myoglobin has the ability to reversibly bind oxygen. Indeed, when the iron binds the globin, it is raised above the heme plane allowing oxygen to bind (6th ligand) and moving the iron to the original position; this induces a conformational change of the globin. A prerequisite for the heme group to covalently bind oxygen and form oxymyogobin (**Figure 1.5**, reaction A) is the existence of an electron donor ligand, and this function is undertaken by the imidazole side chain of histidine 93. A second histidine residue of myoglobin (64 position) contributes to the stabilization of the oxygen molecule. Changes in pH would affect the protonation and deprotonation of the imidazole ring from histidines, influencing electron density and, consequently, the form of myoglobin.

With this in mind, the colour of meat is determined by the ratios of three forms of myoglobin. Myoglobin with no oxygen in its structure and reduced iron (Fe^{2+}) is purple and is named deoxymyoglobin. The covalent complex of ferrous (Fe^{2+}) myoglobin and oxygen, oxymyoglobin, is of bright-red colour, and the oxidation product of deoxymyoglobin in the ferric state (Fe^{3+}) is of brown colour, named metmyoglobin.

Overall, when meat is vacuum packed, the oxygen depleted atmosphere causes meat surface turn purple colour (deoxymyoglobin). If the package is immediately opened and exposed to air (oxygenated), purple surface turns back to red colour (oxymyoglobin). However, additional biochemical changes take place during the vacuum storage of meat through the ageing process, which will depend on the length of storage but also on livestock species and breed.

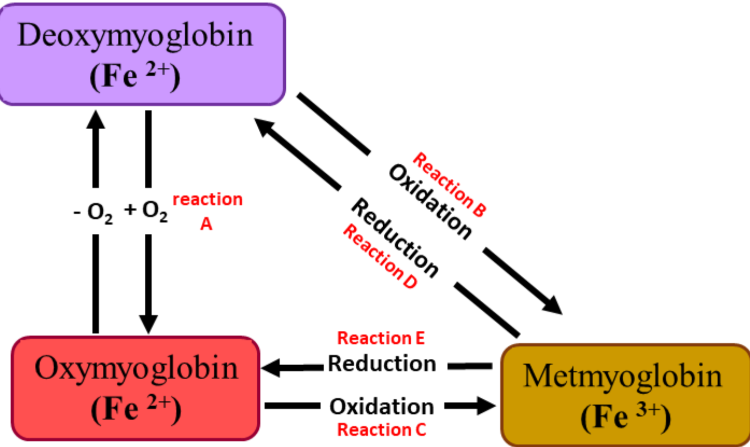

Figure 1.5 Interconversion of myoglobin redox forms in vacuum packed fresh meats. Adapted from Mancini & Hunt (42).

If short times of vacuum storage periods are considered, a redder meat is obtained after ageing and subsequent oxygenation or blooming. This is because initially, in *post-mortem* muscle, oxygen consumption to produce mitochondrial ATP is lower (section 1.3.3.1) and, therefore, more residual oxygen is available to bind deoxymyoglobin. Consequently, a thicker layer of oxymyoglobin is formed under the surface (**Figure 1.5**, reaction A), and thus, meat is redder after oxygenation.

On the other hand, if longer vacuum storage times are contemplated, additional reactions need to be considered. Indeed, under low oxygen partial pressures such as under vacuum, myoglobin and oxymyoglobin are slowly but continuously oxidized to metmyoglobin (**Figure 1.5**, reactions B and C). Contrary to early *post-mortem* situation, mitochondrial reduction activity decreases after extended ageing and, in consequence, these reactions can not be converted back via reduction (**Figure 1.5**, no possibility of reactions D and E). This leads to the formation of a layer of metmyoglobyn giving brownish colour to the meat (43). On the other hand, decreased mitochondrial reduction activity can also result in oxygen available to bind myoglobin and form oxymyoglobin. Overall, the rate and extent of these changes are species-, breed- and muscle-specific, so particular cases should be independently evaluated.

1.3.3.4 Lipid deterioration

Lipids are important constituents of meat and contribute to many of its desirable (and undesirable) characteristics (*i.e.*, flavour, texture). Lipid deterioration is a particularly relevant cause of odour defects in meat, mainly due to the generation of secondary oxidation products. Of course, for vacuum aged meat, deterioration would be reduced or at least delayed. On the one hand, lipolytic enzymes (endogenous or from microbial origin) break down fatty acids (FAs) from triacylglycerols and phospholipids, releasing free FAs, phosphate and glycerol. In meat, free FAs do not have such negative organoleptic consequences compared to other products such as milk. On the other hand, FAs and particularly unsaturated FAs are susceptible to oxidation. The latter is an unavoidable spontaneous process in which unsaturated FAs react with molecular oxygen via reactive oxygen species.

At an early stage of oxidation and due to the interaction with oxygen, a hydrogen atom is removed from unsaturated FAs, leading to the creation of alkyl radicals, that are considered the first oxidation products. These can further react with molecular oxygen and generate several reactive oxygen species such as peroxyl radicals. Reactive oxygen species are highly unstable and find stability in subsequent propagation stages, by removing hydrogen atoms from other susceptible unsaturated FAs. The process is continuously repeated leading to a series of reactions that may occur up to 100 times before two radical species combine and terminate the process by generating a non-radical product. The peroxides that are commonly formed as termination products can further undergo scission to form lower molecular weight compounds such as carbonyls, alcohols, hydrocarbons, and furans. Overall, this process leads to lipid degradation and to the development of oxidative rancidity compounds, which depend on the characteristic lipid composition of the animal, as well as on storage conditions and on the presence of catalysts of the reaction such as haemoglobin and myoglobin. (44,45). In addition, anti or pro-oxidants could naturally occur or be artificially added to the meat matrix, in order to prevent or delay oxidation processes (46).

1.3.3.5 Protein oxidation

Oxidation of meat proteins during ageing has not been as extensively studied as that of lipids. Indeed, due to lack of suitable and specific methodologies, protein oxidation was ignored for years. Nowadays, the process is known to occur when protein radicals are formed. This is believed to happen via a free radical chain reaction similar to that of lipid oxidation but a larger variety of oxidation products. Moreover, secondary lipid oxidation products have been reported to react with muscle proteins, and in this sense, a high correlation between lipid and protein oxidation has been demonstrated in model systems (47).

Protein oxidation may result in the formation of covalent intermolecular crosslinking, amino acid side chain modifications, and/or protein fragmentations (48). Clearly, anaerobic vacuum storage leads to reduced levels of carbonyl formation; however, the process has been demonstrated to happen even when meat is vacuum packed (49).

1.3.3.6 Modification of myofibrillar structure and associated water retention

When meat enters the *rigor mortis* phase, it reaches its toughest state through the formation of crosslinks among muscle filaments (section 1.3.3.1). At that point, water is pushed out of myofibrils due to myofibrillar shrinkage and ultimately out of the muscle cells. However, in extended ageing, myofibrils have the capacity to retain more water (17). Several explanations have been provided for this phenomenon but it is believed to happen as a consequence of *post-mortem* proteolysis of structural proteins. The degradation of crosslinks during ageing has been suggested to leave more space within muscle fibres to retain water (50).'The sponge effect' theory has been also proposed, where myofibrillar proteins are broken down to disturb drip channels increasing the ability of meat to retain water (51). Considering either of these explanations, early *post-mortem* events including pH drop and proteolysis will be key factors for the evolution of myofibrillar structure during ageing.

1.3.3.7 Growth of microorganisms

Meat is a nutrient-rich medium that has the potential to allow microorganism growth and express their metabolic functions, potentially limiting the shelf life of the product (52). During storage, therefore, microorganisms present in meat interact together and with the meat substrate.

Microbial growth is highly dependent on the hygiene practices and storage conditions, and in this sense, a high number of potentially pathogenic microorganism have been related to contamination derived from the lack of good hygiene practices in meat (*i.e*., *Esterichia coli, Staphylococcus, Listeria monocytogenes, Salmonella* or *Clostridium perfringens*). However, if no contamination is present, the environment of vacuum ageing (vacuum at refrigeration temperature) will, in general, decrease or delay the growth of microorganisms. In this sense, under anaerobic conditions, the growth of aerobic bacteria (*i.e.*, strictly aerobic *Pseudomonas*) is supressed. Typically, mesophilic bacteria dominate the initial microflora of vacuum packed meat, while during storage lactic acid bacteria (LAB) will increase their count and dominate (53,54).

Using the carbon sources they consume from meat, LAB produce lactic acid and, consequently, lead to a pH drop that also exerts antimicrobial activity. As they constitute a very large group that encompasses more than 10 genera, LAB growth may have different effects depending on the strains and also on the type of meat. In any case, they are generally considered relatively innocuous regarding the generation of spoilage manifestations (*i.e*., off-odours) (55). Nonetheless, in extended storage, LAB reach maximum counts and their metabolic by-products accumulate until they are organoleptically detectable. Regarding other types of microorganisms such as moulds and yeasts, these are generally not considered as spoilage microorganisms of stored meat even if they constitute a small part of the resident microbiota (56).

1.3.4 Measurement of meat quality attributes affected by *post-mortem* biochemical and physiological events

Biochemical and physiological events taking place in *post-mortem* muscle (**Figure 1.2**) have an impact, sometimes with synergistic effect, on meat quality attributes. In the following sections, changes happening on the most relevant attributes will be addressed, as well as the most extended methods for their measurement. Studies performed in horse meat, although limited, will also be discussed.

1.3.4.1 Meat tenderness

Meat tenderness is considered one of the most relevant organoleptic attributes influencing meat quality (57). It is influenced majorly by several intrinsic factors including animal age, breed (21), muscle type (58), muscle fat content, collagen content and collagen solubility (59). All these parameters contribute to the basal tenderness of meat. On the other hand, it is widely accepted that having the basal tenderness as a starting point, meat is tenderized during the ageing process by the fragmentation of structural and associated proteins integrating the myofibrillar structure, which is mediated by muscle proteolytic systems (30). Improvement (increase) of meat tenderness is the principal reason why the ageing process is carried out (17). The tenderization process is framed in the conversion of muscle to meat (section 1.3.3.1; **Figure 1.3**) while protein oxidation (section 1.3.3.5) has been also proposed to negatively affect the degree of tenderness. However, the exact mechanisms by which protein oxidation affects tenderness have not been completely elucidated yet and it has been suggested that tenderness could decrease by either the creation of crosslinks among myofibrillar proteins or by the inactivation of proteolytic systems (*i.e.*, calpains) caused by protein oxidation (60,61).

Meat tenderization during ageing has been widely studied on the most consumed meat species (*i.e.*, poultry, pork, beef) by measuring tenderness at different time points of the process. For the objective determination of meat tenderness, several devices have been developed to measure the forces required for meat disruption. The most widely used device is the Warner–Bratzler shear, that measures the force required, namely Warner–Bratzler shear force (WBSF), to cut a meat core. Tenderness is usually expressed as a resistance

measurement such as in kilogram (kg) or newton (N), so that low values indicate more tender meat (62).

Other complementary methods have also been used together with WBSF in the study of tenderness development. These are most of the times, based on the measurement of intrinsic determinants of tenderness, or alternatively, based on the measurement of crosslinking, proteolysis or other factors affecting tenderness degree. Some examples are the measurement of sarcomere length by microscopy diffraction, the measurement of collagen amount and solubility, the determination of myofibrillar fragmentation index of muscle proteins and the study of protein profile by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) in one or two dimensional gel electrophoresis (1-DE or 2-DE). Usually, the last two approaches are linked to the characterization of proteins by liquid chromatography (LC) and mass spectrometry (MS) analyses. In addition, the quantification of carbonyl compounds by using the routine 2,4-dinitrophenylhydrazine (DNPH) method has been widely employed for the determination of protein oxidation in meat. This method is based on the derivatization of protein carbonyls with DNPH to form a complex that can be identified and quantified by LC-MS.

Sensory methods are also a useful approach to study tenderness and its evolution during ageing, having even the capacity to carry out the simultaneous evaluation of more than one attribute. Compared to instrumental methods, sensory methods have not been widely used due to the higher variability of the obtained results, the longer time needed for implementation and, in general, higher costs. There are several ways to evaluate meat quality attributes by means of sensory studies using trained panels and untrained consumer tests. Overall, consumer tests usually focus on hedonic measurements where the acceptability of a certain product is measured in a hedonic scale, while sensory descriptive analyses are typically performed by trained panels. The high specialization of trained panels permits the collection of detailed, robust and consistent results. However, creating and maintaining them can be quite expensive and time consuming (63).

Using the variety of methods described above, the optimum duration of the ageing process to reach optimum tenderness values has been agreed for different meat species such as poultry (12-14 hours), pork (5-7 days), lamb (7-10 days) and beef (14 days) (64). Of course,

this is a generalization, because breed- and muscle-specific factors will modulate the evolution of tenderness. In the particular case of horse meat, shear force measurements showed that tenderness improved during the first 10 days of ageing in tenderloin steaks from Jeju horses (29), while other authors reported earlier tenderization rates. This was the case of meat from IHD horses which showed the most tender meat after only 3 days of ageing (24). Intermediate periods of 4 and 8 days have been suggested to obtain tender meat in Galician Mountain x Burguete horses, depending on the feeding strategy (28).

On the other hand, carbonyl content of meat from Galician mountain and HB x Galician mountain horses was analysed up to two weeks of ageing, and no significant differences were found among time points when meat was vacuum aged (22,23). However, another study reported a significant increase in carbonyl content after 9 days of ageing in meat from IHD horses (26). Regarding the sensory evaluation of horse meat tenderness, trained panellists did not report differences between non-aged and aged meat in the two studies carried out in meat from Jeju and IHD horses vacuum aged up to 30 and 21 days, respectively (26,29).

1.3.4.2 Meat colour

It was previously indicated that tenderness is one of the most important attributes to define meat quality especially during consumption. However, colour is the most important one influencing the initial purchase decision (42). A bright cherry-red colour is commonly perceived as an indicator of wholesomeness in fresh meat. On the contrary, meat discoloration results in high economic loses and product wastage (65). In this sense, the duration of the ageing process should be optimized to avoid the irreversible formation of metmyoglobin, together with other factors such as temperature and light that may also alter muscle metabolism. In this context, it is noteworthy to point that a higher myoglobin content compared to other meats has been reported in horse meat (66). This may influence both the colour of non-aged meat and the evolution of colour during the ageing process.

For the instrumental measurement of colour, colorimeters and spectrophotometers are typically used. These two types of equipment differ in the way they measure the reflected light but normally in both cases the obtained results are converted to CIE $L^*a^*b^*$ values.

This allows meat colour to be expressed in a three dimensional space, where *a** corresponds to redness, *b** to yellowness and *L** to lightness. In addition, sensory methods, both based on trained panels and consumer tests, can be used for colour perception and evaluation. In the particular case of evaluating the appearance of meat, sensory methods may be challenging to design because a thorough standardization of sample presentation (cutting, packing) and environment (light, temperature) is needed. For such cases, sensory evaluation of appearance, the use of professional photographs has been proposed as a good alternative (67).

Overall, studies have shown that when beef is vacuum aged between 7 and 14 days it develops a brighter red colour than non-aged beef, but longer periods can lead to discoloration (68). However, previous studies that have measured the evolution of instrumental colour during vacuum ageing of horse steaks have documented very diverse, and sometimes contradictory, trends. On the one hand, increases in redness have been described from non-aged to meat aged for 4 days in meat from Galician Mountain breed (23), and from meat aged for 7 days to meat aged for 14 days in Galician Mountain x HB crossbreeds (22). On the contrary, no differences in redness were observed in meat from IHD horses aged up to 14 days (25). In Jeju horses, no difference in redness was found in the loin while an increase in redness was recorded in tenderloin aged for 20 days. Regarding lightness, either no difference during ageing (25,29) or an increase from non-aged to meat aged for only 4 days have been reported (22,23). With reference to yellowness parameter, it has been described to increase with ageing but at different time points of the process. An increase was observed from non-aged to horse meat aged for 10 (23,29) or 7 (22) days.

There are no scientific records of sensory evaluation of redness during horse meat storage.

1.3.4.3 Meat flavour

Meat flavour is the sensory trait perceived by taste and smell buds. Its perception is a complex phenomenon and it is the odour or aroma of meat that accounts for the dominant sensory trait referred to as flavour (69). The characteristic aroma of cooked meat is an important quality attribute for consumer acceptability (70). Precursors of volatile compounds generated during ageing need to be taken into account in order to understand

and control the overall aroma of cooked meat. Odour defects coming from extended ageing periods need to be avoided. In this regard, lipid oxidation should be a matter of study in the particular case of horse meat ageing, due to its particularly high content of heme-containing myoglobin (section 1.3.4.2) and unique lipid composition rich in essential polyunsaturated FAs (PUFAs) (linolenic, 18:3n-3 and linoleic, 18:2n-6 acids).

Overall aroma of cooked meat has been traditionally evaluated by trained panels and consumer studies. However, estimation of the sensory contribution of each single odour active compound to meat aroma is carried out instrumentally by previously extracting them using steam distillation-extraction or solid-phase microextraction (SPME) followed by their identification using gas chromatography (GC) coupled to MS or flame ionization detectors (FID). These methods allow the detection of meat volatile odour compounds without identifying odour characteristics of detected compounds. For that purpose, results obtained need to be combined with further results obtained from GC-olfactometry as this technique measures human response to odorants previously separated by GC.

In general, studies have described the enhancement of characteristic beef flavour when meat was aged for one to two weeks (71,72), while in other cases two weeks of ageing resulted in unpleasant aromas (73). In order to evaluate the contribution of each compound on these changes, the volatile composition of meat at different stages of the ageing process has been determined in beef (74), lamb (75) and pork (76). However, in studies related to horse meat, only trained (or semi-trained) panels have been used to determine the presence of off-odours (22,23). Investigations regarding the volatile profile of aged horse meat are very recent and limited to meat obtained from IHD horse breed (26,77).

In the particular case of rancid flavour that may be generated from lipid oxidation (section 1.3.3.4), the common methodology for its determination is the spectrophotometric measurement of thiobarbituric acid reactive substances (TBARS) taking into account its direct correlation with rancidity (78). Iodine value is also used, considering it as the equivalent to the amount of lipid double bonds and, thus, relating its decrease with lipid oxidation. Because the conventional TBARS method has insufficient specificity and sensitivity, alternative analytical approaches have been developed, such as extracting hypdroperoxides and maloaldehyde (primary and secondary oxidation products in foods)

under acidic conditions and then determining them by LC. However, because of the longer time needed, alternative methods are not usually used in meat matrix (79). Even when meat is aged under vacuum conditions, TBARS have been reported to increase (80) in beef. Variable results have been obtained when lipid oxidation of vacuum aged horse meat was studied; some studies have reported no increase in TBARS after two weeks while others have reported an increase from the first week of ageing.

Microorganism growth during ageing could also potentially contribute to meat flavour (section 1.3.3.7). It has been generally considered that acceptability of meat is over when values of 7 log colony forming units (CFU) of a certain microorganism per gram of meat are reached (81). Enumeration of microbial groups by using conventional culture methods on agar plates has been used to assess food spoilage due to microbial growth. An investigation performed in beef reported maximum LAB accounts (7 log CFU/g) after 25 days of refrigeration under vacuum, while raw meat started to be rejected by the consumer due to spoilt odour after 8 weeks (82). In horse meat, only two studies have evaluated the most relevant microorganisms during horse meat ageing revealing that LAB were the prevailing bacteria. In these studies, more than 12 or 14 days of vacuum storage at 2 °C were needed to reach LAB accounts of 7 log CFU/g. Understanding of the dynamics of microbial populations will allow to minimize economic losses owing to food spoilage.

1.3.4.4 Water holding capacity

Water-holding capacity (WHC) is defined as the ability of meat to retain water when external pressures are applied to it. It constitutes an important quality characteristic of the raw meat because meat with poor WHC is rejected by the consumer and leads to revenue losses in the meat industry (50). In addition, sensory juiciness (the impression of moisture and lubrication when meat is chewed) of cooked meat is in part determined by WHC, being a meat with a higher WHC considered juicier (83). Changes in WHC during the ageing process are inevitable due to changes in myofibril structure (section 1.3.3.6). In addition, protein oxidation may also negatively affect WHC because protein charges increase the electrostatic repulsion between myofilaments and, consequently, increase the volume of the myofibrils and decrease the available space for the water (60).

In practice, WHC is commonly measured as drip loss, calculating the difference of sample weight before and after storage, and expressing this value relative to sample weight before storage. By this means, studies have shown that an ageing process of more than two weeks improves WHC (reduces drip loss) in beef and venison (51,84), while shorter ageing periods were needed in pork (85). There is no record of drip loss studies in horse meat during ageing. WHC can be also measured after heat application (normally boiling), in such case the water released is described as cook loss. From all WHC measurements, cook loss has the highest correlation with the sensory trait of juiciness. However, the structures and mechanisms that influence water loss during cooking have been less studied and are different to those of raw meat. In this case, cooking temperature and cooking conditions will also influence the results. For the determination of cook loss, meat is weighed before and after cooking, and cook loss is expressed relative to the sample weight before cooking. In beef, and contrary to the observations made on raw meat, a higher cook loss is usually observed after an ageing process (86,87). From the few studies reporting cook loss in horse meat at different ageing times, some of them did not observe any difference (ageing up to 30 days) whereas others observed higher cook losses in horse meat aged for longer (28 *vs.* 14 days) periods of time (29,88).

2. HYPOTHESIS AND OBJECTIVES

Horse production and, therefore, horse meat consumption can be a partial solution to some of the future challenges of the agriculture production system. As indicated before, global population is expected to increase and food demands, especially those for high quality protein, need to be faced in a sustainable manner. Horse meat production under extensive grazing conditions could be a very promising option for certain regions as it contributes to the conservation of mountain ecosystems while the greenhouse gas emissions are considerably lower compared to ruminant species. In addition, the digestive physiology of equines allow them to efficiently transfer beneficial fatty acids, mainly n-3 polyunsaturated fatty acids, from pasture (diverse in plant species) to meat and milk. Horse meat consumption is increasing in several countries but the research available is still scarce in order to understand horse meat quality parameters and both *ante*- and *post-mortem* factors affecting to it. For instance, *post-mortem* factors such as ageing and associated biochemical processes, which are known to strongly influence meat quality parameters in other domestic species, are far from being understood in horse meat. Gaining knowledge about *post-mortem* interventions such as ageing will allow a better understanding and standardization of these actions, providing, in general terms, a more homogeneous final product while avoiding ageing periods longer than the minimum required for quality improvement. Overall, research focused on horse meat will allow its promotion as an alternative red meat.

Under this framework, the overall objective of the present Ph.D. Thesis was to investigate the effect of ageing time (0, 7, 14 and 21 days) on horse meat quality using instrumental and sensory methods.

The present work has been developed following three main specific objectives:

Objective I: To study the effect of ageing time, by instrumental means, on the most relevant horse meat quality parameters such as pH, volatile compounds, colour, cook loss and shear force.

Objective II: To develop an innovative approach, using liquid isoelectric focusing (OFFGEL) coupled to 1-DE, to study changes in the myofibrillar meat sub-proteome as affected by ageing time and its association with meat tenderness.

Objective III: To study the effect of ageing time on consumer preference and sensory description of horse meat.

3. MATERIALS AND METHODS

3.1 Experimental design and sampling

Twenty commercial horse loin samples where purchased from Cárnicas Mutiloa, S.L. (Sangüesa, Navarra) meat industry. These loins came from ten HB breed horses (five females and five males), a well-established breed in northern Spain which is part of the genetic heritage and its conservation is key to maintain mountain ecosystems (**Figure 3.1**)

Figure 3.1 Several horses from Hispano-Bretón breed (not from the experimental design) in Cantabrian Mountain grasslands. Photo courtesy of G. Redondo-Corrales.

After birth, foals suckled their mothers from May to June, 2017, until weaning at 6-8 months of age. Then, they continued freely grazing until, at 11-13 months of age, entered a commercial feedlot where they were finished for 100-120 days on an *ad libitum* concentrate-based diet (13.3 % protein, 2.70 % fat, 7.60 % fibre) composed by barley, wheat bran, wheat, soybean flour, dehydrated alfalfa, corn, beet pulp, soybean hulls, molasses, palm oil and salts.

Horses were slaughtered in a commercial abattoir at 15-17 months of age, following the specifications outlined in the European legislation (89). The average carcass weight was 246.2 ± 14.0 kg (249.9 ± 15.0 kg for females and 242.5 ± 12.5 kg for males). All carcasses were classified as U (conformation) and 2 (fat cover) according to the Community scale for the classification of carcasses of adult bovine animals (90) (**Figure 3.2**).

Figure 3.2 Half carcasses of Hispano-Bretón horses included in the experimental design.

Two horses (female and male) were slaughtered per week during five consecutive weeks. After 48 hours (h) at 4 °C (day 0), both right and left rib joints were removed from each carcass (n=20) and transported to the laboratory under refrigerated conditions. *Longissimus thoracis et lumborum* (LTL) muscle was subsequently excised and sliced into 1.5 cm thick steaks. From each LTL, 27 steaks were cut and destined for different analyses.

The first two steaks beginning from the thoracic side were trimmed, vacuum packed and directly frozen at -80 °C for chemical composition, myoglobin content and total fatty acid composition determinations. In addition, a portion of subcutaneous (SC) fat taken from the thoracic region of each LTL sampled (n=20) was vacuum packed and frozen at -80 °C for total FA composition analysis.

The rest of the steaks were individually vacuum packed, randomly assigned to an ageing period (0, 7, 14 or 21 days, d) and aged at 4 °C without illumination. After the corresponding ageing time, steaks were frozen at -80 °C and destined to other analyses such as the determination of volatile compounds (steaks 3 to 6), study of myofibrillar sub-proteome (steaks 7 to 10), consumer study (steaks 11 to 18), instrumental colour, cook loss determination, shear force (steaks 19 to 22) and pH measurement (steaks 23 to 26). Several actions such as taking the photographs for the consumer study or pH and instrumental colour measurements were immediately performed after reaching the corresponding ageing time. Moreover, several steaks aged for 21 days (n=4; steak 27) were microbiologically analysed in order to evaluate their safety prior to using samples aged for 21 days in the consumer study. In this sense, it was assured that samples met the established safety requirements for consumption (91).

The aforementioned sampling procedure for different ageing times was considered a full-randomized block design with slaughter day as a blocking factor and ageing time as a split plot factor. As indicated, ageing time was randomly assigned (within each type of analysis) to different individual steaks obtained from each LTL. The experimental unit (loin) was considered as a plot and the steaks were subplots (sampling units) in which ageing time (factor) was assessed. Animal sex, carcass side and carcass weight were distorting variation sources controlled by the experimental design in order to minimize the residual variation.

3.2 Methodology

3.2.1 Chemical composition of horse meat

Chemical composition of horse meat was determined in overnight thawed (4 °C) steaks (n=20) by using standard procedures for dry matter (92), crude protein (93), ether extract (94) and ash determinations (95).

3.2.2 Myoglobin content of horse meat

Total myoglobin content was determined by the method of Faustman and Phillips with minor modifications (96). Steaks (n=20) were thawed overnight (4 °C) and minced in a

domestic grinder. Five grams of minced horse meat were homogenized in ice-cold sodium phosphate buffer (40 mM, pH 6.8) and set aside in the dark for 1 h at 4 °C. Samples were then centrifuged at 32,000 *g* for 45 minutes (min) and filtered through a n° 1 Whatman filter paper (Whatman PLC, Maidston, UK). Absorbance was read at 525 nm in a spectophotometer (Model UV 1280, Shimadzu Corporation, Kioto, Japan). The total myoglobin concentration was estimated applying the extinction coefficient of 7.6 mM^{-1} cm^{-1} as proposed by Bowen (97).

3.2.3. Fatty acid composition of horse meat

3.2.3.1 Lipid extraction and derivatization

From SC fat (n=20), 50 mg of adipose tissue were freeze-dried and directly methylated with sodium methoxide (Methanolic-Base, 0.5 N; Supelco Inc., Bellefonte, PA, USA) at 50 °C for 15 min, using 1 mL of internal standard (IS) (4 mg/mL of 23:0 methyl ester in toluene).

For muscle tissue, steaks (n=20) were freeze-dried and grinded prior to lipid extraction. Total muscle lipids were extracted from 1 g of freeze-dried meat using a chloroform:methanol mixture (2:1 v/v) according to Folch *et al*. (98) and improved by Blight & Dyer (99). After removal of the solvent, lipids were gravimetrically weighted and redissolved in 15 mL of chloroform. Then, aliquots of 10 mg of lipids were derivatized using 2 % H_2SO_4 (Sigma Aldrich, Madrid, Spain) in anhydrous methanol (99.8 % purity, Sigma Aldrich) (100). Prior to methylation, 1 mL of IS solution (1 mg/mL of 23:0 methyl ester in toluene; Nu-Check Prep Inc., Elysian, MN, USA) was added. Acidic methylation provided the conversion of acyl and N-acyl lipids into fatty acid methyl esters (FAMEs) and alk-1-enyl ethers of plasmalogenic lipids into dimethylacetals (DMAs).

3.2.3.2 Gas chromatography-flame ionization detector (GC-FID) analysis

Both, FAMEs and DMAs, were analysed by GC-FID (Model 7890A, Agilent Technologies, Madrid, Spain) coupled to an automatic injector (Model 7693, Agilent Technologies). Injection volume was set at 1 μL. Separation was carried out in a Supelco

SP-2560 capillary column (100 m x 0.25 mm I.D., 0.2 m coating; Supelco Inc.), following the 175 ºC program previously described (101). Hydrogen (99.999 % purity, Air Liquid, Madrid, Spain) was used as a carrier gas at a flow rate of 1 mL/min, and injector and detector ports were set at 250 °C. Chromatographic data were registered using Agilent ChemStation software (version B.04.03, Agilent Technologies).

Peak identification was performed using commercial reference standards such as #463 and #603 mixtures, individual 21:0, 23:0, 20:3n-9 FAMEs and a conjugated linoleic acid (CLA) mixture #UC-59-M composed by 9 *cis* (*c*),11 *trans* (*t*)-/ 8*t*,10*c*-/ 11*c*,13*t*-/ 10*t*,12*c*-/ 8*c*,10*c*-/ 9*c*,11*c*-/ 10*c*,12*c*-/ 11*c*,13*c*-/ 11*t*,13*t*-/ 10*t*,12*t*-/ 9*t*,11*t*-/ 8*t*,10*t*-18:2 obtained from Nu-Check Prep Inc., and a bacterial mixture purchased from Matreya, Pleasant Gap, PA, USA). In addition, identifications were confirmed using FAME fractions obtained from silver-ion solid-phase extraction glass cartridges as previously described (102,103) and following retention times and elution orders reported in the literature (100,101,103,104). Chromatographic areas were corrected according to theoretical response factors (105) and IS was used to calculate quantitative data (mg per 100 g fresh meat) and then percentages (% of total quantified).

3.2.4 Volatile compound composition of horse meat

The volatile compound composition of horse meat was analysed in horse meat steaks aged for 0, 7, 14 and 21 days (n=80) following the general workflow depicted in **Figure 3.3**.

3.2.4.1 Meat cooking

Steaks were cooked (grilled) according to AMSA recommendations (106). Prior to cooking, steaks were thawed overnight (4 °C) and kept at room temperature for 2 h covered with an oxygen permeable polyvinylchloride (PVC) film (oxygen permeability of 580 mL/m^2/h). Cooking was carried out in plane double clamp electric grills (Model Dalyko MB-30, Sogo, Barcelona, Spain) and the same grill was used for the steaks of the same ageing time (0, 7, 14 or 21 days). Grills were set at 200 °C and meat cooked until an internal temperature of 71 ± 1 °C was reached (9-13 min), monitored individually with multichannel thermocouples (Lutron electronic, Coopersburg, PN, USA). Cooked steaks were allowed

to cool at room temperature, minced, vacuum packed and frozen (-80 °C) until volatile compound analysis.

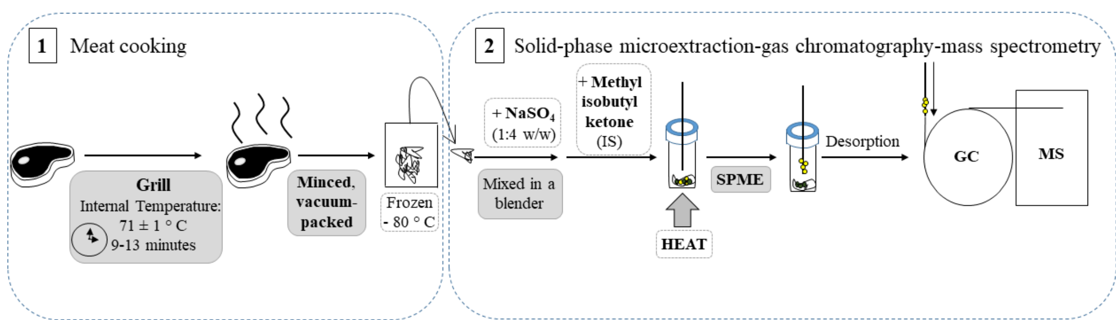

Figure 3.3 Volatile compound analysis workflow using solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS).

IS, internal standard; GC, gas chromatography; MS, mass spectrometry.

3.2.4.2 Solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) analysis

After thawing for 2 h at room temperature, SPME-GC-MS analyses were performed as previously described (107), with minor modifications. A total of 10 g of minced meat were mixed with anhydrous sodium sulphate (Sigma Aldrich) (4:1 w:w) and, subsequently, homogenized in a blender. From the mixture, 2.5 ± 0.001 g were weighted in a 10 mL amber vial, to which 20 µL of an aqueous IS solution (1 g/L of methyl isobutyl ketone, 99.8 % purity, Sigma Aldrich) were added. Vials were sealed with a polytetrafluoroethylene septa and steel magnetic cap (Agilent Technologies).

Prior to injection, volatile compounds were extracted by SPME on a 30/50 µm DVB/Carboxen/PDMS fibre (Supelco Inc.) using a PAL RSI 85 autosampler (CTC Analytics AG, Zwingen, Switzerland). Extraction was performed over 50 min at 80 °C, after 15 min of pre-equilibration time at the same temperature. Volatiles trapped onto the fibre were desorbed in the injection port during 25 min at 240 °C in splitless mode (split valve was opened at 200 mL/min after 30 min of injection).

Analyses were performed using a GC equipment (Model 7820A, Agilent Technologies) coupled to a MS detector (Model 5975E, Agilent Technologies). The separation of volatile compounds was carried out in a Supelcowax-10 capillary column (60 m x 250 µm I.D., 0.25 µm film thickness; Supelco Inc.) using the following temperature gradient: oven temperature was held at 40 °C for 10 min, then increased at a rate of 5 °C/min until 240 °C, and finally held at 240 °C for 15 min. Helium (99.999 % purity, Air Liquid) was used as a carrier gas and volatiles were transferred to a MS detector throughout a transfer line at 280 °C. Chromatographic data were registered with the MSD Chemstation Data Analysis software (version 5.52, Agilent Technologies), and MS detector operated at 150 °C in full scan mode (1.4 scan/s; m/z range 26-350) using 70 eV as total current. Two replicates were analysed from each meat sample.

Volatile compounds were quantified using the chromatographic peak area. The limit of detection (LOD) was established as two times the average baseline noise, which was estimated in three chromatographic regions from the analysis of 10 blanks (empty vial). The relative abundance of volatile compounds was calculated using peak areas of volatile compounds present in both replicates (>LOD) and in over 70 % of the samples from each ageing time, according to the following equation:

$$\text{Relative abundance} = \frac{\text{peak area}}{\text{IS area}} \cdot \frac{2.5\ \text{g}}{\text{mixture weight (g)}} \cdot 100$$

Tentative identifications of volatile compounds were performed by comparing the mass spectra of detected peaks with those of the NIST 2.0 library (National Institute of Standards and Technology, Gaithersburg, MD, USA), using a matching factor of >700. In addition, when available, commercial standards of high purity were used (1-penten-3-ol, 1-pentanol, 1-octen-3-ol, 1-heptanol, 6-methyl-2-heptanone, 1-octanol, (*E*)-2-octen-1-ol, 1-tetradecanol, 1-tridecanol, acetaldehyde, hexanal, 1-hexanol, (*E*)-2-hexenal, heptanal, (*E*)-2-octenal, (*E*)-2-nonenal, (*E*)-2-decenal, 2-butyl-2-octenal, toluene, benzaldehyde and 1-hexadecene obtained from Sigma Aldrich and 1-propanal, 2-methylbutanal, 3-methylbutanal, octanal and 2-heptanone obtained from Honeywell-Fluka Research Chemical-Fisher Scientific (Madrid, Spain). Experimental liner retention index (LRI) of commercial standards were calculated, as well as those from horse meat samples, using C7-

C24 alkanes (Sigma Aldrich). Additionally, experimental LRI values of compounds detected in horse meat samples were compared to values from the literature.

3.2.4.3 Odour impact ratio (OIR) calculation

Odour impact ratio (OIR) was used as an indicator of the odour intensity of each volatile compound detected by SPME-GC-MS. It was calculated as previously described in Abilleira *et al*. (108) with minor modifications, following:

$$\text{OIR} = \frac{\text{mean relative abundance}}{\text{OT } (\mu\text{g/kg})}$$

In addition, OT values (calculated in water) available in the literature (109) were used to calculate OIR values from each compound. In this case, the mean relative abundance of areas obtained from the chromatographic peak of each volatile compound detected in horse meat samples from all ageing times (0, 7, 14 and 21 days) was used for the calculation.

3.2.5 pH measurements in horse meat

Triplicate pH measurements were taken in steaks aged for 0, 7, 14 and 21 days (n=80) using a portable pH meter (Model HI99163, Hanna Instruments, Woonsocket, RI, USA) equipped with a penetrating glass electrode (Model FC232D, Hanna Instruments).

3.2.6 Instrumental colour measurements in horse meat

Instrumental colour was determined in steaks aged for 0, 7, 14 and 21 days (n=80). When each ageing time was reached, steaks were covered with an oxygen-permeable PVC film (oxygen permeability of 580 $mL/m^2/h$) and exposed to air (bloomed) for 1 h at 4 °C. Instrumental colour was recorded using a Minolta CR-200 colorimeter (Konica Minolta, Tokyo, Japan), with a D65 illuminant and a10° visual angle. L^*, a^* and b^* values were measured according to the CIEL^*a^*b colour space by a total of 5 spectral readings per sample taken in different parts of the steaks (110). Other two additional parameters were calculated: hue angle (h^*) = arctangent (b^*/a^*) which defines colour, and chroma or saturation index (C^*) = $(a^{*2} + b^{*2})^{1/2}$ (111).

3.2.7 Cook loss determination and shear force measurements in horse meat

Cook loss and shear force were determined in steaks aged for 0, 7, 14 and 21 days (n=80). Steaks were thawed overnight (4 °C), set for 1 h at room temperature and individually weighted after eliminating the excess moisture. Cooking was performed in batches of eight steaks (2 sets of 4 ageing times). Steaks were introduced in open plastic bags and cooked in a water bath at 80 °C (SV Thermo, Orved, Italy) until they reached an internal temperature of 71 ± 0.1 °C, monitored by temperature probes (SV Thermo). Then, they were removed from the water bath, cooled at room temperature for 1 h, moisture eliminated and individually weighted. Cook loss was determined by calculating the weight difference between raw and cooked steaks, and expressing it relative to the sample weight before cooking (112).

For shear force measurements, eight cuboids of approximately 1 x 1.5 x 1 cm^3 were cut parallel to the muscle fibres and maximum WBSF of each cuboid was measured using a TA-XT2i texture analyser (Stable Micro Sytems, Godalming, UK) connected to an IBM-compatible Foxen computer, with an AutenticAMD-K6 ™ 3D microprocessor. The texture analyser was equipped with a WBSF device (cutting blade at a constant speed of 1.70 mm/s and a load cell of 300 N). The Texture Expert software version 1.22 for Windows (Stable Micro Sytems) was used.

3.2.8 Study of myofibrillar sub-proteome of horse meat

The study of the myofibrillar sub-proteome of horse meat was carried out using 1-DE followed by liquid isoelectric focusing (OFFGEL) that had been previously optimized in beef samples (**Figure 3.4**; **Publication II**, Appendix II), with minor modifications.

For the study of myofibrillar sub-proteome, eight loins (right loins from four female and four male horses) were selected. From these, all the ageing times (0, 7, 14 and 21 days) were analysed by 1-DE, while the two extreme ageing periods (0 and 21 days) were selected for OFFGEL fractionation.

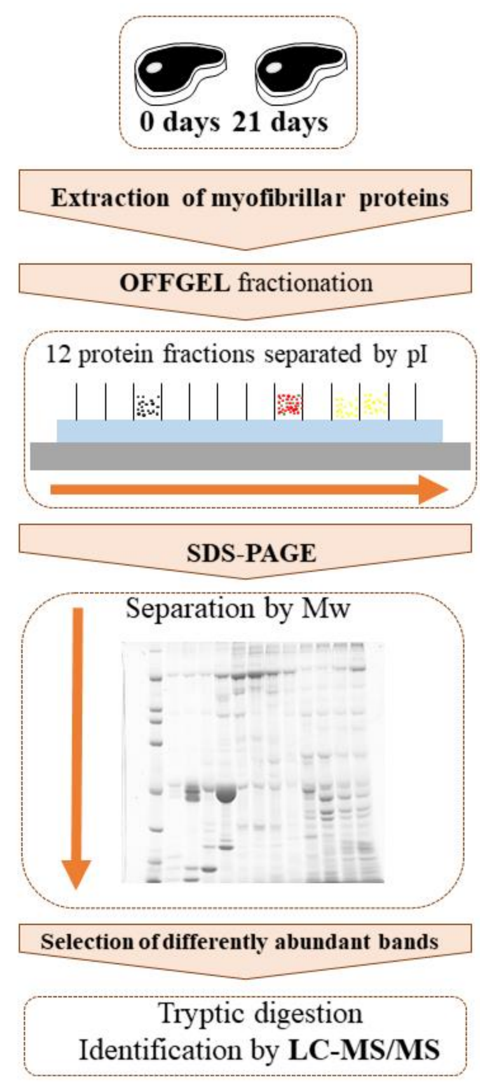

Figure 3.4 General workflow of liquid isoelectric focusing based approach for the separation of myofibrillar sub-proteome of horse meat samples.

SDS-PAGE, Sodium Dodecyl Sulphate-Polyacrilamide Gel Electrophoresis; Mw, molecular weight; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry.

3.2.8.1 Extraction of myofibrillar proteins

Myofibrillar sub-proteome was extracted from steaks aged for 0, 7, 14 and 21 days (n=32). From the centre of the steak, 10 g were taken from which half a gram was weighted and cut in small pieces. Then, meat was homogenized in 5 mL of 10 mM Tris (tris-hydroxymethyl-aminomethane) buffer pH 7.6, 1 mM EDTA, 0.25 M sucrose containing 25 µL of protease inhibition cocktail (Sigma Aldrich) using a Ultra-Turrax Yellow Line Di (IKA-Werke GmbH, Staufen, Germany) and centrifuged at 20,000 *g* for 20 min at 4 °C. The obtained

precipitate was redissolved in the same buffer and centrifuged again, under the same conditions, to efficiently wash the pellet from residual soluble proteins. The washed precipitate was further redissolved in 10 mM Tris buffer pH 7.6 containing 7 M urea (Scharlab S.L., Barcelona, Spain), 2 M thiourea (Scharlab S.L.) and 2 % CHAPS (Sigma Aldrich) and, then, centrifuged under the same conditions described above. The supernatant containing the myofibrillar protein fraction (*i.e.*, the myofibrillar sub-proteome) was filtered through glass wool and stored (-80 °C) until analysed.

3.2.8.2 Total protein content determination

Total protein quantification of myofibrillar protein fraction was determined using the Bio-Rad Protein Assay Kit (Bio-Rad laboratories, Madrid, Spain) following the Bradford method (113). A calibration curve ranging from 0.05 to 0.8 mg/mL of BSA was used and samples were 1:20 diluted.

3.2.8.3 OFFGEL fractionation of myofibrillar sub-proteome

Myofibrillar sub-proteome from non-aged and 21 days aged meat samples (n=16) was fractionated by OFFGEL. A total of 3 mg of proteins were separated using an Agilent 3100 OFFGEL fractionator and following manufacturer's instructions. Briefly, protein extracts were first diluted to a final volume of 2 mL using 1.25X protein OFFGEL stock solution containing 50 % glycerol v/v, 7 M urea, 2 M thiourea and 65 mM dithiothreitol (DTT) obtained from Scharlab S.L. and specific immobilized pH gradient gel (IPG) buffer obtained from Cytiva, Marlborough, MA, USA. Then, IPG strips with a linear gradient ranging from 3.0 to 10.0 (GE Healthcare, Uppsala, Sweden) and corresponding to a 12-well frame size were fixed on the tray and rehydrated for 5 min with 1.25X protein OFFGEL stock solution. A total of 150 µL of each diluted sample extracts were loaded into each well and fractionated under a constant electric current of 50 mA and 20 °C, to reach 20 kVh in about 18 h. Proteins were automatically separated according to their isoelectric point (pI) values across 12 liquid fractions as illustrated in **Figure 3.5**. Fractions were individually recovered by pipetting and stored at -20 °C until further analysis.

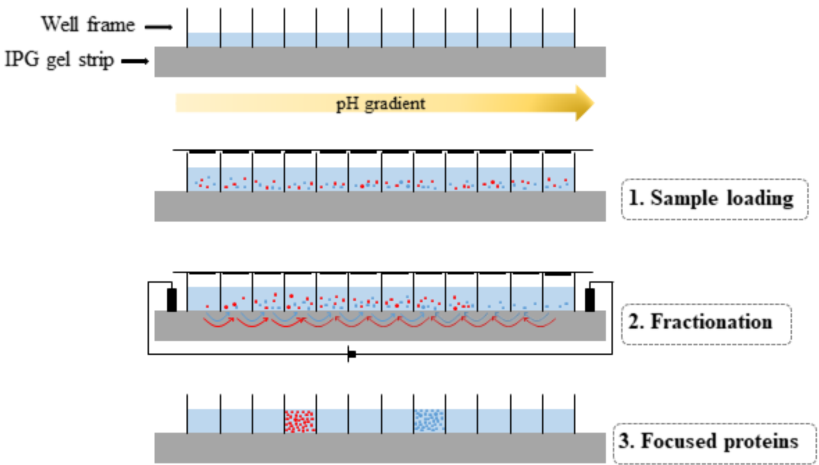

Figure 3.5 Visual diagram of the liquid isoelectric focusing protein fractionation steps: sample loading, fractionation and focused proteins.

IPG, Inmobilized pH gradient

3.2.8.4 SDS-PAGE

Total myofibrillar sub-proteome from samples aged for 0, 7, 14 and 21 days aged (section 3.2.8.2; n=32) was resolved by SDS-PAGE in 12 % polyacrylamide gels. In addition, OFFGEL fractions from non-aged and samples aged for 21 days (section 3.2.8.3; n=16) were resolved in 5-16 % polyacrylamide gradient gels that were previously cast using a SG-100 gradient maker (Hoefer, San Francisco, CA, USA). All analyses were carried out in duplicate for each of the samples.

In both sets, total myofibrillar sub-proteome and individual OFFGEL fractions, each sample was individually mixed at a 50:50 ratio with 0.088 M Tris-HCl pH 6.8 sample buffer solution containing 10 % glycerol v/v, 2 % SDS w/v (Sigma Aldrich), 0.2 DTT and 0.02 % bromophenol blue (Sigma Aldrich) and heated at 95 °C for 4 min to denature proteins. Samples were then centrifuged and subsequently loaded onto 1.5 mm x 8 cm x 9 cm polyacrylamide gels. Electrophoretic separation was carried out simultaneously for two gels at a constant current of 50 mA using a Mighty Small II SE260 electrophoretic unit (Hoefer). After electrophoresis, gels were fixed in a solution containing 10 % trichloroacetic acid (TCA) for 1 h, rinsed, and stained overnight with colloidal Coomassie

(114). Then, gels were destained with bidistilled water and stored under refrigeration conditions.

3.2.8.5 Gel image analysis

Destained gels were digitalized using an Amersham ImageQuant 800 (GE Healthcare) biomolecular imager and images were further processed and analysed using ImageQuant TL 8.2 software (GE healthcare). Intensity of bands was quantitatively determined using 1-DE gel analysis after rolling ball background subtraction with the radius set at 200 pixels. For an accurate correction of images, individual band intensities were normalized with respect to the total band volume per lane (for total myofibrillar sub-proteome) or per gel (for individual OFFGEL fractions). Molecular weight of protein bands was determined by the software comparing band migration results of sample proteins with those from the commercial Bio-Rad 1610317 broad-range molecular mass protein standards (Bio-Rad).

3.2.8.6 In-gel trypsin digestion of selected protein bands

Protein bands that were found to be significantly ($p < 0.05$) different in abundance among ageing times, both for total myofibrillar sub-proteome and for individual OFFGEL fractions, were digested with trypsin in order to elucidate their protein identity. First, bands were excised from the gels, cut into small pieces, washed three times during 10 min with 50 mM ammonium bicarbonate pH 8.0 (Sigma Aldrich) and dehydrated twice with acetonitrile (ACN) (Scharlab S.L.) until shrinking and turning opaque. After removing the remaining liquid using a Savant SPD121P Speed-Vac concentrator equipped with a RVT400 refrigerated vapour trap (Thermo Scientific, San Jose, CA, USA), 15 µL of a 12.5 µg/mL solution of trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate pH 8.0 were added. Then, additional 15 µL of the same trypsin solution were added in order to cover the gel pieces. Finally, tubes were incubated at 37 °C overnight.

After incubation, the supernatant was transferred to a clean Eppendorf tube, and subsequently, the remaining peptides were recovered by adding ACN/0.1 % formic acid (FoA) (50:50) and sonicated for 10 min in a Ultrasonic 40000-00301 equipment (Ovan, Barcelona, Spain). This supernatant was combined with the previous one and the liquid

phase was evaporated in a Speed-Vac. Samples were then acidified with 0.1 % FoA and transferred into glass vials.

3.2.8.7 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis

Analyses were carried out in a Thermo Surveyor Plus system (equipped with a cooled autosampler, quaternary pump, column oven and vacuum degasser) coupled to a Thermo LCQ Advantage ion-trap MS analyser with an electrospray ionization (ESI) probe operating in positive mode (Thermo Scientific). The separation of peptides was carried out through a Luna Omega PS C18 column (150 mm × 2.1 mm, 3 µm particle-size; Phenomenex Inc., Torrance, CA, USA). Mobile phases were solvent A containing 0.1 % FoA (v/v) in ultrapure water and solvent B containing 0.1 % FoA (v/v) in ACN; with the following separation conditions: initially 100 % A, held for 2 min, linear 0-80 % B in 23 min, 95 % B in 0.1 min, held for 4.9 min for washing, 100 % A in 0.1 min, and column equilibration for 14.9 min. Total run time was 45 min with a flow rate of 200 µL/min; injection volume was 25 µL. Autosampler and column temperatures were set at 10 and 23 °C, respectively.

Operating parameters of the ion trap detector were the following: capillary temperature was set at 250 °C and collision energy was normalized to 35 %. Spray and capillary voltages were 4.0 kV and 42.0 V, respectively. The analysis combined two scan events: First scan event was full-MS detection in the m/z range 400–2000. The second scan event was a dependent MS/MS analysis of the most intense ions with charges from +1 to +4 with the following parameters: exclusion list, 25 masses including those from background provided a blank injection; exclusion time, 3 min; exclusion mass width, 3 amu; repeat count for MS/MS of most intense ion, 2; repeat count duration, 0.3 min; minimum MS/MS ion intensity threshold, $1x10^5$. Control of the MS analysis platform and manual data processing was performed using Thermo Xcalibur v2.04 software (Thermo Scientific).

Peptide identification was done by interrogating MS/MS data obtained from tryptic peptides against NCBIprot protein database using Mascot search engine v2.7 with the following settings: enzyme, trypsin; no fixed or variable modifications but 'Error tolerant'

option enabled; mass accuracy 1.2 and 0.8 Da for full MS and MS/MS analyses, respectively and Mammalia as taxonomy restriction parameter. Estimation of false positive rates by means of false discovery rate threshold 1 % was achieved through the activation of the 'Decoy' option. Only those results having a protein score derived from individual ion scores indicating identity or extensive homology ($p < 0.05$) were considered as true protein identifications.

3.2.9 Consumer study of horse meat

A consumer study was carried out in order to study the sensory quality – in mouth and visual evaluation– of steaks aged for 0, 7, 14 and 21 days.

3.2.9.1 Recruitment and session organization

Consumers were recruited using regular participant databases from the Sensory Laboratory of the University of the Basque Country (LASEHU). In addition, other potential participants were reached by e-mail, posters, broadcast, social media and personal contacts. Initially, interested consumers filled out a basic recruitment questionnaire in which they stated their gender, age and horse meat consumption frequency. From them, 120 volunteers from Vitoria-Gasteiz (northern Spain) and surrounding areas were recruited, balancing as much as possible for age and sex.

Sessions were organized in LASEHU facilities, which are equipped with individual booths and computers. Ten sessions of 12 consumers each were carried out during three consecutive days. Sessions lasted approximately 1 h and were organized as follows: first, in-mouth acceptability (IMA) and check-all-that-apply (CATA) were performed, followed by visual acceptability (VA) and CATA, and a final questionnaire (gender, age and horse meat consumption frequency). In-mouth and visual tests were alternated in order to avoid consumer fatigue and to have enough time to cook the second set of samples for in-mouth CATA.

For in-mouth evaluation test, each consumer tasted samples from all the ageing periods assayed (0, 7, 14 and 21 days) belonging to the same animal, twice: first for IMA and then

for CATA. For this reason, 8 steaks from each LTL were needed (steaks 11 to 18; n=160). Half of the steaks were used for IMA and the other half for in-mouth CATA.

3.2.9.2 In-mouth evaluation (acceptability and CATA)

For in-mouth evaluation (IMA and in-mouth CATA), batches of 16 steaks were employed in each session. Samples were thawed overnight (4 °C) and, then, the 16 steaks used for the session were unpacked, covered with an oxygen-permeable PVC film (to avoid surface dryness) and kept at room temperature for 2 h. Samples were cooked in two rounds as described for volatile compounds (section 3.2.4.1): a first cooking round for IMA and a second one for CATA.

Cooked steaks were immediately trimmed; cut into 2 x 1.5 x 2 cm cuboids, wrapped in aluminium foil and labelled with random three-digit codes. Samples were kept in heaters (heat diffusion by glass microspheres, Indoterm, San Cugat, Barcelona, Spain) at 60-65 °C until serving (maximum of 5 min). For IMA and in-mouth CATA, samples were provided randomly and monadically. Consumers were provided with water and unsalted crackers to clean palate between samples. Tasting was performed under red light (16 ± 1 lx) in order to avoid colour bias.

Evaluation of IMA was performed by answering the question 'How much do you like this steak?' in a 10 cm continuous hedonic scale with an extra centimetre in both sides, that ranged from 'I extremely dislike' to 'I very much like'. For in-mouth CATA, descriptive terms were selected after an in-depth bibliographical search (115,116) and discussion. A total of 19 terms were finally selected: 'cowshed', 'fatty', 'intense', 'livery', 'low intensity', 'rancid', 'roasted' and 'unpleasant' for odour/aroma category; 'chewy', 'dry', 'easily dissolving', 'high residue', 'juicy', 'tough' and 'tender' for texture category; and 'bitter', 'metallic', 'salty' and 'sweet' for taste and trigeminal sensations category. Consumers were asked to tick off all the terms that considered appropriate to describe each sample After assessing the 4 samples for in-mouth CATA, consumers were asked to tick off all the attributes that relate to the 'ideal horse meat' sample, using the same list of terms. In all cases, terms from the three categories were presented mixed and randomly ordered for each sample.

3.2.9.3 Visual evaluation (acceptability and CATA)

Visual acceptability and visual CATA were assessed evaluating the photographs taken from steaks of each ageing time. When each ageing time was reached, steaks were unpacked, put in a while plate and covered with an oxygen-permeable PVC film for 1 h (4 °C). Then, three photographs were taken from each steak under controlled artificial illumination (911 ± 10 lx) using a professional digital camera (Nixon-700, Nikon Corporation, Tokyo, Japan). In order to avoid differences in contrast, intensity or direction of the illumination, the same white plate was used as background. From the whole set of photographs, 4 were selected as representatives from each ageing time by a group of experts, taking into account the variability among animals, sexes and LTL sides (right and left) (**Figure 3.6**). Same batch of selected photographs were used for visual acceptability and visual CATA in all sessions.

In the booths, a correct position and a fixed distance to the screen were established in order to avoid as much as possible differences among consumers while no illumination was provided. For VA, consumers were asked to consider the average impression of the batch of the selected photographs by answering the question 'How much do you like this steak?' with the same scale as for IMA. Visual CATA terms were also defined beforehand, and consisted on the following: 'brownish', 'dark', 'fresh', 'maroon', 'not uniform colour', 'pinkish', 'red', 'spoilt' and 'uniform colour'. Consumers were asked to tick off all the terms that considered appropriate to describe each sample. After assessing the samples for visual CATA, consumers were asked to tick off all the attributes that relate to the 'ideal horse meat' sample, using the same list of terms. In all cases, terms from the three categories were presented mixed and randomly ordered for each sample.

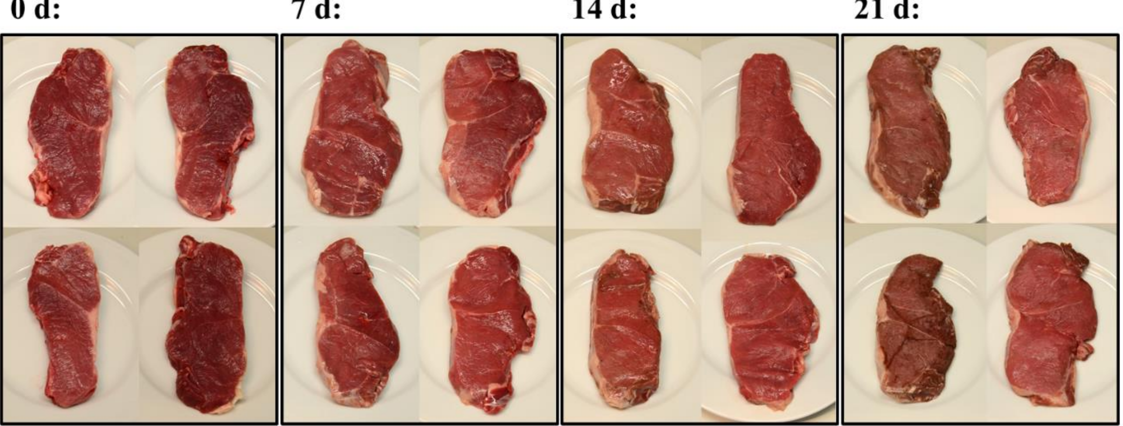

Figure 3.6 Compilation of four selected photographs of each ageing time (0, 7, 14 and 21 days) for visual acceptability and CATA tests.

3.3 Data treatment and statistical analysis

Statistical analyses were conducted using SPSS statistical software (version 26.0, Armonk, NY, USA) unless otherwise indicated.

3.3.1 General Linear Models

The General Linear Model (GLM) of ANOVA was used to determine, separately, significant differences among the variables studied. Normality and homoscedasticity of the variables were checked by Shapiro-Wilk and Leven´s test, respectively. The effect of ageing time (0, 7, 14 and 21 days) on volatile compounds, instrumental colour, cook loss, shear force and pH was investigated using the linear Model 1, that included the corresponding main factor (ageing time, AT) and the controlled distorting variation factors (animal sex, AS and carcass side, CS) as fixed effects, carcass weight (CW) as a covariate and slaughter day (D) as a random effect. Slaughter day was considered as a blocking factor, and furthermore, it was a distorting factor of uncontrolled variation coming from several sources. Among them, at least, individual animal, feeding, and transport and/or slaughter conditions. In addition, binary interactions among all factors were included in the model.

Model 1: $Y_{ijkl} = \mu + AT_i + AS_j + CS_k + D_l + (AT*AS)_{ij} + (AT*CS)_{ik} + (AT*D)_{il} + (AS*CS)_{jk} + (AS*D)_{jl} + (CS*D)_{kl} + CW + \varepsilon_{ijkl}$

A modification of Model 1 was used to compare the FA composition of studied fat tissues, with tissue (T) as fixed effect, instead of AT.

For the statistical study of the myofibrillar sub-proteome, Model 1 was simplified to Model 2 as samples from a single carcass side were analysed.

Model 2: $Y_{ijk} = \mu + AT_i + AS_j + D_k + (AT*AS)_{ij} + (AT*D)_{ik} + CW + \varepsilon_{ijk}$

In the particular case of data from the consumer study, session (S) and animal (A) were included as a fixed factors and consumer (C) as a random factor, giving rise to Models 3 and 4 for IMA and VA, respectively:

Model 3: $Y_{ijkl} = \mu + AT_i + AS_j + (AT*AS)_{ij} + C_k(A_l(AS_j)) + A_l(AS_j) + \varepsilon_{ijkl}$

Model 4: $Y_{ijk} = \mu + AT_i + C_j(S_k) + \varepsilon_{ijk}$

When needed, Eta square (η^2) was used for effect size estimation and Fisher's least significance difference test of estimated marginal means was used for pairwise comparisons among ageing levels. Three significant figures were used to express data and significance was declared at $p < 0.05$.

3.3.2 Agglomerative hierarchical clustering and cluster analysis

Agglomerative hierarchical clustering (AHC) was performed on the IMA and VA scores in order to identify groups of consumers with different patterns (117). Euclidean distance and Ward's agglomeration methods were employed. The number of selected clusters was determined by the observation of the dendrogram (118). Then, the non-parametric *Chi*-squared test ($p < 0.05$) was applied in order to explore relationships among the information gathered from consumers (questionnaires) and the clusters selected.

3.3.3 Non-parametric analysis for results from CATA

A contingency table was generated by counting the number of consumers that used each CATA term to describe meat from each ageing time. Following the criteria proposed by other authors, only terms with effective sample size equal or over 24 were considered in the statistical analysis (119). In order to evaluate the correct effective sample size (number of assessors for each term), assessors who tick one term in all four samples and those who did not tick any of them were automatically excluded. Then, the non-parametric Cochran's Q test ($p < 0.05$) was applied to determine the presence or absence of significant differences in the in-mouth and visual CATA scores among ageing times (120) and McNemar´s test ($p < 0.05$) was applied for multiple comparisons (121).

3.3.4 Multivariate analysis

For volatile compound data analysis, partial least square discrimination analysis (PLS-DA) was performed using Unscrambler X software version 10.3 (Camo Asa, Oslo, Norway) in order to discriminate among ageing times. The categorical Y-variable was AT while weighted volatile relative abundances (used as the inverse of standard deviation) were used as X-variable. Full (leave-one-out) cross validation with an uncertainty test was applied to extract the optimal number of model factors. Additionally, variable importance in projection scores were used to estimate the X-variables contribution to PLS-DA model and uncertainty limits were used to estimate the significance of the weighted coefficients that correlated X-variables to categorical Y-variables (122).

For the integrated analysis of several of the variables determined in the present Ph.D. Thesis, principal component analysis (PCA) with Varimax rotation was performed on the covariance matrix of the following variables: volatile compound composition, pH measurements, instrumental colour, cook loss, shear force, IMA and VA for the whole sample set (n=80). Kaiser criterion (eigenvalue > 1) was applied to extract the principal components and bi-plot graphs were used to depict the associations among ageing times and studied variables.

4. RESULTS AND DISCUSSION

Overall, horse meat quality has been less investigated in comparison to other livestock species. In this regard, research addressing the association of *pre*- and *post-mortem* factors with the final quality of horse meat will contribute to provide the market with a known and a more homogeneous product that will satisfy consumer demands. Regarding *post-mortem* interventions, it is known that the ageing process plays a pivotal role in the quality of meat due to the biochemical and physiological changes occurring during it. Ageing of horse meat, however, has not been well studied yet and, therefore, three ageing periods (7, 14 and 21 days) together with non-aged meat (0 days) were considered in the present Ph.D. Thesis. To evaluate the effect of ageing on horse meat quality, several attributes were determined using instrumental and sensory methods.

In terms of structure, the present section has been divided in two parts. In the first one, non-aged horse meat samples have been characterized by means of chemical and FA composition, together with myoglobin content determination. In the second part, attention has been focused on studying the effect of ageing time (non-aged, 7, 14 and 21 days) on principal horse meat quality attributes (volatile compound composition, pH, instrumental colour, cook loss, shear force, myofibrillar sub-proteome, and consumer preference and sensory description). Finally, all results have been integrated in a joint discussion supported by a multivariate analysis.

4.1 Characterization of non-aged horse meat

4.1.1 Chemical composition and myoglobin content of horse meat

Chemical composition and myoglobin content have been reported in **Table 4.1**. Mean moisture was comparable to values described in the literature for meat from horses of a similar age (123-125). Similarly, protein content was comparable to values obtained in previous studies using different horse breeds, ages and production systems (29,123-131).

Muscle fat content obtained here was comparable to other studies in which horses were extensively reared, finished on concentrates and slaughtered at similar age (123,125,126). With reference to ash content, results obtained were in line with those obtained for meat from horses slaughtered at similar age (123,124). In general, ash content is reported to vary with the age of the animals and mineral feed content (132).

Table 4.1 Chemical composition (%) and myoglobin content (mg/g) of horse *Longissimus thoracis et lumborum* muscle.

	Mean	Min	Max	SEM
Moisture	75.3	72.9	78.1	0.3
Crude protein	20.4	19.1	22.3	0.2
Fat (ether extract)	3.31	1.87	5.13	0.19
Ash	1.03	0.750	1.17	0.02
Myoglobin	3.47	2.76	4.69	0.60

Min, minimum value; Max, maximum value; SEM, standard error of the mean.

Myoglobin content of horse meat samples was of special interest in the present work as it determines meat colour via its chemical forms (section 1.3.3.3). Myoglobin chemistry is species-specific and, in this regard, horse meat has been previously described as a myoglobin-rich meat compared to other meats (133). Although different forms of myoglobin were not determined in the present work, results obtained for total myoglobin (3.47 mg/g of meat) were comparable to those reported in extensively reared horses slaughtered at similar ages (131,134) and slightly higher than those described for beef cattle of similar age (3 mg/g on average) (135) (**Publication III**, Appendix III).

4.1.2 Fatty acid composition of horse meat

Horse meat characterization was carried out following determination of total FA composition in two adipose tissues: SC and intramuscular fat tissues (**Publication III**, Appendix III). To take a broad view of obtained results, data were presented in absolute and percentage basis, and discussed considering results reported in the literature.

4.1.2.1 Fatty acid composition of muscle tissue

When results were calculated in absolute basis (mg per 100 grams of meat; **Table 4.2**), wide concentration ranges were observed for the different groups of FAs. The reason for

this relies in the differences that may arise during the management of animals within a semi-controlled commercial study, which leads to variable total lipid contents among animals. Animal-related factors (*i.e.*, breed, age, feeding) were already reported to generate variable lipid contents in horse meat surveyed at retail level (136), capturing significant differences among collection period and regions surveyed.

Table 4.2 Total fatty acid composition (mg/100 g of meat) of horse *Longissimus thoracis et lumborum* muscle.

	Mean	Min	Max	SEM
Total FA	2427	893	4238	199
SFA	956	333	1743	85
BCFA	4.98	2.32	7.97	0.35
MUFA	1054	380	1877	93
cis-MUFA	1051	378	1872	92
trans-MUFA	3.32	1.07	5.42	0.29
CLA (18:2)	2.75	1.42	4.36	0.21
NC-dienes (18:2)	1.40	0.814	2.91	0.11
Trienes (18:3)	0.522	0.105	1.14	0.05
PUFA	345	147	546	22
n-6	266	113	390	15
18:2n-6	231	96	350	14
n-3	78	25	177	8
18:3n-3	56.1	15.6	154	8
DMA	60.3	26.6	84.7	3.3

Min, minimum value; Max, maximum value; SEM, standard error of the mean; FA, fatty acid; SFA, saturated FA; BCFA, branched-chain FA; MUFA, monounsaturated FA; CLA, conjugated linoleic acids; NC, non-conjugated; PUFA, polyunsaturated FA; DMA, dimethylacetals (sum of DMA, alk-1-enyl methyl ethers and fatty aldehydes).

In both, absolute and percentage basis (**Tables 4.2** and **4.3**), monounsaturated FAs (MUFAs) represented the major group of FAs (1054 mg/100 g; 42.9 %). While *c*-MUFAs represented 99.7 % of MUFAs (9*c*-18:1, 9*c*-16:1 and 11*c*-18:1 being the most abundant), *t*-MUFAs accounted only for 0.32 % (9*t*-18:1 being the most abundant). Indeed, *t*-MUFAs have not generally been reported in horse meat studies, because it is known that their content is low compared to ruminant products. Saturated FAs (SFAs) (333-1743 mg/100 g; 88.9 %) accounted for similar proportions as those described in previous studies, being palmitic acid (16:0) the major FA, followed by stearic (18:0) and myristic (14:0). Interestingly, branched-chain FAs (BCFAs) that are normally not discussed in horse meat studies were also found (4.98 mg/100 g; 0.21 %), being *iso*-16:0 the major one as also reported in previous horse meat studies by our group (130). Something similar occurs with CLA; they are minor in horse compared to ruminant meats, as they are primarily produced

from PUFAs by rumen microbiota. However, a low accumulation of these compounds was confirmed, representing 0.12 % of total FAs and in line with previous observations (137).

Table 4.3 Effect of fat tissue (muscle and subcutaneous fat) on total fatty acid composition (%) of horse meat.

	Muscle		**Subcutaneous**		***p* value**
	Mean	**SEM**	**Mean**	**SEM**	
SFA	38.9	0.3	39.5	0.5	0.383
12:0	0.206	0.006	0.224	0.008	0.079
14:0	3.65	0.09	4.33	0.09	<0.001
15:0	0.274	0.015	0.410	0.017	<0.001
16:0	29.3	0.3	29.9	0.4	0.256
17:0	0.357	0.015	0.505	0.020	<0.001
18:0	4.79	0.09	3.85	0.14	<0.001
BCFA	0.211	0.006	0.285	0.012	<0.001
i-16:0	0.0890	0.0023	0.0844	0.0037	0.297
MUFA	42.9	0.6	44.3	0.4	0.074
cis-MUFA	42.8	0.6	44.2	0.4	0.076
9*c*-14:1	0.412	0.013	0.389	0.016	0.295
7*c*-16:1	0.182	0.008	0.328	0.014	<0.001
9*c*-16:1	8.09	0.27	7.58	0.23	0.174
9*c*-18:1	31.4	0.4	32.7	0.4	0.025
11*c*-18:1	1.93	0.04	1.51	0.03	<0.001
13*c*-18:1	0.0965	0.0027	0.0531	0.0017	<0.001
11*c*-19:1	0.0675	0.0027	0.0738	0.0042	0.201
11*c*-20:1	0.335	0.008	0.470	0.016	<0.001
trans-MUFA	0.136	0.006	0.149	0.005	0.143
9*t*-18:1	0.0970	0.0026	0.0625	0.0026	<0.001
CLA (18:2)	0.118	0.007	0.0931	0.0050	0.008
9*c*,11*t*-	0.0535	0.0022	0.0581	0.0043	0.318
NC-dienes (18:2)	0.0605	0.0033	0.0475	0.0030	0.008
Trienes (18:3)	0.0220	0.0016	0.0263	0.0020	0.099
PUFA	15.0	0.7	15.6	0.5	0.455
20:3n-9	0.0263	0.0019	0.0125	0.0014	<0.001
n-6	11.7	0.7	12.5	0.6	0.424
18:2n-6	10.1	0.6	12.0	0.5	0.026
20:2n-6	0.206	0.012	0.281	0.012	<0.001
20:3n-6	0.240	0.015	0.0338	0.0022	<0.001
20:4n-6	0.946	0.071	0.0631	0.0053	<0.001
22:4n-6	0.0705	0.0067	0.0194	0.0021	<0.001
22:5n-6	0.0470	0.0054	ND	-	-
n-3	3.19	0.21	3.18	0.23	0.975
18:3n-3	2.26	0.19	2.98	0.21	0.018
20:3n-3	0.110	0.007	0.106	0.008	0.714
20:5n-3	0.125	0.0134	0.0119	0.0010	<0.001
22:5n-3	0.503	0.027	0.0561	0.0034	<0.001
22:6n-3	0.115	0.0084	0.0120	0.0010	<0.001
n-6/n-3	3.97	0.38	4.30	0.45	0.565
P/S	0.386	0.020	0.399	0.016	0.609
DMA	2.63	0.11	ND	-	-

SEM, standard error of the mean; SFA, saturated fatty acid (FA); BCFA, branched-chain FA; *i*, *iso*; MUFA, monounsaturated FA; *c*, *cis*; *t*, *trans*; CLA, conjugated linoleic acids; NC, non-conjugated; PUFA, polyunsaturated FA; P/S, polyunsaturated FA/ saturated FA; DMA, dimethylacetals (sum of DMA, alk-1-enyl methyl ethers and fatty aldehydes).

One of the most significant peculiarities of horses is their capacity to absorb dietary PUFAs before microbial hydrogenation, allowing their efficient deposition in horse tissues. This is

why, overall, horse meat has been described as rich in PUFAs, especially in essential linoleic (18:2n-6) and linolenic (18:3n-3) acids (12,138). In the present work, an average PUFA content of 345 mg/100 g of meat was found, accounting for the 15 % of all FAs. This percentage was not as high as that reported by others in the literature, where values up to 46 % have been reported in 2 year old grass fed Galician Mountain horses (139). The lower PUFA content found in comparison to other studies was related to a lower n-6 content, especially of linoleic acid, which is the major n-6 PUFA representing 86 % of this group. On average, linoleic acid accounted for 10.1 % of total FAs, a percentage that is below to those usually reported in horses slaughtered at similar ages and managed under semi-extensive systems (123,131). Presumably, the grain-finishing period of the animals studied in the present Ph.D. Thesis caused a remarkable decrease of linoleic acid in favour of MUFAs. Regarding the rest of n-6 PUFAs, arachidonic acid (20:4n-6) was the second most abundant, in agreement with previous works.

With respect to n-3 PUFAs, which are the ones majorly present in pasture, values from 1.53 % of total FAs in 36 month old Bretón horses up to 24.4 % in 9 month old extensively reared Galician Mountain horses have been reported in the literature (12). In the present work, an average value of 3.19 % n-3 PUFA (78 mg/100 g of meat) was observed, evidencing again that the grain-finishing (rich in 18:2n-6) limited the n-3 PUFA intake and deposition. On average, linolenic acid accounted for 2.26 %, and the next major long-chain n-3 PUFA were DPA (22:5n-3), EPA (20:5n-3), DHA (22:6n-3) and 20:3n-3. These four FA are seldom reported in horse meat studies. When compared with other livestock species, the percentage observed for n-3 in horse meat from the present work was similar to those typically described in non n-3 enriched chicken (3.03 %) or rabbit (3.40 %) and higher than non n-3 enriched pork (1.22 %) (140,141).

4.1.2.2 Muscle *vs*. subcutaneous fat tissue

It is known that SC fat is a sensitive indicator of FA profile in different species (104). However, very few studies have reported FA results from SC tissue in horse meat (12) and, therefore, a comparison of both adipose tissues (muscle and SC) was reported (**Table 4.3**). Several significant differences were found between both tissues. As expected, SC tissue showed a higher content of individual SFAs than muscle, and the same happened with total

and major MUFAs. This is because SC tissue is mainly composed of triacylglycerols that are components of neutral lipids, which in general exhibit high amounts of SFAs and MUFAs, and low PUFA depositions (130). As an exception, 18:0 showed a significantly higher content in muscle than in SC fat, because this FA is mainly esterified to polar lipids of horse meat as previously reported (130). No difference was found in total PUFA content between the tissues, but most of the long-chain n-6 and n-3 PUFAs were significantly higher in muscle than in SC fat.

4.2 Effect of ageing time on horse meat quality attributes

4.2.1 Effect of ageing time on volatile composition of horse meat

The aroma of cooked meat is an important quality attribute for consumer acceptability that is well known to change during the ageing process (70,71). In this section, volatile compounds from cooked horse meat are reported together with their estimated contribution to the overall aroma and the subsequent analysis of their evolution over the ageing process (**Publication IV**, Appendix IV).

4.2.1.1 Nature and origin of volatile compounds detected in cooked horse meat

In the volatile fraction of cooked horse meat (0, 7, 14 and 21 days of ageing), a total of 77 compounds were identified by SPME-GC-MS, which were initially sorted by their chemical families (**Table 4.4**). Most of the compounds were aldehydes (30 compounds), followed by alcohols (13), non-aromatic hydrocarbons (11), ketones (8), benzenoic compounds (4), furans (2) and a sulphur compound. In addition, another 8 volatile compounds were detected although their chemical nature could not be established because their MS signal was not clear enough. These were labelled unknowns. In terms of relative abundance, aldehydes were the most abundant (around 75 %) as observed in previous studies of meat from different species (76,142,143). From them, hexanal was the major aldehyde, followed by nonanal. The second major chemical family was that of alcohols (10 %), while the remaining families together accounted for around 13 % of the total volatile abundance.

It is noteworthy that some of the chemical families reported in relatively high concentrations in cooked meat from horse and other species were minor or absent in our results, namely short-chain FA, esters or nitrogen and sulphur compounds (only dimethyl trisulphide was detected). The reasons for the absence could be several, but the cooking procedure until a medium degree of doneness (144) and extraction conditions employed in SPME could the most relevant.

Regarding the chemical origin of detected volatiles, most of them derived from the thermal degradation of lipids during cooking. In this regard, PUFAs are known to increase the susceptibility of meat to undergo lipid oxidation and the subsequent formation of volatile compounds, as proved in other species (76,145,146). Moreover, experiments performed in meat-like model systems proved that the presence of n-3 and n-6 PUFAs reduced the formation of Maillard derived aromatic compounds, presumably due to the reaction of PUFAs with intermediate compounds from Maillard reaction (147). In this sense, the unsaturated FA content of raw meat is relevant to understand the volatile profile of cooked meat. For example, it is known that heptanal, octanal, nonanal, decanal, (*E*)-2-decenal and (*E*)-2-undecenal are major oxidation products of oleic acid, which is the predominant MUFA in horse meat (31 %; **Table 4.3**). The most relevant volatile compounds detected in the present study have been reported to be thermal oxidation/degradation products of oleic, linoleic and linolenic acids. However, some compounds formed via Strecker amino acid degradation, namely acetaldehyde and 2- and 3- methylbutanal, were also identified **(Table 4.4)**.

4.2.1.2 Contribution of volatile compounds to cooked horse meat aroma

After classifying the volatile compounds into their families and analysing their origin, the contribution of each compound to the aroma of cooked horse meat was estimated by the calculation of OIR values. Odour descriptors were also considered; these were retrieved from previous investigations or from specialized flavour databases (**Table 4.4**). Overall, aldehydes showed the highest OIR values in cooked horse meat, specially C6-C10 linear aldehydes and hexadecanal (OIR >34) and, in consequence, their contribution to cooked meat aroma was very relevant. These compounds are, in general, related to grassy and fruity odours. The contribution of alcohols, on the other hand, was, in general low (with the

exceptions of 1-hexanol, 1-heptanol and 1-octen-3-ol), and the same happened with ketones and non-aromatic hydrocarbons. From the rest of the volatile compounds, benzaldehyde and dimethyl trisulphide could be highlighted as they have been related to unpleasant odours in meat from different species (40,148). In our samples, dimethyl trisulphide showed a high OIR value (173) so it seems feasible that this compound contributed significantly to cooked horse meat aroma.

Table 4.4 Relative abundance after 0, 7, 14 and 21 days of ageing and estimated mean odour threshold, odour impact ratio (109) and odour description of volatile compounds of cooked horse meat analysed by solid-phase microextraction coupled to gas chromatography-mass spectrometry. Mean relative abundance values were calculated from volatile compounds detected in aged and cooked horse meat samples (n=80).

Volatile compound	IMe	LRI	Mean relative abundance	OT	OIR	SEM	0 d	7 d	14 d	21 d	SEM	*p* value	Odour description	Ref.
Aldehydes														
Acetaldehyde	P	704	7.07	25	0.283	0.009	7.71	6.66	6.80	7.09	0.30	0.791	Fruity	(149)
Propanal	P	796	5.78	145	0.0399	0.0025	5.60	5.25	6.49	ND	0.26	0.085	Nut like	(150)
2-Methylbutanal	P	911	2.88	4.40	0.655	0.115	ND	1.88^{b}	3.28^{a}	3.49^{a}	0.17	≤0.001	Cinnamon, toast	(151)
3-Methylbutanal	P	915	7.04	1.20	5.87	1.18	ND	4.23^{b}	8.17^{a}	8.73^{a}	0.40	≤0.001	Chocolate, caramel, green, nutty	(148)
Pentanal	T	981	46.5	12.0	3.88	0.09	48.4	46.2	48.0	43.5	1.7	0.187	Almond, malt, pungent, acrid	(40)
Hexanal	P	1078	501	1.00	500	32	564^{a}	527^{a}	497^{ab}	415^{b}	21	0.005	Grassy, tea, vegetable, lemony, sour, beefy	(40)
2-Methyl-2-butenal	T	1085	6.72	458	0.0147	$8.02 \cdot 10^{-4}$	7.44	6.12	7.27	6.06	0.38	0.294	Coffee like	(152)
Heptanal	P	1183	95.2	2.80	34.0	0.9	98.6	95.4	98.5	88.1	4.0	0.527	Fruity, nutty	(148)
(*E*)-2-Hexenal	P	1223	5.20	110	0.0473	0.0045	ND	4.70	5.70	ND	1.91	0.357	Eucalyptus, fruit/flower, potato, toast	(151)
Octanal	P	1282	134	0.700	191	6	122	139	143	133	6	0.094	Soap/orange	(151)
(*E*)-2-Heptenal	T	1333	31.8	13	2.44	0.08	34.6	30.2	32.3	29.9	1.5	0.165	Fishy	(151)
2-Methyl-2-heptenal	T	1363	5.60				6.40^{a}	6.12^{a}	6.05^{a}	3.82^{b}	0.39	0.013	-	-
Nonanal	T	1398	278	2.80	99.2	0.6	277	274	280	280	11	0.866	Grassy, tea, vegetable, lemony, sour, beefy	(153)
(*E*)-2-Octenal	P	1441	44.2	75.0	0.590	0.017	46.1	44.0	46.0	40.8	2.0	0.480	Green, nut, fat	(40)
Decanal	T	1505	13.3	1.50	8.88	0.12	13.8	13.0	13.2	13.3	0.5	0.765	Powerful, waxy, aldehydic, orange, citrus peel	(40)
(*E,E*)-2,4-Heptadienal	T	1509	8.65	15.4	0.562	0.036	8.22	8.04	10.3	8.04	0.43	0.147	Roast meat, fried potatoe	(154)

Table 4.4 (*continuation*)

Volatile compound	IMe	LRI	Mean relative abundance	OT	OIR	SEM	0 d	7 d	14 d	21 d	SEM	*p* value	Odour description	Ref.
(*E*)-2-Nonenal	P	1550	69.5	0.0800	868	21	72.8	67.5	71.9	65.7	2.9	0.364	Earthy, fermented,	(40)
(*E*)-2-Decenal	P	1662	171	0.350	487	11	177	166	177	162	8	0.179	Tallow, orange	(40)
2-Butyl-2-octenal	P	1683	36.8	20.0	1.84	0.22	45.9[a]	37.6[a]	39.1[a]	24.4[b]	3.4	0.003	-	-
Dodecanal	T	1722	53.6	55	0.974	0.026	51.7	57.6	53.5	51.4	2.8	0.357	Onion, green, vomit	(145)
(*E,E*)-2,4-Nonadienal	T	1726	43.1	0.100	430	1	43.0	ND	43.2	ND	2.8	0.594	Meaty, burnt, chocolate	(148)
(*E*)-2-Undecenal	T	1774	217	1.40	155	2	225	213	221	210	9	0.549	-	-
(*E,Z*)-2,4-Decadienal	T	1786	11.1	0.070	158	7	12.0	10.0	12.0	10.4	0.5	0.203	Fried onion, lemon	(155)
Tridecanal	T	1831	108	70	1.54	0.05	115	108	108	98.9	4.5	0.327	Nutty	(156)
(*E,E*)-2,4-Decadienal	T	1839	60.1	0.0270	2226	58	59.8	57.1	64.5	59.0	2.8	0.624	Fruity	(157)
Tridecanal	T	1831	108	70	1.54	0.05	115	108	108	98.9	4.5	0.327	Nutty	(156)
(*E,E*)-2,4-Decadienal	T	1839	60.1	0.0270	2226	58	59.8	57.1	64.5	59.0	2.8	0.624	Fatty, fried potatoe	(157)
Tetradecanal	T	1940	219	53	4.14	0.07	224	217	227	210	10	0.730	Roasted, fried meat	(158)
(*E,E*)-2,4-Undecadienal	T	1955	14.1	1.00	14.1	0.4	15.0	13.8	14.4	13.0	0.6	0.907	-	-
Pentadecanal	T	2049	222	1000	0.222	0.004	230	218	226	214	10	0.715	Hot timber	(145)
Hexadecanal	T	2156	96.9	0.910 (14)	106	17	71.2[c]	74.5[c]	102[b]	140[a]	5.5	≤0.001	Sweet	(145)
cis-11-Hexadecenal	T	2189	17.3				16.6	16.2	18.1	18.2	1.0	0.719	Waxy	(159)
Ketones														
2-Heptanone	P	1181	8.74	140	0.0624	0.0061	ND	9.59	7.88	ND	1.79	0.695	Rancid, flower, vinegar, soap/orange	(151)
6-Methyl-2-heptanone	P	1242	9.71	8.10	1.20	0.09	11.6	9.69	9.43	8.11	0.51	0.021	Cloves, menthol, eugenol	(40)
3-Octanone	T	1259	4.17	24	0.174	0.024	5.78	4.14	3.49	3.27	0.46	0.130	Herbal	(152)

Table 4.4 (*continuation*)

Volatile compound	IMe	LRI	Mean relative abundance	OT	OIR	SEM	0 d	7 d	14 d	21 d	SEM	*p* value	Odour description	Ref.
5-Methyl-3-hepten-2-one	T	1342	35.8				45.1[a]	37.7[ab]	33.7[bc]	26.6[c]	2.1	≤0.001	-	-
(*E*)-3-Octen-2-one	T	1415	7.18	250	0.0287	0.0008	6.73	7.33	7.70	6.98	0.35	0.895	Nut, crushed bug, earthy, spicy, herbal, sweet, mushroom, hay	(40)
(*E,E*)-3,5-Octadien-2-one	T	1586	13.5	125	0.108	0.008	12.2[b]	13.5[b]	16.4[a]	11.8[b]	0.7	0.013	Fruity, green, grassy	(159)
6,10-Dimethyl-(*E,E*)-5,9-undecadien-2-one	T	1870	12.0	60	0.200	0.011	12.7	11.3	ND	ND	0.54	0.166	Fresh, green, fruity, waxy, rose, woody, magnolia tropical	(159)
2-Pentadecanone	T	2038	8.65				ND	7.63[b]	8.92[ab]	9.41[a]	0.42	0.001	Fresh, jasmin, celery, fatty, oily, waxy, burnt	-
Alcohols														
1-Penten-3-ol	P	1055	0.674	400	$1.69 \cdot 10^{-3}$	$6.50 \cdot 10^{-5}$	0.708	0.631	0.730	0.629	0.026	0.239	Flower, burnt, meaty	(151)
1-Pentanol	P	1251	36.5	$4.00 \cdot 10^{3}$	$9.12 \cdot 10^{-3}$	$4.78 \cdot 10^{-4}$	40.5	38.3	35.3	31.7	1.6	0.052	Mild odour, fuel oil, fruit, balsamic	(40)
1-Hexanol	P	1352	14.8	5.60	2.64	0.11	15.2[a]	15.2[a]	15.6[a]	13.0[b]	0.6	0.035	Woody, cut grass, chemical-winey, fatty, fruity, weak metallic	(40)
1-Octen-3-ol	P	1446	83.3	1.50	55.5	1.8	88.4	85.4	83.8	75.6	3.2	0.249	Fishy, fatty, mushroom, grassy	(160)
Heptanol	P	1453	42.6	5.40	7.88	0.11	42.9	42.0	44.0	41.4	1.6	0.766	Fragant, woody, oily, green, fatty, winey, sap, herb	(40)
2-Ethyl-hexan-1-ol	T	1488	2.79	$2.54 \cdot 10^{4}$	$1.09 \cdot 10^{-4}$	$4.74 \cdot 10^{-6}$	2.60	2.75	2.66	3.14	0.11	0.191	Resin, flower, green	(40)
1-Octanol	P	1555	76.8	190	0.404	0.010	77.2	78.6	80.0	71.4	2.9	0.894	Fatty, waxy, citrus, oily, walnut, moss, chemical, metal, burnt	(40)
3,5-Octadien-2-ol	T	1581	6.47				7.16	ND	6.03	6.23	0.65	0.499	-	-
(*E*)-2-Octen-1-ol	P	1613	24.7	20	1.24	0.05	26.9	24.7	24.1	23.1	0.9	0.075	Green, citrus	(40)

Table 4.4 (*continuation*)

Volatile compound	IMe	LRI	Mean relative abundance	OT	OIR	SEM	0 d	7 d	14 d	21 d	SEM	*p* value	Odour description	Ref.
9-Decen-2-ol	T	1781	8.04				9.75	6.32	ND	ND	0.59	0.016	-	-
1-Dodecanol	T	1966	18.8	158	0.119	0.005	20.5[a]	19.3[ab]	18.7[ab]	16.9[b]	0.8	0.037	Earthy, soapy, waxy, fatty, honey, coconut	(159)
1-Tridecanol	P	2083	10.8				11.9	9.87	10.6	10.6	0.55	0.595	Musty	(159)
1-Tetradecanol	P	2174	27.4				28.1	27.7	28.7	25.2	1.5	0.345	Fruity, waxy, coconut	(159)
Non-aromatic hydrocarbons														
2,2,4,6,6-Pentamethyl-heptane	T	958	5.12				ND	5.1	ND	ND	0.4		-	-
Butyl-cyclopentane	T	1038	2.24				ND	2.2	ND	ND	0.1		-	-
Tridecane	P	1297	14.5	$2.14 \cdot 10^{3}$ (17)	$6.75 \cdot 10^{-3}$	$2.13 \cdot 10^{-4}$	14.6	13.3	15.6	14.3	1.0	0.829	Alkane	(39)
3-Ethyl-2methyl-1,3-hexadiene	T	1430	23.0				22.2	23.0	23.7	22.9	1.1	0.697	-	-
Pentadecane	P	1498	23.7	$1.30 \cdot 10^{7*}$	$1.82 \cdot 10^{-6}$	$3.10 \cdot 10^{-8}$	24.6	22.7	23.5	23.9	1.2	0.946	Waxy	(159)
1- Pentadecene	T	1525	8.14	$3.60 \cdot 10^{3}$	$2.26 \cdot 10^{-3}$	$1.08 \cdot 10^{-4}$	8.84[a]	8.60[a]	8.05[ab]	7.09[b]	0.36	0.012	-	-
5,5-Dimethyl-1,3-heptadiene	T	1591	7.58	$1.30 \cdot 10^{7*}$	$5.87 \cdot 10^{-7}$	$5.98 \cdot 10^{-8}$	9.14	7.95	7.81	5.43	0.64	0.126	-	-
Hexadecane	P	1598	12.1	500*	0.0242	0.011						0.187	Mild waxy	(159)
1-Hexadecene	T	1647	11.3	$8.00 \cdot 10^{3}$	$1.42 \cdot 10^{-3}$	$4.65 \cdot 10^{-5}$	9.20	7.51	9.52	8.16	0.50	0.468	-	-
1-Heptadecene	T	2270					11.6	10.3	11.5	12.0	0.4	0.099	-	-
1,15-Hexadecadiene	T	958	5.12				23.2	22.0	23.6	23.6	1.0	0.937	-	-

Table 4.4 (*continuation*)

Volatile compound	IMe	LRI	Mean relative abundance	OT	OIR	SEM	0 d	7 d	14 d	21 d	SEM	*p* value	Odour description	Ref.
Benzenoic compounds														
Toluene	P	1035	3.78	42	0.0900	0.0032	ND	3.54	4.01	3.79	0.14	0.472	Chemical solvent aroma	(161)
Benzaldehyde	P	1545	75.9	$7.51\cdot10^{-4}$	$1.01\cdot10^{5}$	$4.92\cdot10^{3}$	67.6^{c}	72.1^{bc}	79.8^{ba}	83.9^{a}	2.5	≤0.001	Almond oil, bitter almond, burning aromatic taste	(40)
3-Ethyl-benzaldehyde	T	1743	109				115	108	111	102	3	0.387		
4-Pentyl-benzaldehyde	T	2057	23.3				25.0	23.4	23.0	21.8	1.0	0.442		
Furans														
2-Ethyl-furan	T	950	3.14	8000	$3.92\cdot10^{-4}$	$5.59\cdot10^{-5}$	ND	2.69	3.58	ND	0.18	0.022	Sweet corn	(162)
2-Pentyl-furan	T	1231	50.2	5.80	8.65	0.19	ND	52.3	49.6	48.7	2.5	0.174	Green, vean, butter	(40)
Miscelaneous														
Dimethyl trisulphide	T	1394	17.4	0.100	173	53	32.0	8.90	18.3	10.3	4.73	0.488	Sulphury, burnt,onion	(148)
Unknown$^{m/z: 97, 55, 41, 71, 84}$		1471	80.5				61.5^{b}	87.1^{a}	85.2^{a}	88.2^{a}	3.9	≤0.001		
Unknown$^{m/z: 67, 95, 41, 81, 12}$		1630	7.70				7.86	7.68	8.00	7.26	0.35	0.736		
Unknown$^{m/z: 43, 84, 71, 57, 128}$		1696	16.5				16.8	16.2	16.7	16.1	0.8	0.979		
Unknwon$^{m/z: 121, 91, 77, 150, 65}$		1889	70.5				69.0	67.7	75.6	69.6	3.0	0.560		
Unknwon$^{m/z: 95, 81, 43, 55, 67}$		1918	24.9				27.0^{a}	25.8^{a}	27.0^{a}	19.6^{b}	1.2	0.032		
Unknwon$^{m/z: 43, 41 57, 83, 69}$		1997	13.2				15.6	12.7	12.1	12.4	0.7	0.051		
Unknown$^{m/z: 55, 43, 69, 83, 97}$		2070	27.3				28.6	28.1	27.5	25.2	1.3	0.357		
Unknown$^{m/z: 45, 55, 67, 73, 41}$		2295	22.5				21.1	21.9	25.4	21.5	1.3	0.172		

d, days; IMe, identification method; SEM, standard error of the mean; P, positive identification; T, tentative identification, ND, not detected; OT was expressed as µg/kg of water; *OT, expressed as µg/kg of oil; Ref, reference; Means with different superscripts indicate statistically significant differences among ageing days ($p < 0.05$).

4.2.1.3 Effect of ageing time on cooked horse meat volatile composition

The last step in the analysis of horse meat aroma consisted on studying changes the in the volatile composition caused by the ageing process (0, 7, 14 and 21 days). In this regard, changes in the concentration of volatile compounds may indicate chemical, enzymatic or microbial degradation of precursors such as peptides, amino acids, sugars and lipids, depending mainly on the packaging type used during the process and on temperature (section 1.3.3.2). From the 77 individual volatile compounds detected, the abundance of 15 changed significantly ($p < 0.05$) during the ageing process. The relative abundance of some compounds, including three aldehydes (hexadecanal and 2- and 3- methylbutanal), benzaldehyde and 2-pentadecanone, increased with ageing time. Interestingly, both 2- and 3- methylbutanal were not detected in non-aged meat samples but their relative abundance increased significantly from 7 to 14 days of ageing. These branched aldehydes were already reported to increase in beef samples aged for 28 days (163), and the reason for their increase was suggested to be an increase in free leucine and isoleucine content related to the proteolysis occurring during ageing (164).

Among the rest of compounds that showed increasing concentrations over the ageing period, benzaldehyde had already been described to increase during horse meat ageing (77,131) whereas hexadecanal, as it has been related to sweet odour notes, could be the responsible for the sweeter aroma of aged compared to non-aged horse meat. The increase of this latter compound is likely related to the oxidative degradation of lipids. Autooxidation, however, did not seem to be relevant during the ageing process of horse meat because other compounds typically derived from lipid oxidation did not increase, and even decreased their concentrations. Indeed, some volatile compounds related to lipid oxidation such as hexanal, 2-methyl-2-heptenal, 2-butyl-2-octenal, hexanal, dodecanol, 5-methyl-3-hepten-2-one, (*E,E*)-3,5-octadien-2-one, and 1-pentadecene significantly decreased over time in the cooked horse meat samples.

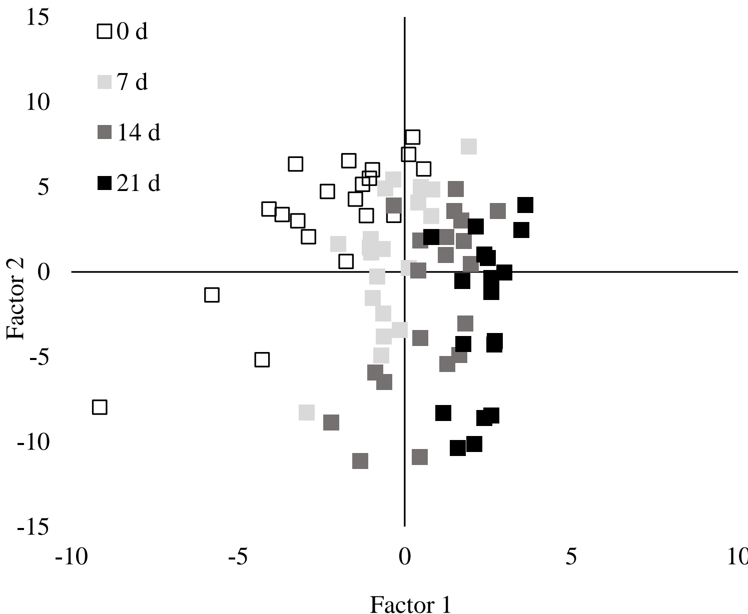

Figure 4.1 Partial least squares- discrimination analysis (PLS-DA) factor scores depicting cooked horse meat sample distribution according to ageing time (0, 7, 14 and 21 days). Y-variable: ageing time, X-variable: weighed relative abundance of volatile compounds.

Factor 1: explained 18 % of X variance; explained 19 % of Y variance. Factor 2: explained X-variance 36 %; explained Y-variance 7 %. d, days.

The decrease in the concentration of several compounds during ageing does not agree with what is usually reported in the literature about aged and cooked meat, where an increase of lipid oxidation compounds has been described (77). In agreement with our results, decreasing contents of several oxidation products such as aldehydes and ketones during the ageing period were reported in cooked meat from n-3 enriched grazed lambs (75). This was attributed to the presence of antioxidants coming from pasture botanical species (155), and may also be the case of results observed in the present work. Another possible explanation, as proved to occur in other meats, could be the reaction or interaction among proteolysis and lipid oxidation products during the ageing process, giving rise to other non-volatile compounds and leading to a decrease in volatile compound abundance (75,76).

The PLS-DA methodology clearly confirmed that horse meat volatile profile was affected by ageing time. Indeed, when scores calculated for horse meat samples in the two-dimensional plot formed by the PLS-DA model were checked, non-aged samples clearly separated from the ones aged for 14 and 21 days. Similarly, samples aged for 7 days clearly

separated from samples aged for 21 (**Figure 4.1**), but samples aged for 7 and 14 days were very close to each other. Therefore, under the present conditions, a period of at least 14 days would be necessary to result in a differentiated volatile profile.

4.2.2 Effect of ageing time on pH of horse meat

It is known that evolution of muscle pH is indicative of *post-mortem* metabolism (section 1.3.3.1) and it is helpful to understand the evolution of other meat quality attributes. Initial pH value measured at non-aged meat (5.60 ± 0.09 at 48 h *post-mortem*) was similar to that reported by others in loin samples from other horse breeds (29,124,125). This would mean that a normal acidification was achieved during *post-mortem* metabolism (**Figure 4.2**). Indeed, defects coming from an anomalous *post-mortem* metabolism have scarcely been reported in horse meat literature of. Observed initial pH value was slightly lower than the one typically reported in beef (165,166). This could be related to the higher glycogen reserves reported in horse meat compared to beef (167). For this reason, horse muscle would retain more residual glycogen after reaching the *rigor mortis* and that would result in lower pH values at 48 h *post-mortem*.

In addition to the effect of the ageing time (0, 7, 14 and 21 days), the statistical analysis revealed that slaughter day (D; 5 consecutive days) and its interaction with ageing time were significant ($p < 0.01$), which would be attributed to other uncontrolled pre-slaughter factors, especially at the first slaughter day, affecting the pH of meat (**Figure 4.2**). Focusing on the effect of ageing process, in general, pH values decreased from 0 to 7 days and then, stayed stable until the end of the ageing period established. However, only in samples collected during the first slaughter week, pH continued decreasing until the second week of ageing. This evolution was not easy to explain but according to the literature, the pH decrease could be potentially related to the action of LAB, typical microflora found in vacuum aged meat (section 1.3.3.7). In this line, a slight increase of LAB in vacuum packed horse meat from Galician Mountain breed during the first 7 days of ageing has been reported, which did not entail the development of spoilage odours (22). In contrast, pH

increases have been also reported by others in meat aged for 14 (22,23) and 20 days (29) (**Publication III**, AppendixIII).

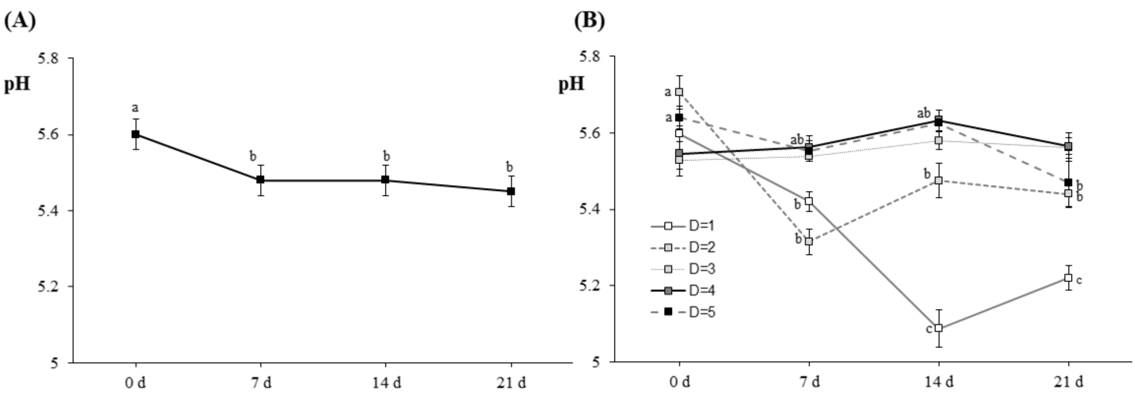

Figure 4.2 Effect of ageing time (0, 7, 14 and 21 days) on mean pH values (**A**) and on mean pH values for each slaughter day (5 consecutive weeks) (**B**) in horse *Longissimus thoracis et lumborum* muscle.

d, days; D, slaugher day. Different letters indicate significant differences among ageing times ($p < 0.05$).

4.2.3 Effect of ageing time on instrumental colour of horse meat

The colour of horse meat was initially analysed instrumentally using a colorimeter (**Publication III**, AppendixIII). As indicated in section 1.3.4.2, colour is the most important quality attribute influencing purchase decisions from consumers, and it is known to change during meat ageing. In this regard, evolution of colour indexes needs to be considered in order to establish an optimum ageing period. In general, the range of instrumental values obtained in the present Ph.D. Thesis for the different colour measurements was in good agreement with horse meat studies reported in the literature (23,129,168). Ageing time significantly ($p < 0.01$) affected all colour measurements, while slaughter day and their interaction (ordinal interaction) were also significant for some measurements. In these cases, however, their effect size (measured by η^2) was smaller than the effect of ageing time.

Our results indicate that L^* significantly increased by ageing (**Figure 4.3**). According to literature, L^* values would not vary so much during *port-mortem* storage, but they may

show slight increases due to changes in light scattering associated to muscle shrinkage and fluid expulsion to the extracellular space (169). Regarding *a** values, redness reached its highest value after 14 days of ageing. The lowest values were found for non-aged meat, whereas intermediate values were observed in meat aged for 7 and 21 days. In this respect, other authors have reported increasing redness values of aged horse meat over 14 (23) and 30 days (29) of ageing. Finally, *b** value increased from non-aged to meat aged for 14 days as reported by others (29), while no difference was found between 14 and 21 days (**Figure 4.3**).

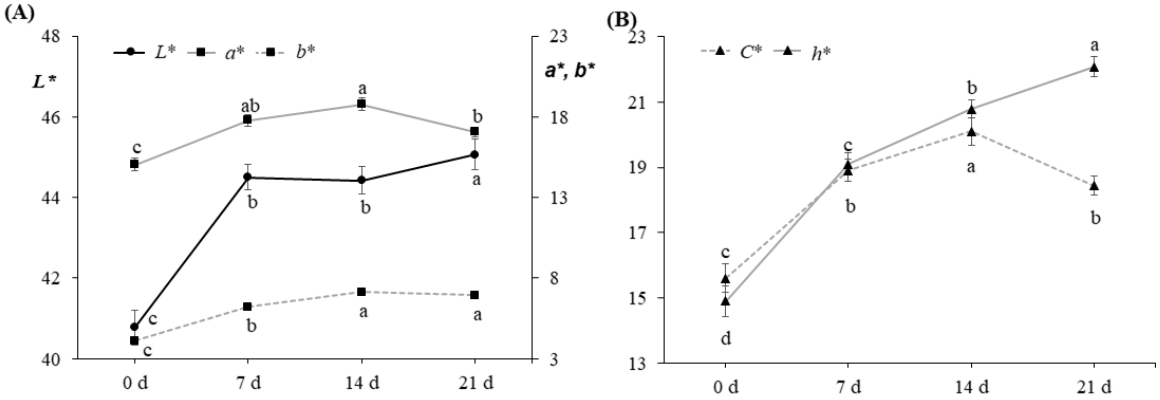

Figure 4.3 Effect of ageing time (0, 7, 14 and 21 days) on mean values of instrumental colour measurements in horse *Longissimus thoracis et lumborum* muscle. *L** (left axis), *a** and *b** (right axis) values (**A**), and *C** and *h** values (**B**).

d, days. Different letters indicate significant differences among ageing times ($p < 0.05$).

Colour indexes are helpful to visualize colour evolution during the ageing period. Particularly, the decrease observed in *a** but not in *b** between 14 and 21 days of ageing resulted in a significant increase of *h** values from 20.8 to 22.1, in the yellow (+*b**) direction of the CIE *L*a*b** space. At the same time, a decrease in *C** from 20.1 to 18.4 was observed, being recognized as a more grey (dull yellow) perceived as brown. This would indicate that horse meat started to turn brownish between 14 and 21 days of ageing, due to the formation of metmyoglobin and the impossibility to convert it back to deoxy and/or oxymyoglobin. These changes are well illustrated in the photographs of horse meat steaks taken in the present Ph.D. Thesis for the consumer study (**Figure 3.6**), where four representative photographs of each ageing time were gathered.

4.2.4 Effect of ageing time on cook loss and shear force of horse meat

Texture is the quality attribute that is most affected by meat ageing process, primarily because of an increase of tenderness throughout the process. Being tenderness one of the most important quality attributes for the consumer, its evolution during the ageing process needs to be understood. In the present Ph.D. Thesis, instrumental tenderness was estimated by WBSF measurements. In addition, WHC, a quality attribute that is closely related to juiciness (section 1.3.4.4), was also evaluated by determining cook loss (83). As expected, ageing time significantly ($p < 0.01$) affected both quality attributes, and slaughter day was also significant for these determinations (**Publication III**, Appendix III).

4.2.4.1 Effect of ageing time on cook loss of horse meat

It is known that cooking contributes to texture changes in meat and that cook loss is time and temperature dependant. Under the assay conditions of the present thesis, cook loss values obtained ranged between 20.3 and 23.0 %, being these similar to those reported by others in cooked horse loins (29,170) (**Figure 4.4A**). With regard to ageing, cook loss decreased from non-aged to meat aged for 7 days and then remained constant, meaning that aged meat (7, 14 or 21 days) would have a higher WHC and would be considered juicier. To this respect, the two physiological explanations that have been provided for the higher capacity of aged meat to retain water (section 1.3.3.6) associate this phenomena to the evolution of *post-mortem* pH. In our case, pH changes were also observed between 0 and 7 days of ageing (section 4.2.2). In this regard, meat aged for 7 days or longer underwent transformations that led to a higher WHC. Other authors that have investigated cook loss in horse meat over the ageing process have not observed differences (29) or have observed an increase in cook loss in a later stage of the ageing process (day 14) (170). Overall, few studies that have reported data about cook loss in horse meat have yielded a great variability of results.

4.2.4.2 Effect of ageing time on shear force of horse meat

Values of WBSF ranged between 68.5 N for non-aged and 32.1 N for meat aged during 21 days (**Figure 4.4B**), being the mean value for non-aged meat comparable to those reported

previously in the literature. As expected, WBSF values decreased significantly during the first two weeks of ageing, with no significant changes from here until the end of the process. Considering the WBSF-dependant classification proposed for beef loin (as there is no homologous classification for horse meat) (171), non-aged meat would be classified as 'tough', meat aged for 7 days as 'intermediate' and meats aged for 14 and 21 days as 'tender'.

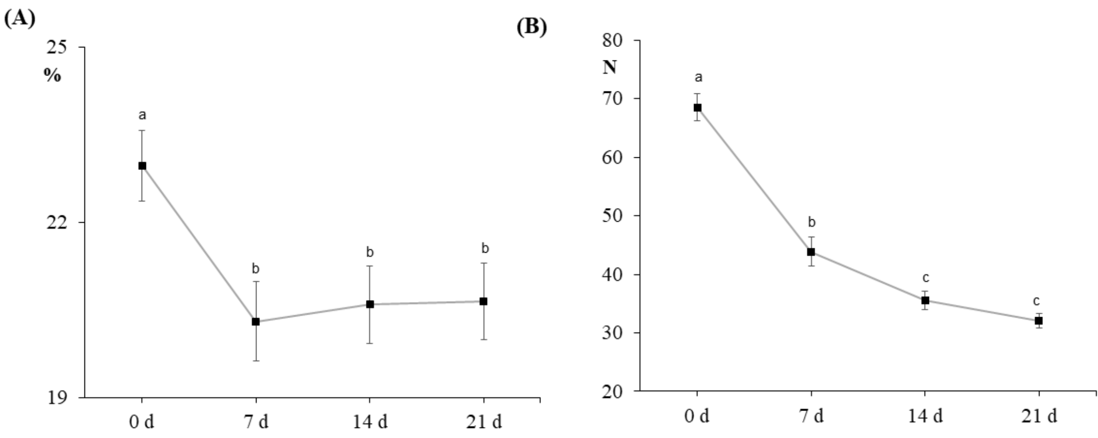

Figure 4.4 Effect of ageing time (0, 7, 14 and 21 days) on cook loss **(A)**, and Warner-Bratzler shear force (**B**) in horse *Longissimus thoracis et lumborum* muscle.

d, days; WBSF, Warner Bratzler shear force. Different letters indicate significant differences among ageing times ($p < 0.05$).

Essentially the ageing period, and particularly the first two weeks, improved horse meat tenderness. Comparing with literature, some authors have reported a decrease in WBSF values during the first 10 days of ageing (29), while others have reported an earlier tenderization, obtaining significantly more tender meat after only three days of ageing (24).

4.2.5 Effect of ageing time on the myofibrillar sub-proteome of horse meat

In order to delve in the tenderization process of horse meat during ageing, the myofibrillar sub-proteome was studied to further understand the degradation of this highly organized structural network during this period (section 1.3.3.1) (**Publication V**, Appendix V). The myofibrillar fraction of muscle proteome was specifically chosen because it contains mainly structural (insoluble) proteins, responsible for the mechanical resistance of meat, rather than metabolism (soluble) proteins. Despite this, the presence of soluble proteins in

the myofibrillar fraction has been described (172) due to changes in protein solubility at early *post-mortem* stages induced by pH reduction and high temperature. Initially, non-aged meat and meat aged for 7, 14 and 21 days were investigated by 1-DE. Subsequently, the optimised approach based on OFFGEL fractionation coupled to 1-DE was used to further investigate differences between non-aged meat and meat aged for 21 days.

4.2.5.1 Study of the myofibrillar sub-proteome by 1-DE

Separation of myofibrillar sub-proteome by 1-DE evidenced ten protein bands (A to J) that were significantly ($p < 0.05$) affected by ageing time (**Figure 4.5**). These bands were further identified by LC-MS/MS, revealing that seven of them contained more than one protein. This is not surprising considering the resolution limitations of 1-DE technique (173). Simultaneous identification of co-eluting proteins was enabled by tandem MS (**Table 4.5**). Although the trend observed in these seven bands could not be attributed to a single protein, an overview of the major changes occurring during ageing was feasible. On the other hand, certain bands were considered protein fragments when they were correctly identified but showed remarkable inconsistency between their theoretical and apparent molecular weights.

For bands A-J, most changes in abundance among ageing periods were observed between days 0 and 7. This was the case of five of the bands (B, E, F, G and H). In addition, four bands changed in abundance between 14 and 21 days. On the contrary, none of the bands considered showed statistically significant differences between 14 and 21 days of ageing. This was in accordance to that observed by WBSF measurements (section 4.2.4.2), evidencing an increase in tenderness from 0 to 14 days, with no significant increase after this period. Thus, from these data we could infer that the third week of ageing did not improve tenderness, presumably because no further significant degradation of structural proteins occurred.

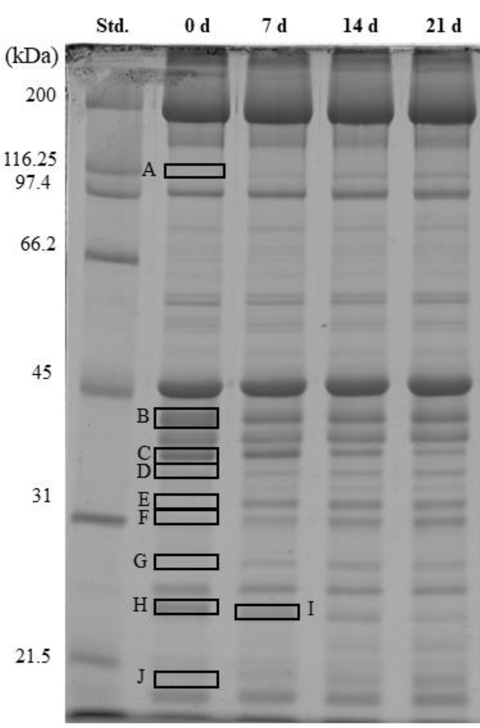

Figure 4.5 12 % SDS-PAGE of myofibrillar extracts from 0, 7, 14 and 21 days aged horse *Longissimus thoracis et lumborum* samples.

d, days.

It is noteworthy to point that most bands that changed in abundance among ageing times had molecular weights below 45 kDa, as already observed by other authors in *post-mortem* proteomes of horse and bovine species (24,172). The exception was a fragment of MYBPC1 (band A, see Protein Nomenclature), whose abundance increased with ageing. MYBPC1 protein is known as one of the accessory components of muscle contraction. Its detailed mechanism of action and properties remain poorly characterised and there are no previous reports about changes in MYBPC1 abundance during *post-mortem* horse meat storage.

A single protein (MYL1) was identified in band I. Its abundance decreased after two weeks of ageing and then remained constant. In line with this, it had been hypothesized that MYL1 is released from the myofibrillar to the soluble fraction during ageing as a consequence of *post-mortem* proteolysis, acting as an indicator of this phenomenon (174). The behaviour of MYL1 would be, again, consistent with the fact that the third week of ageing did not

result in a further increase in tenderness. Concerning the remaining eight bands, those that increased their abundance throughout ageing time (bands E, F and G) consisted of protein fragments generated by *post-mortem* proteolytic activity.

Table 4.5 LC-MS/MS identification and mean values of abundances (in arbitrary units) obtained by image analysis in 0, 7, 14 and 21 days aged samples for proteins found in A-J bands (Figure 4.5).

Band	PROTEIN ID	0 d	7 d	14 d	21 d	SEM
A	MYBPC1 **fragment**	148^{b}	332^{ab}	614^{ab}	822^{a}	81
B	TPM2 ALDOA	10944^{a}	7461^{b}	7466^{b}	8261^{ab}	409
C	GAPDH CAPZA2	6936^{a}	5261^{a}	2912^{b}	1765^{b}	391
D	LDHA MDH2	1015^{c}	1340^{bc}	1851^{ab}	2096^{a}	109
E	TNNT3 **fragment** VDAC3 ATP5F1C	421^{b}	4131^{a}	4286^{a}	3864^{a}	248
F	CA3 TNNT3 **fragment** PGAM2	507^{c}	2965^{b}	3294^{ab}	4006^{a}	196
G	MYL3 **fragment**	558^{b}	1504^{a}	1824^{a}	1732^{a}	100
H	TNNI2 ATP5PO	4185^{a}	275^{b}	ND	ND	291
I	MYL1	2957^{a}	4823^{a}	1732^{b}	1624^{b}	257
J	MYLPF TNNI2	596^{b}	810^{b}	1641^{a}	2296^{a}	135

SEM, standard error of the mean. Different superscripts indicate statistically significant differences among ageing times ($p < 0.05$).

d, days.

4.2.5.2 Study of the myofibrillar sub-proteome by OFFGEL and 1-DE

In order to overcome the resolving limitations of 1-DE technique and with the objective to analyse individual protein changes, OFFGEL fractionation was incorporated as a preliminary protein fractionation step for which an optimized approach was utilized. Since most protein bands showing significant changes in 1-DE analysis had molecular weights below 45 kDa, 5-16 % polyacrylamide gradient gels were used to analyse OFFGEL fractions, as these were the gels showing the highest resolving power for that molecular weight range. As expected, results from the combination of OFFGEL with SDS-PAGE fractionation techniques favoured, in most cases, the isolation of single protein bands (**Figure 4.6**). In some cases, proteins appearing at the same position in neighbouring SDS-PAGE lanes (bands A, B, C and M) were collected together (same protein) for quantitative analysis.

Most of the bands identified after OFFGEL and 1-DE fractionation were structural and muscle contraction proteins (**Table 4.6**). Among them, abundance of MYL1 (band A) was higher in non-aged meat than in meat aged for 21 days because, as discussed in section 4.2.5.1, this protein is released *post-mortem* from the myofibrillar to the sarcoplasmic fraction. Similarly, abundance of TUB4A decreased by ageing. TUB4A, a protein that is constituent of the microtubules and with an important role in cellular processes, had already been linked to tenderization in beef (175). According to results of the present work, it may also play a role in the tenderization of horse meat. TNNT3 (identified in bands C_1 and C_2) also decreased with ageing, giving rise to a degradation product of about 31 kDa identified in bands M_1 and M_2 that, consequently, was more abundant in meat aged for 21 days. Such degradation of TNNT3 *post-mortem* and the appearance of 30 kDa fragments has been well recognized to be associated to development of meat tenderness in beef (32) and, more recently, also in horse meat (24). All this, together with present results, make it reasonable to think that TNNT3 could be investigated as a good biomarker of horse meat tenderness. A similar result was found for TNNI2 (band J) whose abundance decreased after ageing, giving rise to two fragments of about 16 and 19 kDa (bands K and L). Other authors suggested that degradation of TNNI2 during the first 24 hours *post-mortem* could be indicative of the tenderization rate in beef (176). In our case, however, further research would be necessary to corroborate this assumption.

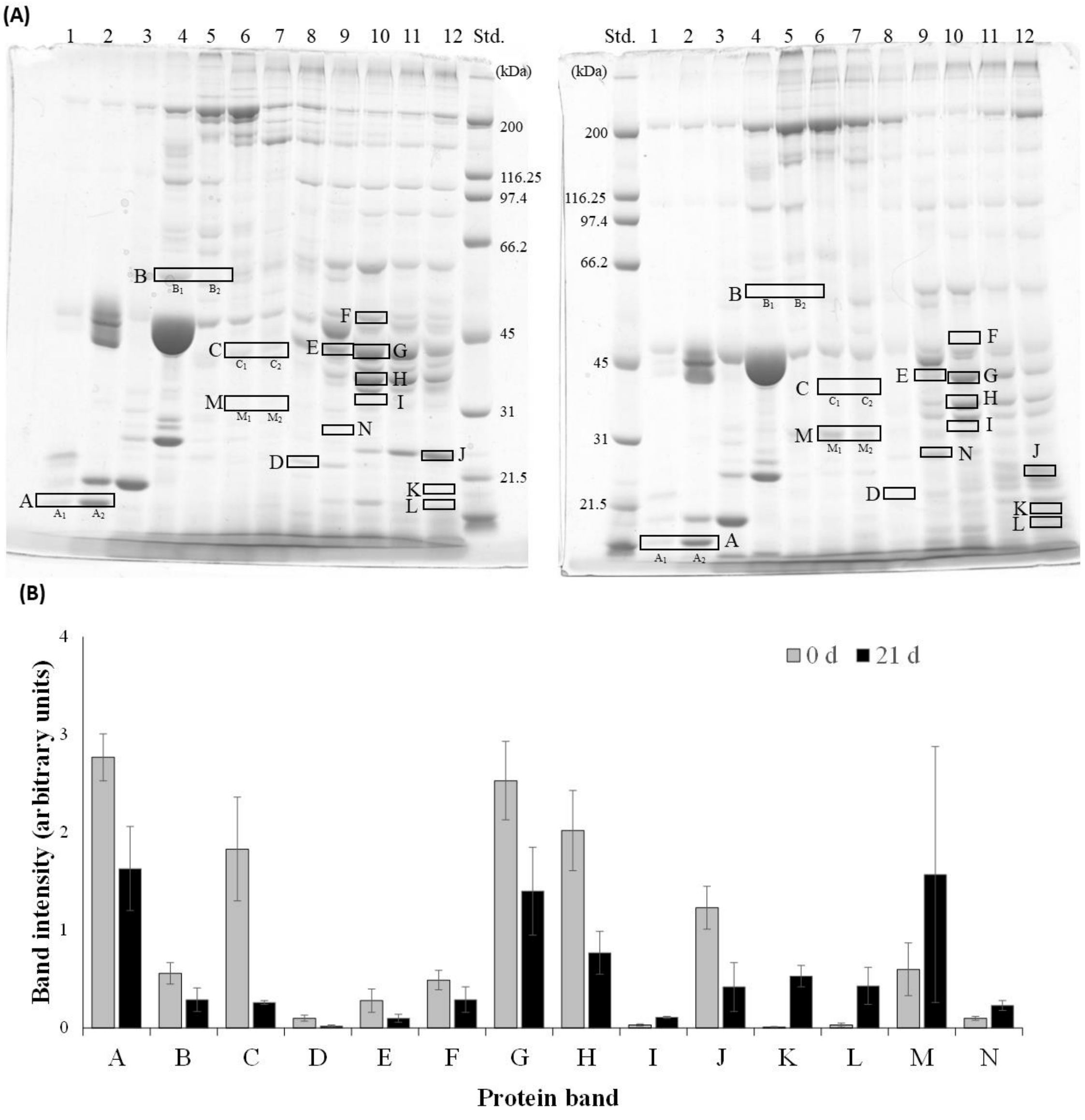

Figure 4.6 5-16 % SDS-PAGE gradient gels of the myofibrillar extracts obtained after OFFGEL fractionation (3-10 pH range) from non-aged (left) and 21 (right) days aged **(A)**, and quantification by gel image analysis of SDS-PAGE protein bands A-N corresponding to non-aged (■) and 21 d aged (■) **(B)** horse meat samples.

d, days. Error bars indicate the standard deviation of the means for each group.

As indicated earlier in this section, not only structural proteins were observed to change in abundance during ageing. Band D, for instance, was identified as CRYAB, a small heat shock protein that binds to myofibrils and protects skeletal muscle from protein degradation. It has been suggested that decrease in heat shock proteins could be related to the evolution of tenderness, claiming that they have no longer the ability to prevent damage

of muscle structure (177). Accordingly, CRYAB was more abundant in non-aged than in meat aged for 21 days.

Table 4.6 LC-MS/MS identification, theoretical and apparent molecular weight and isoelectric point of proteins found in A-N bands (**Figure 4.6**).

Band	PROTEIN ID	Theoretical[a] Mw (kDa)	Apparent[b] Mw (kDa)	Theoretical[a] pI	Apparent[b] pI
A1	MYL1	16.68	17	4.62	3.59-4.08
A2	MYL1	16.68	17	4.62	4.08-4.56
B1	TUB4A	49.55	63	4.78	5.05-5.53
B2	TUB4A	49.55	63	4.78	5.53-6.02
C1	TNNT3	37.67	38	6.13	6.02-6.50
C2	TNNT3	37.67	38	6.13	6.50-6.98
D	CRYAB	20.02	23	6.76	6.98-7.46
E	CKM	43.16	43	6.79	7.46-7.94
F	ENO3	47.02	47	8.05	7.94-8.42
G	ALDOA GAPDH	39.43 35.80	42	8.30 8.22	7.94-8.42
H	GAPDH	35.80	37	8.22	7.94-8.42
I	LDHA	36.57	32	8.17	7.94-8.42
J	TNNI2	21.38	23	8.86	8.9-9.38
K	TNNI2 **fragment**	21.38	19	8.86	8.9-9.38
L	TNNI2 **fragment**	21.38	16	8.86	8.9-9.38
M1	TNNT3 **fragment**	37.67	31	6.13	6.02-6.50
M2	TNNT3 **fragment**	37.67	31	6.13	6.50-6.98
N	CKM **fragment**	43.16	27	6.79	7.46-7.94

Mw, molecular weight; pI, isoelectric point.

Proteins related to energy metabolism were also identified. It is the case of CKM (band E), which is one of the most cited tenderness biomarkers in literature. This enzyme is involved in the initial *post-mortem* metabolism and has been reported to be progressively degraded during ageing until its complete inactivation (178). This seemed to be the case in *post-mortem* horse muscle, since abundance of band E decreased after ageing for 21 days. Likewise, bands F, G and H decreased from non-aged to meat aged for 21 days, being identified as glycolytic enzymes ENO3, ALDOA and GAPDH, respectively. Being glycolysis an important biochemical pathway affecting *post-mortem* meat quality, it seems clear that *post-mortem* evolution of these proteins may affect meat tenderization, regardless of not having a direct link with tenderness. The *post-mortem* decreasing abundance of these proteins was already observed in horse meat (25). Band I was also identified as a glycolytic enzyme (LDHA), but this time its abundance increased after three weeks of ageing. This could be explained because of a higher LDHA activity or a loss in solubility of this protein as the ageing process went on. In general, further research would be required to understand the relation of glycolytic enzymes with meat tenderization.

4.2.6 Effect of ageing time on consumer preference and sensory description of horse meat

Evaluation of the effect of ageing time in horse meat quality was made by the consumer (**Publication VI**, Appendix VI). They evaluated the acceptability of meat and provided a detailed description of samples by the novel CATA method, both in-mouth and visual. Interestingly, many of the attributes instrumentally measured in the previous sections of this thesis were well reflected in the description made by consumers in CATA.

4.2.6.1 In-mouth and visual acceptability

Both IMA and VA were affected by the ageing time (**Figure 4.7**). In addition, as it is usual in this type of studies, consumer had a significant effect over acceptability when nested with other factors. This was the case when animal sex was nested within animal and subsequently within consumer, C(A(AS)); and also, when the session was nested within the consumer, C(S) (section 3.3.1, Models 3 and 4).

With regard to IMA, meat aged for 7 days obtained significantly higher scores than non-aged meat, while meat aged for longer periods obtained intermediate scores. According to literature, meat tenderness is one of the most important attributes influencing the IMA of meat. In relation with this, in the present work, meat aged for 7 days (highest acceptability) was more tender than non-aged meat (section 4.2.4.2). However, horse meat aged for 14 days was even more tender but this was not translated into higher acceptability scores. Therefore, some other factors would have influenced the overall acceptability.

Visually, meat aged for 7 days was also better scored than non-aged meat while meat aged for 14 days obtained intermediate scores. Interestingly, meat aged for 21 days was remarkably bad scored by the consumer, and this could be related to the turn of meat colour to brownish as also measured instrumentally (**Figure 3.6;** section 4.2.3).

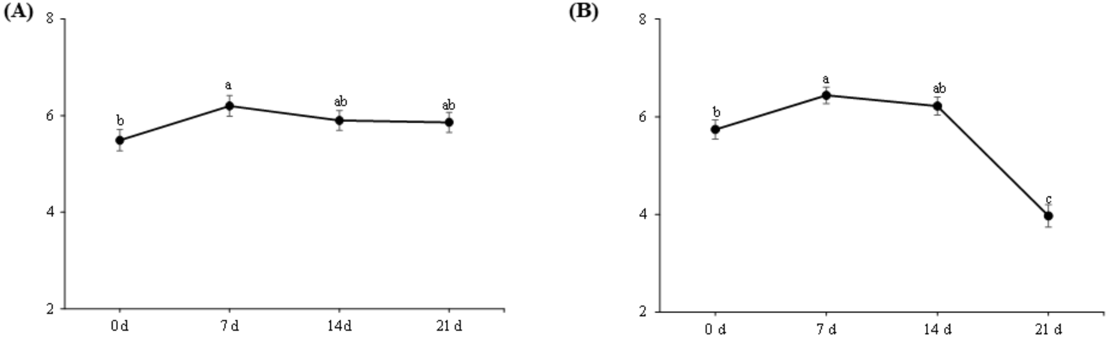

Figure 4.7 Mean in-mouth **(A)** and visual (**B**) acceptability scores during the ageing process (0, 7, 14 and 21 days, d) of horse meat.

Error bars indicate the standard deviation of the means for each group.

In order to identify different consumer preference patters AHC data analysis was performed using IMA and VA scores separately (**Table 4.7**). Three consumer clusters were identified using IMA. Consumers from the first cluster, overall, scored meat aged for 7, 14 and 21 days better than non-aged meat, while consumers from cluster 2 rated all the samples with high scores but preferred non-aged meat over meat aged for 21 days. Clusters 1 and 2 discriminated aged *vs.* non-aged horse meat. On the other hand, cluster 3 did not appreciate significant changes among ageing periods of meat, and scored all samples quite negatively. When cluster composition was matched to the aspects asked to the participants in the questionnaire, consumption frequency was significantly different ($p < 0.05$) among clusters. Consumers from clusters 2 and 3 seldom consumed horse meat (once a year or have never tried). On the contrary, consumers from cluster 1 consumed horse meat once a week or month. The lack of familiarity with horse meat consumption may have been the reason for the low discrimination and low rating of horse meat by consumers in cluster 3. However, the reason seemed to be different for consumers included in cluster 2.

When consumers were clustered by VA, four clusters were obtained. Clusters 1 and 3 rated horse meat aged for 21 days with lower scores than the rest, although scores from cluster 3 were more extreme, with scores between 6 and 7 for meat aged for 0, 7 and 14 days and an average score of only 1.50 in meat aged for 21 days. Consumers from cluster 2 preferred the visual appearance of horse meat aged for 7 and 14 days, compared to non-aged horse meat or horse meat aged for 21 days. Finally, consumers included in cluster 4 rated quite

or very positively the visual appearance of all horse meat samples and did not discriminate among samples. In this case, none of the information gathered matched with the information collected in the final questionnaire and no relationship was established.

Table 4.7 In-mouth and visual acceptability scores of horse meat after 0, 7, 14 and 21 days of ageing depending on clusters (agglomerative hierarchical clustering).

Acceptability	Cluster	n	0 d	7 d	14 d	21 d	SEM
In-mouth	1	31	4.55^{c}	7.55^{a}	5.53^{bc}	6.62^{ab}	0.18
	2	54	7.54^{a}	6.94^{ab}	7.10^{ab}	6.66^{b}	0.12
	3	35	3.14^{a}	3.83^{a}	4.26^{a}	3.91^{a}	0.16
Visual	1	33	3.95^{a}	4.70^{a}	4.16^{a}	2.51^{b}	0.16
	2	44	5.90^{cb}	6.70^{ab}	6.92^{a}	5.62^{c}	0.13
	3	30	6.35^{a}	7.07^{a}	6.36^{a}	1.50^{b}	0.24
	4	13	7.52^{a}	9.80^{a}	9.68^{a}	8.00^{a}	0.15

d, days; SEM, standard error of the mean; n, number of consumers in the clusters. Different superscripts indicate statistically significant differences among ageing times ($p < 0.05$).

4.2.6.2 Horse meat description by CATA: in-mouth and visual

In order to evaluate and understand the acceptability scores obtained, results from CATA method were analysed (**Table 4.8**). In the case of in-mouth description, from the 19 terms proposed (section 3.2.9.2), 13 showed an effective sample size equal or over 24. In other words, at least 24 consumers discriminated among horse meat samples using these terms (179). In relation to odour/flavour-related terms, 'intense' and 'roasted' presented higher citation frequency (CF) in steaks aged for 21 days compared to non-aged or 7 days aged meat, while meat aged for 14 days obtained intermediate values. In contrast, the opposite trend was observed for 'low intensity' term. These results are logical since, as observed in the volatile composition analysis of horse meat (section 4.2.1), a period of at least 14 days was needed for the development of a differentiated volatile profile which would translate into a differentiated aroma. This result differs from that of other consumer studies, which have not detected aroma differences in aged beef (180,181). The term 'livery', despite not showing statistically significant differences among ageing times, was considerably cited. In this regard, 'livery' would be considered merely descriptive as a typical aroma of horse meat, as already described by others (125).

The terms included in the texture category, 'juicy', 'tender' and 'easily dissolving' showed similar trends: lowest CF in non-aged samples ($p < 0.01$) while no significant differences

among aged samples. The opposite trend was observed for 'dry', 'tough' and 'chewy' terms. These results clearly reflect the improvement of tenderness during the first week of ageing, which is presumably responsible for the increase of IMA. Although between days 7 and 14 a significant decrease in shear force (section 4.2.4.2) and a significant protein degradation (section 4.2.5) was observed, those have not been statistically reflected in 'tender' term but a numerical increase was observed in CF value. In the case of 'juiciness', which is known to be related to cook loss (section 1.3.4.4), its citation clearly reflected changes observed when cook loss was instrumentally measured. Indeed, CF of 'juiciness' increased during the first week of ageing, period in which cook loss decreased (section 4.2.4.1).

Table 4.8 CATA term citation frequencies of horse meat samples aged for 0, 7, 14 and 21 days and of 'ideal horse meat' (%).

Terms	ESS	0 d	7 d	14 d	21 d	*p*-value	Ideal horse meat (%)
IN-MOUTH							
Odour/aroma							
Intense	67	24^{b}	28^{b}	35^{ab}	44^{a}	0.004	72.5
Livery	58	14	24	18	23	0.250	5.0
Mild	73	43^{a}	36^{a}	26^{ab}	22^{b}	0.004	9.2
Roasted	71	23^{bc}	18^{c}	33^{ab}	39^{a}	0.003	57.5
Texture							
Easily dissolving	86	20^{b}	44^{a}	48^{a}	45^{a}	≤0.001	87.5
Dry	99	63^{a}	46^{b}	44^{b}	45^{b}	0.027	0.0
High residue	80	42^{a}	26^{b}	41^{a}	30^{ab}	0.031	1.7
Juicy	87	25^{b}	49^{a}	45^{a}	41^{a}	0.003	98.3
Chewy	95	71^{a}	38^{b}	35^{b}	29^{b}	≤0.001	0.8
Tender	91	14^{b}	45^{a}	53^{a}	52^{a}	≤0.001	94.2
Tough	75	59^{a}	21^{b}	12^{b}	19^{b}	≤0.001	0.0
Taste sensations							
Bitter	24	6	6	7	12	0.289	0.0
Sweet	46	23	23	21	15	0.335	37.5
VISUAL							
Brownish	77	16^{b}	15^{b}	12^{b}	64^{a}	≤0.001	2.5
Dark	87	61^{a}	22^{b}	4^{c}	69^{a}	≤0.001	14.2
Fresh	107	68^{b}	88^{a}	81^{a}	10^{c}	≤0.001	96.7
Maroon	81	63^{a}	33^{b}	19^{c}	25^{bc}	≤0.001	20.8
Not uniform colour	94	46^{b}	44^{b}	25^{c}	62^{a}	≤0.001	3.3
Pinkish	50	10^{c}	20^{b}	37^{a}	6^{c}	≤0.001	20.8
Red	100	43^{b}	69^{a}	64^{a}	17^{c}	≤0.001	83.3
Spoilt	105	31^{b}	11^{c}	20^{bc}	97^{a}	≤0.001	0.0
Uniform colour	95	47b	43b	68a	20c	≤0.001	89.2

d, days; ESS, effective sample size. Means with different superscripts indicate statistically significant differences by Cochran´s Q test ($p < 0.05$). Ideal horse meat (%) = (citation frequency for ideal horse meat/number of consumers)

For the description of the 'ideal horse meat' (**Table 4.8**), in the odour/flavour category, 'intense' and 'roasted' were the most cited terms (72.5 % and 57.5 % of the consumers, respectively). However, attributes that obtained the highest CF were from the texture

category being, therefore, critical for the consumer. Within this category, the terms 'juicy', 'tender', and 'easily dissolving' were the most cited terms (98.3, 94.2 and 87.5 %, respectively), and conversely, 'dry', 'tough', 'chewy' and 'high residue' were almost not included in the description of the 'ideal foal meat'.

For visual CATA, all terms that were selected at the beginning of the experiment resulted in an effective sample size over 24 and showed statistically significant differences among ageing times ($p < 0.05$). Meat samples aged for 21 days obtained the highest CF for undesirable terms such as 'brownish', 'not uniform in colour' and 'spoilt', confirming that meat was not visually attractive for the consumer. This agrees with the instrumental colour measures obtained in the present work, which showed that after 14 days meat colour started to turn brownish (section 4.2.3). Conversely, terms like 'fresh' and 'red' obtained significantly higher CF in meat aged for 7 and 14 days, and 'maroon' was more cited in non-aged horse samples compared to others. Considering obtained results together with the fact that the term 'dark' obtained highest CF in non-aged meat and in meat aged for 21 days, it was considered that the consumer understood 'dark' as intense maroon in non-aged samples and as intense brown in horse meat aged for 21 days.

Regarding the consumer description of 'ideal horse meat' (**Table 4.8**), 'fresh', 'uniform colour' and 'red' were the most cited terms (96.7 %, 89.2 % and 83.3 %, respectively), while 'not uniform colour', 'brownish' and 'spoilt' were the least desired terms (below 3.5%).

4.2.7 Multivariate analysis

In order to analyse the effect of ageing time in horse meat quality using an overall approach, a multivariate analysis was performed. In this sense, a multivariate sample distribution plot using the two-dimensional coordinate system defined by PCs 1 and 3 has been depicted in **Figure 4.8**. For that, the variables analysed in the present work were taken into account (section 4.2). As an exception, data retrieved from the analysis of the myofibrillar sub-proteome of horse meat (section 4.2.5) were excluded from the PCA because such analyses were not performed in the whole sample set (n=80) but in a limited sample size (n=32). From the consumer study, acceptability results were the ones included, as data obtained from CATA method (section 4.2.6.2) correspond to frequency data subjected to non-

parametric analysis. In the case of data from volatile compounds (section 4.2.1.3), chemical families instead of individual compounds were considered for simplification purposes.

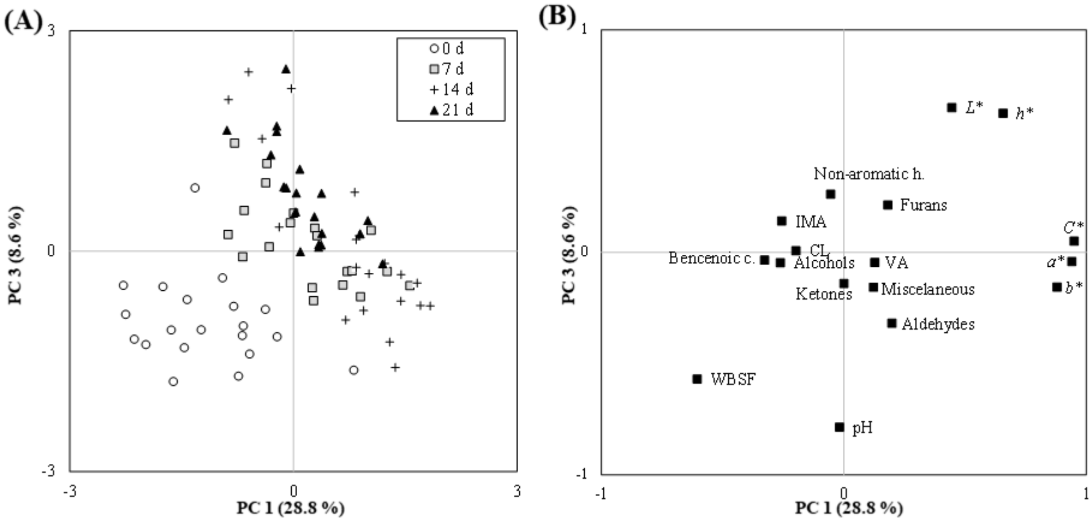

Figure 4.8 *Longissimus thoracis et lumborum* samples aged for 0, 7, 14 and 21 days (**A**) and loading variable distribution **(B)** according to the principal components 1 and 3.

d, days; non aromatic h., non aromatic hydrocarbon; IMA, in-mouth acceptability; CL, cook loss; bencenoic c., bencenoic compounds; WBSF, Warner-Bratzler shear force; *L**, lightness; *a**, redness; *b**, yellowness; *C**, chroma; *h**, hue.

Principal components 1 and 3 explained 37.4 % of the total variability corresponding to the samples studied. Non-aged horse meat samples were well differentiated from the rest and associated with WBSF (negative loading in both PC1 and 3) and pH (negative loading on PC1) measurements. On the contrary, colour parameters (*C**, *a** and *b**) were the ones with positive loading on the PC3. These were associated to part of the samples aged for 7 and 14 days, and a low proportion of samples aged for 21 days. Overall, aged sample groups were not well differentiated from each other and were, as expected, further from WBSF measurements and closer to the space defined by volatile compound families and colour parameters. This would indicate an improvement in tenderness and changes in pH, colour and aroma during ageing time. Particularly, samples aged for 7 days were closer to non-aged samples, while the ones aged for 14 and 21 days were further located.

From them, meat samples aged for 14 days were the most scattered ones, having both positive and negative loadings on PC1 and 3. On the contrary, samples aged for 21 days

(with few exceptions) not influenced by PC1 but positively related to PC3, being the latter mostly defined by some colour parameters (L^* and h^*) and the volatile families of non-aromatic hydrocarbons and furans. This shows that the third week of ageing did not imply a further evolution in texture, but changes happening in colour and aroma during this period make them become a more homogeneous group. Variables related to acceptability did not define any sample group as some of the samples aged for 7 and 14 days were close to the space defined by IMA while other samples of 14 and 21 days of ageing were close to the space defined by VA. Cook loss had little weight on the PCA.

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SECTION II

5. CONCLUSIONS

Based on the research conducted on Hispano-Bretón horse meat in the present thesis, the following conclusions were drawn:

1. Horse meat, concretely Hispano-Bretón horse meat, was lean (3.31 % fat) and contained more n-3 polyunsaturated fatty acids (78 mg/100 g; 3.19 %) than values usually described in ruminant derived products. These results, together with the environmental and social advantages provided by extensive equine breeding, confirmed the potential of horse meat as an interesting red meat alternative.

2. A total of 77 volatile compounds were found in the headspace of cooked horse meat using solid-phase-microextraction coupled to gas chromatography and mass spectrometry. The analysis of non-aged and aged meat revealed that the abundance of several odorant aldehydes, such as hexanal, and 2- and 3- methylbutanal, was higher in meat aged for 21 days compared to non-aged meat affecting meat odour. Overall, periods longer than 14 days are needed to obtain significant changes in the volatile profile of horse meat.

3. Myoglobin content of horse meat was slightly higher (3.47 mg/ g) than values reported for beef cattle of similar age. Instrumental colour assessment of horse meat throughout the ageing period revealed that meat aged for 14 days was the reddest meat. Afterwards, colour properties started to deteriorate since meat aged for 21 days shifted to brownish colour. In agreement with this, the consumer study revealed that the appearance of meat aged over 14 days was rejected (average score of 3.97 out of 10).

4. Shear force measurement of horse meat throughout the ageing period confirmed that the improvement in tenderness took place mainly during the first two weeks, with no significant changes after that. In this sense, the consumer highlighted the relevance of texture attributes in overall acceptability of horse meat although,

according to CATA method, changes were only perceived after the first week of ageing.

5. The development of an approach based on liquid isoelectric focusing (OFFGEL) and one dimensional gel electrophoresis (1-DE) proved to be successful for investigating myofibrillar muscle sub-proteome in a simple and reliable way. This approach was successfully adapted to the particular case of horse muscle myofibrillar sub-proteome and constitutes a promising alternative to methodologies based on traditional two-dimensional gel electrophoresis..
6. Several proteins from horse loin myofibrillar sub-proteome were found to change during ageing suggesting their role in tenderness development. Most relevant proteome changes happened during the first two weeks, correlating well with shear force measurements. The abundance of some structural and muscle contraction proteins such as myosin light chain 1 and troponins T and I, decreased during ageing while peptide fragments generated from troponins (T, I) and myosin binding protein C increased. This opens up an opportunity for considering these proteins as potential biomarkers of horse meat tenderness.

7. The optimum ageing time recommended for Hispano-Bretón horse meat was established at 7 days. During this period, colour and tenderness attributes and consumer acceptability improved significantly. Further improvement of these attributes during the second week of ageing were not perceived by the consumer, and ageing periods over 14 days resulted in colour deterioration and generation of a different odour profile without improving consumer acceptability. Therefore, longer ageing periods than 7 days are not justified from the consumer point of view.

8. Overall, obtained results provide accurate and valuable information about the quality of horse meat throughout the ageing period. Considering instrumental and sensory methods utilized, these results will help stakeholders to establish an optimum ageing period for the production of a high-quality and homogeneous horse meat.

SECTION III

APPENDIX I

Publication I

Insausti, K., **Beldarrain, L.R.**, Lavín, M.P., Aldai, N., Mantecón, A.R., Sáez, J.L., Canals, R.M. (2021). Horse meat production in northern Spain: ecosystem services and sustainability in High Nature Value farmland. *Animal Frontiers*, 11(2), 47-54.

https://doi.org/10.1093/af/vfab003

ISSN: 2160-6056

IF (2020): 5.304

Category: Agriculture, Dairy & Animal Science (Q1)

Feature Article

Horse meat production in northern Spain: ecosystem services and sustainability in High Nature Value farmland

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Implications

- Pasture-based, extensive livestock systems play an important role in the preservation of the rural landscape, and the many ecosystem services associated.
- Spain is the major horse meat producer at EU level, based on traditional extensive systems and a subsequent fattening outside the origin region.
- The movement of animals from raising to fattening areas entails a loss of added value in the regions of origin, which decreases farm profitability.
- The social recognition of the environmental role of extensive rearing systems based on native breeds and the local production and valorization of the horse meat are crucial objectives that need to be addressed and developed in the following years.

Key words: ecosystem services, extensive livestock systems, horse rearing, meat production, valorization and promotion strategies

Introduction

The natural environment provides many benefits to humans, which are assessed in terms of ecosystem services. In most mountain regions of Europe, the rural exodus and the abandonment of the traditional practices have led to deep changes in the landscape, decreasing the characteristic mosaicism and the diversity of inherited ecosystems (Martínez-Fernández et al., 2015; Múñoz-Ulecia et al., 2021). As a consequence, many ecosystem services are being affected, such as the biodiversity, the carbon sequestration, and the provisioning value of food for herbivores, among others (Durán et al., 2020; Oggioni et al., 2020). In addition, the current situation of climate change seriously aggravates the problem of land abandonment, especially in areas of high plant productivity. Forest expansion and shrub encroachment are leading to a loss of open spaces, a homogenization of the landscape and an accumulation of fuels that, in a situation of high temperatures and drought, entail high environmental risks.

Europe, as other regions of the world, has undergone in the last decades profound changes in their fire regime, and extreme wildfires are becoming increasingly frequent due to fuel accumulations and to drier and hotter climatic conditions than decades before (Krawchuk et al., 2009; Leys and Carcaillet, 2016). As a consequence, extreme wildfires are one of the most important threats Europeans face nowadays, due to its destructive capacity and its affliction on both human lives and the natural environment (San-Miguel-Ayanz et al., 2013). Until recently, fire policies have mostly focused and invested on fire suppression and have assigned a minor role to fire prevention and to fuel management techniques. However, the high costs of preventing fuel buildups through periodic mechanical clearings of biomass cannot be accomplished with the limited budgets of the public administrations, and a social engagement and an active landscape management are necessary (Sande et al., 2010; Otero and Nielsen, 2017). It is in this context that the role of domestic herbivores and their associated extensive livestock systems become crucial. The capacity of herbivores to ingest high amounts of biomass may constitute an effective tool for reducing vegetation fuels in critical areas of the landscape (Canals, 2019).

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doi: 10.1093/af/vfab003

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Equine farms play an important socioeconomical role in the sustainable development of mountain areas in many regions of southern Europe, and they play a key role as preservers of landscapes. Of the range of domestic herbivores suitable for environmental grazing, horses have several important advantages. Despite being monogastric, they can digest cellulose efficiently, the main organic component of plants, and their big frame size and rapid digestion process ensure a high intake capacity. Their digestive tract is designed to take in small and frequent meals of forage, and they exert a selective pressure on grasslands that results in a characteristic "lawn and rough" pattern according to their dietary preferences (Williams et al., 2020). Horses are estimated to spend about 75% of the daytime and 50% of the nighttime grazing (or a total of 60% of a 24-hr day, Fleurance et al., 2001). In addition, thanks to their size and heavy complexion, they can also gain a control on lignified vegetation through the trampling (personal observation).

Regarding the environment, equines adapt very satisfactorily to extreme climates (cold temperatures, high rainfall, snowfall events) and to rough landscapes, particularly when native breeds are used (Canals, 2019). In the current situation of climate change and the priority to reduce greenhouse gas emissions, horses also have an important advantage over ruminants due to their particular digestive physiology that reduces the production of methane. The emission factor for methane (kg CH_4 per head) coming from enteric fermentation is significantly much lower in equines than in ruminants (14 kg CH_4 per head of equine vs 48 kg CH_4 per head of cattle; FAO, 2020). Similarly, the energy losses in horses due to CH_4 production average 3.5% of the feed digestible energy compared to 10% to 13% in adult ruminants (Vermorel, 1997).

Horse Meat Production in Spain

Worldwide, horse meat production (741,003 tonnes and 4,803,585 carcasses) is currently far below the rest of meat-producing species (0.25% of the total, even below goat and rabbit meats), whereas pork (36%), poultry (34%), and bovine (21%) are the most produced and consumed meats.

Spain stands out as the major horse meat producer in the EU (17%), followed closely by Italy (16%), Romania (14%), Poland (11%), and France (8.2%; FAO, 2020). According to the national statistics, the current horse censuses account for 630,703 heads (MAPA, 2020a), distributed in 189,452 farms (Figure 1b), which are mostly concentrated in southern and western Spanish regions such as Andalusia (34%), Extremadura (6.4%), Castile and Leon (12%), Galicia (6.4%), and Asturias (6.2%) (Figure 1a).

Among the national registered horses, most animals are raised for leisure activities and only 6.1% are focused on meat production. The latter corresponds to 38,200 animals slaughtered in 2019, which means 0.4% of the total Spanish livestock production and a value worth of 78M€ (MAPA, 2020a). From the total registered equine farms, 15.3% horses are bred for meat production, and most of them (88%) are located in the north-western regions of Spain (Asturias, Castile and Leon, Galicia, Basque Country, and Cantabria; Figure 2a). In the last decade, horse meat production has increased in the country by 51%, and over 73% of the meat produced goes to European markets. National economical balance for international trade is positive for equine meat (MAPA, 2020b), with higher exports (27M€ and 7,074 tonnes) than imports (0.51M€ and 302 tonnes). The level of local consumption is low (average supply per capita below 0.10 kg, Belaunzaran et al., 2015), but it is increasing in the last years. All these data indicate the potential of this product for both exportation and local consumption.

The traditional meat production system is based on the raising of native breeds, which were used as draft animals in the past and that were reoriented for meat production after farm mechanization. For decades, equine breeding innovation depended on the weight, which established genetic improvement programs, artificial insemination, etc. When these activities were transferred to the regional governments, the development and promotion of the horse breeds displayed a high variability among regions. As a consequence, a great breed variability can be found nowadays, which is mainly linked to geographical areas: heavy horse breeds (mare live weight > 650 kg) such as Hispano-Bretón (Castille and Leon; Cantabria; and Huesca), Burguete (Navarre), Basque Mountain Horse (Basque Country), and Catalan Pyrenean Horse (Catalonia); medium-frame breeds (351–650 kg) such as Jaca Navarra (Navarre) and crossbred animals; and small-frame breeds (<350 kg) such as Galician Mountain Horse (Galicia), Asturcón (Asturias),

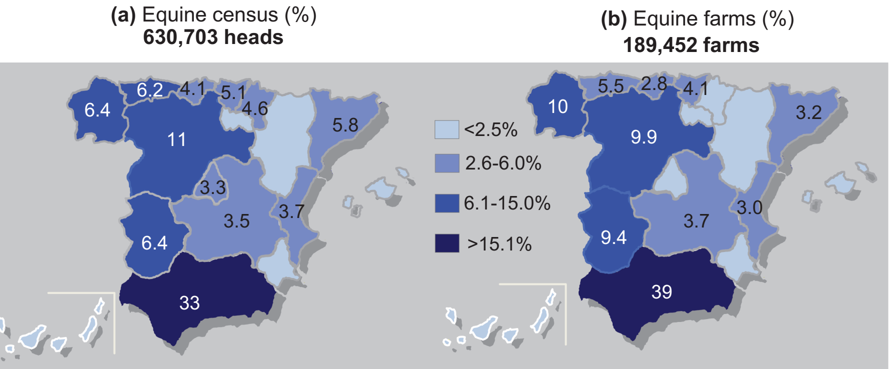

Figure 1. Spanish animal census (a) and equine farms (b) distribution. Source: MAPA (2020a).

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(a) Distribution of breeding farms (%)
29,090 horse meat farms

(b) Distribution of feedlots (%)
249 feedlots

Figure 2. Distribution of horse meat farms: (a) breeding farms and (b) feedlots. Source: MAPA (2020a).

Losino (Castile and Leon), Monchino (Cantabria), and Pottoka (Basque Country) (MAPAMA, 2015).

Due to the high rusticity and adaptation of the local breeds, the breeding systems mostly rely on the extensive grazing of the natural resources provided by mountain areas and on the feeding with preserved forages in the most extreme winter months (Figure 3). Animals are capable to adapt to harsh conditions making an effective use of mountain and valley grasslands, shrublands, and open forests (of communal use in some cases). Only occasionally, under adverse climatological conditions, animals are supplemented or remain stabled during short periods. In most of the production areas, animals freely graze in high-altitude pastures from spring until autumn. Then, foals are weaned, and animals move to valley pastures before the first snowfalls of the year, closer to villages but without housing. This is the common production system in small family farm units in which horse farming provides an additional income to the regular cattle farming or even as an alternative when the livestock activity (or main activity) has been left. In some regions such as Galicia, equine production is performed under total free grazing conditions. This traditional system in which herds are kept wild in communal pastures constitutes a tourist attraction of the region when, once a year, horses are collected ("Rapa das Bestas").

Until the second half of the 20th century, horse meat available in the markets came from old animals that were not properly finished. Nowadays, a high-quality horse meat derived from fattened foals is produced. After weaning, foals are usually moved to other regions for fattening and slaughter. In Spain, 249 farms are classified as feedlots, focused on foals fattening and representing 0.13% of the total equine farms in the country. Those are primarily located in the east of Spain (Figure 2b). Catalonia (28%) and Valencia (16%), together with Castile and Leon (13%), Basque Country (11%), Navarre (9.2%), Aragon (9.2%), and La Rioja (5.6%) regions, concentrate 92% of the Spanish horse feedlots (MAPA, 2020c).

In the primary production areas, fattening facilities are normally small-size family farms or farms directly owned by local butchers. In these units, 20–25 native animals are reared, and after weaning and a short adaptation period, foals are fed on concentrates and forage until slaughter. Commonly, animals are slaughtered at the age of 12 mo, but depending on marbling and butchers' requests, meat from 6-mo-old foals is also commercialized. On the other hand, bigger industrial feedlots with capacity for 100–800 animals can be found, which are exclusively dedicated to fattening and meat trading. In these feedlots, a high breed variability is usually found, depending on the region from which suckling foals are purchased (Tragsega, 2003). Foals are generally fattened until 12–15 mo or up to 24 mo of age, and they are intended for bigger consumption areas and/or international exports.

As a result of the current animal management and movements, 81% of the horse meat produced in Spain (9,527 tonnes) is concentrated in four regions: Navarre, 38%; Aragón, 21%; Valencia, 14%; and Catalonia, 7.6% (Figure 4a; MAPA, 2020b), despite these regions accounting for only 8.2% of the breeding farms. The average carcass weight is 261 kg, but significantly heavier carcasses are produced in the fattening regions compared to the extensive production regions (Figure 4b). In the latter, local breeds are usually small sized, and foals are slaughtered at younger ages with carcass weights averaging 150 kg.

The movement of foals from the north-western to the fattening and slaughtering areas in the north-eastern regions involves a loss of added value in their regions of origin. The finishing of foals in their original regions, besides an opportunity gain for the rural economy, will also ensure an optimal and complete use of the natural resources, and the traceability and highest quality for the final product, the meat. As a consequence, any action that helps and gives support to the local production and valorization of the final product would be beneficial for the producers and for the rural economy.

Nutritional Composition of Horse Meat

Horse meat, as any other meat, is a nutritionally valuable foodstuff. It constitutes a significant source of high-value proteins, iron, zinc, B type vitamins, and selenium with a greater bioavailability compared with that found in other foods (Lorenzo et al., 2014). However, in contrast to other more consumed meat species (i.e., bovine, poultry, or pig), few scientific studies have focused on horse meat quality although it has been recognized as a healthy meat (Lorenzo, 2013; Belaunzaran et al., 2015).

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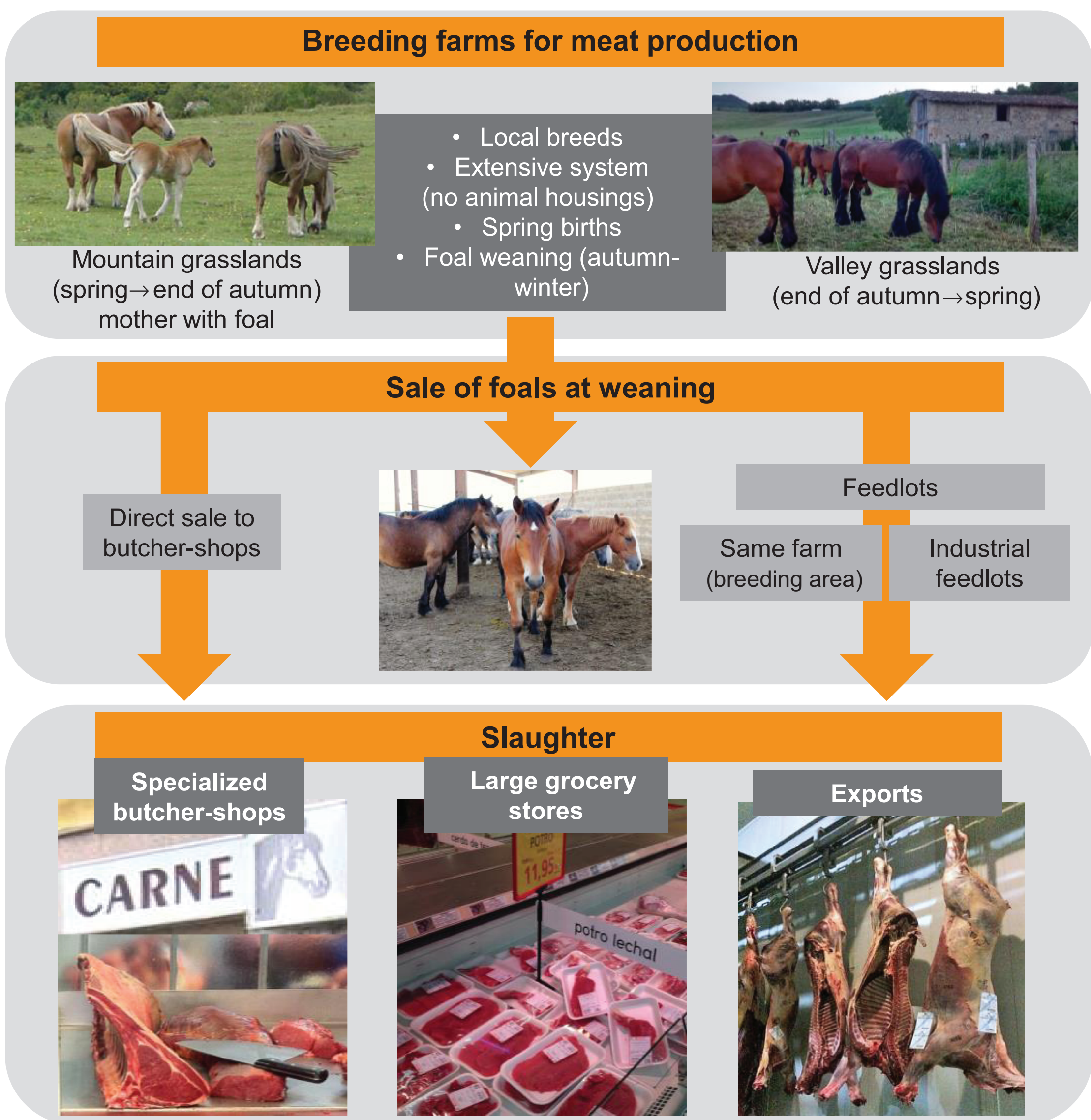

Figure 3. Scheme of the Spanish horse meat production system.

Horse meat has a low-fat content and a significant proportion of n-3 polyunsaturated fatty acids (**PUFA**), such as linolenic (18:3n-3) and other long-chain n-3 fatty acids (**FA**), that have been reported to have beneficial properties for preventing chronic diseases (Weylandt, 2016). In horses, considering their digestive track, dietary FA are absorbed before the anaerobic microbial hydrogenation occurring in the hindgut (cecum and colon). Thus, the postgastric localization of digestive chambers allows them for an efficient absorption and deposition of n-3 PUFAs coming primarily from pastures. In addition, even though the horse is a nonruminant herbivore, due to a light microbial fermentation taking place in the hindgut, the formation and accumulation of several *trans*-18:1 and conjugated linoleic acid isomers have been reported (Clauss et al., 2009). The low *trans*-FA level in horse tissues is not surprising since the formation of nonindustrial *trans*-FA normally occurs in ruminant species through biohydrogenation processes in the rumen (see review by Aldai et al., 2013).

The aforementioned considerable n-3 PUFA transfer efficiency from pasture to muscle tissue in horses was described in the 1950s (Gupta and Hilditch, 1951), and more recently, several studies have brought up new peculiarities of these animals. Even though horses do not have a gall bladder, the continuous secretion of biliary salts, together with lipase-rich pancreatic juices, provides them the ability to efficiently digest high amounts of dietary lipids in the small intestine. In this regard, a specific pancreatic lipase (pancreatic lipase related to protein 2) that is absent in pigs, turkeys, or ruminants has been described in horses, and has been linked to its capacity to hydrolyze the linolenic acid (18:3n-3) esterified in galactolipids of plants (see recent review by Sahaka et al., 2020). In this line, several studies have pointed out that horse tissues constituted

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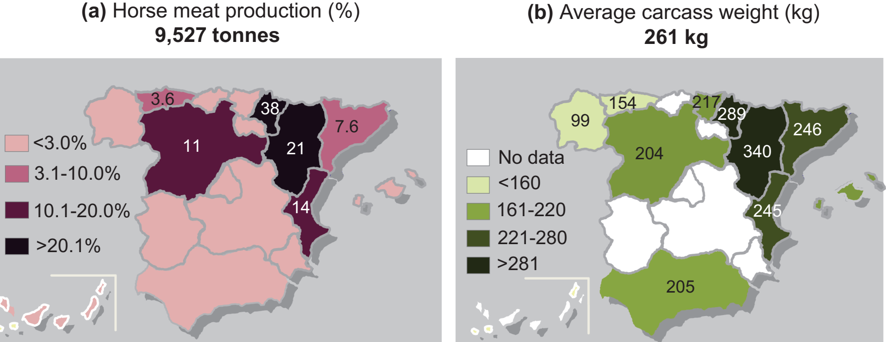

Figure 4. Distribution of the horse meat production (a) and average carcass weights (b) in Spain. Source: MAPA (2020b).

a valuable source of energy and PUFA in the diet of humans in the Paleolithic and the Neolithic, when plants and marine foods were scarce due to recurrent glaciations (Guil-Guerrero et al., 2013), and this could be directly related to the preferential deposition of 18:3n-3 in neutral compared to polar lipids (i.e., subcutaneous fat; Belaunzaran et al., 2017).

Valorization of Horse Meat Production Systems

The concept of High Nature Value (**HVN**) farming in Europe constitutes a recognition of the fact that the conservation of biodiversity depends on the continuation of low-intensity farming systems and the environmental services and positive externalities they provide. But, for its maintenance, these systems need to be profitable to farmers and rural development is necessary (González-Díaz et al., 2019). Existing support mechanisms such as the agri-environment measures have helped to slow down the loss of these systems, but they are insufficient to make HNV farms commercially viable and ensure its survival in the rural economic structure (Rodríguez-Ortega et al., 2018).

The recognition and valuation of the ecosystem services provided by extensive farming systems is necessary to raise social awareness and design effective communication strategies that contribute to improve the market value of the animal-derived products (Faccioni et al., 2019). That is why new efforts are being carried out to valorize and promote the extensive production system itself, as well as the final product (horse meat), based on its important role in the sustainable development of mountain areas at economic, environmental, and social levels (i.e., the European project Open2preserve, https://open2preserve.eu/en/). Anyway, giving a monetary value to these externalities is a challenge, and many attempts of measurable criteria for the valuation of the ecosystem services provided by pasture-based farming systems are being developed in the last years to that purpose (Bernués et al., 2014; Rodríguez-Ortega et al., 2014; Maldonado et al., 2019; Durán et al., 2020).

In the same line, the Payments for Environmental Services can be a suitable arrangement to remunerate producers for the positive externalities associated to the environmental grazing and to compensate farmers for the complexity of the management and/or the potential decrease in productivity (Sattler et al., 2013; Varela et al., 2018). In the south of Spain, there is a long-lived and successful example of the implementation of payments to shepherds for practicing an extensive and targeted grazing for the maintenance of firebreaks areas. The Red de Áreas Pasto-Cortafuegos de Andalucía (RAPCA) program rewards extensive breeders for their biomass control services in fuel breaks located in public forests. The payments received depend on the size, location, and difficulty of grazing of the area assigned to each shepherd, as well as the degree of accomplishment of the task. The political will, the stable commitment from the public administrations, and the well-designed system of biomass monitoring have been crucial for the success of the program (Varela et al., 2018).

Conclusions

In a scenario of climate change, the preservation of resilient landscapes that encompass as many ecosystem services as possible is necessary, and low-intensity farming systems are to play a key role in them. Pasture-based, extensive equine breeding produces a traceable and remarkable high-quality meat, while offering many positive externalities such as the preservation of mosaic landscapes and its associated biodiversity, the reduction of fuels for wildfire prevention, the recovery of endangered breeds, the maintenance of a primary activity that fixes population, and the low-water and -carbon footprint linked to pasture-based livestock productions with a low-methane emission. From now on, an important work to promote the local production of horse meat and the investment in effective diffusion and communication tools that raise social awareness and contribute to improve the consumption and the market value of these animal-derived products is necessary.

Acknowledgments

The authors thank the financial support provided by Interreg Sudoe OPEN2PRESERVE (SOE2/P5/E0804), UPV/EHU ZALDITRANS (EHUA 13/29) projects, and Basque Government (IT944-16). Also, we appreciate the support provided by Jone Lizarza from INTIA.

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APPENDIX II

Publication II

Beldarrain, L.R., Aldai, N., Picard, B., Sentandreu, E., Navarro, J.L., Sentandreu, M.A.. (2018). Use of liquid isoelectric focusing (OFFGEL) on the discovery of meat tenderness biomarkers. *J. Proteomics*, 183, 25-33.

https://doi.org/10.1016/j.jprot.2018.05.005

ISSN: 1874-3919

IF (2018): 3.537

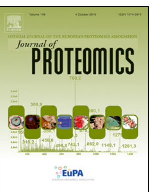
Category: Biochemical Research Methods (Q1)

Journal of Proteomics 183 (2018) 25–33

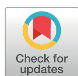
Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot



Use of liquid isoelectric focusing (OFFGEL) on the discovery of meat tenderness biomarkers



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ARTICLE INFO

Keywords:
OFFGEL
Protein fractionation
Proteome analysis
Meat tenderness
Biomarkers

ABSTRACT

Protein biomarkers of meat tenderness are known to be of primary importance for the prediction of meat quality, and hence, industry profitability. Proteome analysis was performed on meat from 8 Main Anjou beef cattle, previously classified as tender or tough meats by Warner Bratzler shear force measurements. Myofibrillar fraction of *Longissimus thoracis* muscle was separated by a novel fractionation approach based on liquid isoelectric focusing (OFFGEL) and further analyzed by SDS-PAGE and liquid chromatography coupled to tandem mass spectrometry.

Obtained OFFGEL fraction profiles were reproducible allowing the comparison of both meat qualities and revealing 7 protein bands capable to discriminate between tender and tough samples. The proteins present in these bands were troponin T, Heat Shock protein beta-1, creatine kinase, actin, troponin C, myosins 1 and 2 and myozenin-1. The latter protein has not been previously reported as a marker of meat tenderness.

Significance: This study introduces an innovative proteomic approach for the study of muscle proteome. The fact of obtaining fractions in liquid state after OFFGEL fractionation allows for a faster analysis of proteins by mass spectrometry, being an interesting alternative to more classical proteomic approaches based on two dimensional gel electrophoresis (2-DE).

1. Introduction

Tenderness is considered one of the most important organoleptic traits that determine beef quality, together with juiciness and flavor [1]. In order to satisfy consumer demands, it would be profitable for the beef industry to be able to predict and control the tenderization level of the final product in order to estimate the potential sensory quality by using biomarkers [2,3]. Moreover, a deeper study of these biomarkers would contribute to the understanding of the complex biological changes taking place during the conversion of muscle into meat, which still represents an unclear field. The discovery and smart measurements of reliable protein biomarkers is still highly demanded [4], generating a big interest in this research field.

Meat tenderness is the result of different factors like muscle fiber characteristics, sarcomere shortening during rigor development and collagen amount and solubility [5,6]. In addition, the effect of breed [7] or muscle type [8] have also been reported in several studies, together with exogenous factors like animal transport and handling [9]. Moreover, it has been widely reported that development of tenderness is also dependent on the *post-mortem* proteolysis of key proteins [4]. As consequence, proteomic strategies have been extensively developed and employed in this field during the last 30 years (see [10] for review). More concretely, comparative proteomics based on two dimensional electrophoresis (2-DE) combined with the use of mass spectrometry [11] are among the most employed strategies in the search of biomarkers of meat tenderness.

The term proteome refers to the total amount of proteins accumulated by a particular cell or tissue at a certain time point. It is considered the link between the genome and the functional quality characteristics and it is constantly changing with environmental/processing factors [12]. Proteome characterization difficulties arise from its variability over time, sample complexity and/or low resolution power of classical methods. This has been partially overcome by the optimization of methodologies including innovative combinations of different proteomic techniques adapted to meat studies, from preliminary fractionation to further protein separation and identification [13].

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https://doi.org/10.1016/j.jprot.2018.05.005
Received 2 February 2018; Received in revised form 25 April 2018; Accepted 7 May 2018
Available online 09 May 2018
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2-DE allows the separation of a high number of spots, and different isoforms or differential post-translational modifications can also be distinguished. However, the time and effort required for a 2-DE based study is considerable and pre fractionation is usually required in order to reduce the complexity of each compartment and facilitate spot identification for a comprehensive analysis of the proteome. In this line, a wide range of methods for protein fractionation have been proposed. These methods separate a complex mixture obtaining fractions containing a decreased number of proteins prior to 2-DE or mass spectrometry for a better observation of possible differences. Reported methods focus mainly on cell organelle differential centrifugation, chromatographic approaches (ion affinity, ion-exchange) or electrophoretic methods as reviewed by Righetti et al. [14]. The latter include all electrokinetic methodologies performed in free solution based on isoelectric focusing steps. In this study, one of these methods is performed prior to 1-DE as an alternative to traditional 2-DE: liquid isoelectric focusing (OFFGEL).

Gel-free based approaches like OFFGEL fractionation have received several good feedbacks from the proteomic-related scientific community [15], including some research in the field of meat safety and fraud detection [16]. As reviewed by Moreda Piñeiro et al. [17], OFFGEL fractionation has been proposed as a preparative stage before further separation. In liquid isoelectric focusing, the instrument uses an immobilized pH gradient (IPG) in a tray with a frame giving rise to 12 or 24 wells over the strips. Proteins are forced to migrate through the IPG strips, as there is no fluidic connection among wells. Focusing occurs through the strips where each protein migrates until it is positioned at the right well at the pH value corresponding to its pI. At this point, the migration stops as the protein has no longer net charge. The time required for the fractionation can vary from few up to 24 h depending on sample composition. In addition, multiple samples can be fractionated in the same analysis.

The major advantage of OFFGEL compared to 2-DE electrophoresis consists on the direct recovery of the proteins from the liquid phase, and the possibility to further analyze them by either 1-DE or directly by gel-free strategies such as liquid chromatography coupled to mass spectrometry (LC-MS). Also, improved pI resolution [18] and low influence of OFFGEL reagents in further separation techniques have been reported [19], thus being a step forward in the design of new protocols for proteomic analysis.

Hence, the objective of this study was to test the suitability of a novel fractionation approach based on liquid isoelectric focusing for the study of muscle proteome. More concretely, we focused on the characterization of samples that were previously classified as tender and tough from animals belonging to *Rouge des Près* breed, which is included in the PDO "Protected Designation of Origin" *Maine-Anjou*. This breed was selected for being associated to provide meat of excellent quality and hence it can be a representative example of the interest that meat industry can have in appropriately certify the quality of such a product.

2. Materials and methods

2.1. Sample selection

For the present work, 8 meat samples previously classified as tender or tough were selected. These meat samples corresponded to muscle *Longissimus thoracis* (LT) (mixed fast oxido-glycolytic muscle) taken from the 6th to 7th rib section and excised from the right half carcasses of 8 cows from PDO *Maine-Anjou* using the *Rouge des Près* breed. Animals from a cooperative of livestock farmers located in the department of Maine-et-Loire (France) were slaughtered in a registered commercial abattoir (Elivia, Lion d'Angers, 49,220, France) at an average age of 67 months following the standard commercial practices in compliance with French welfare and EU regulations (Council Regulation (EC) No 1099/2009). After slaughter, carcasses were not electrically stimulated and they were chilled at 4 °C during 24 h. At this time, tenderness of selected meat samples was measured by Warner Bratzler (WB) shear force using a 1.016 mm Warner Bratzler probe in a TA.TX Plus Texture Analyzer (crosshead speed 20 mm·min^{-1} and a 50 kg load cell, 40 mm distance, calibration weight 10 kg – Stable Micro Systems Ltd., Surrey UK). Samples yielding WB values below 31 N were considered tender, whereas those yielding values over 60 N were considered as tough. From the 8 samples, 4 were tough (mean WB value of 69.6 ± 7.64 N) and the other 4 were tender (mean WB value of 27.9 ± 2.13 N).

From each sample, the epimysium was carefully eliminated and about 110 *g* of muscle sample were immediately frozen in liquid nitrogen at 24 h *post-mortem* and stored at −80 °C until further analysis.

2.2. Extraction of myofibrillar proteins

Half gram of muscle sample from each selected animal was homogenized in 5 mL of 50 mM Tris buffer, pH 8.0 using an IKA DI 25 yellow line homogenizer (IKA-Werke GmbH, Staufen, Germany). The homogenate was then centrifuged at 10,000 *g* for 20 min at 4 °C. The obtained precipitate was redissolved in 50 mM Tris buffer, pH 8.0 and centrifuged again under the same conditions in order to efficiently wash the pellet from residual soluble proteins. The washed precipitate was further redissolved in 50 mM Tris buffer, pH 8.0 containing 6 M urea and 1 M thiourea, and then centrifuged under the same conditions described above. The supernatant, integrated by the myofibrillar protein fraction, was filtered through glass wool and protein concentration was determined by the method of Bradford, using the Biorad protein assay kit, then stored at −80 °C until use.

2.3. Protein OFFGEL fractionation

From the myofibrillar extract of each animal sample (4 tough and 4 tender), the volume containing 1 mg of total protein was fractionated by liquid isoelectric focusing using an Agilent 3100 OFFGEL fractionator (Agilent Technologies, Palo Alto, CA, USA) following manufacturer's instructions. Proteins were focused based on their pI using 13 cm IPG gel strips with a linear pH gradient ranging from 4.0 to 7.0 (Agilent Technologies) that was distributed along 12 well frames. A total of 150 μL of sample was loaded in each well, and the fractionation was carried out at a constant electric current of 50 mA. The focusing step was automatically managed by the fractionator. The total focusing time was 18 h and provided 12 protein fractions covering the mentioned pH range that were separately recovered and stored at −20 °C until further analysis.

2.4. SDS-PAGE and image analysis

The 12 protein fractions obtained from each OFFGEL fractionation were individually mixed with sample buffer solution (0.088 M Tris-HCl pH 6.8, 10% glycerol, 2% *w/v* SDS, 0.2 M DTT and 0.04% bromophenol blue) at a 50:50 ratio and then heated at 95 °C for 4 min. Samples were then centrifuged prior to loading onto 1.5 mm × 8 cm × 9 cm 12% polyacrylamide gels. Electrophoretic separation was carried out for two gels simultaneously at a constant current of 50 mA using a Hoefer Mighty Small II SE260 electrophoresis unit (San Francisco, CA, USA). After electrophoresis, gels were placed in a fixing solution containing 10% trichloroacetic acid (TCA) for 1 h, rinsed, and then stained overnight with colloidal Coomassie [20]. Gels were then destained with bidistilled water and rinsed before being digitalized using a LAS-1000 Luminescent Image Analyzer with Intelligent Dark Box II (FUJIFILM, Barcelona, Spain). After background substraction, the intensity of each band was quantitatively determined using the freeware Gel Analyzer 2010 software (http://www.gelanalyzer.com). In order to correct differences between images, individual band intensities were normalized with respect to the total band volume per gel. The Bio-Rad Precision

Plus™ Protein Standards kit (Hercules, CA, USA; cat. No. 161-0374) was used to calculate the molecular weight of the obtained bands. Estimated pI and Mr of bands were determined from their position in the gel. SDS-PAGE analyses were performed in duplicate for each of the eight individuals. In order to avoid instrumental drift and differences between batches, samples were randomized in technical stages, as suggested by Oberg and Vitek [21].

2.5. In-gel trypsin digestion of selected protein bands

Protein bands found to be significantly different between tender and tough samples ($p < .05$) were excised from the gels, introduced into 0.5 mL Eppendorf tubes and washed 3 times for 10 min with 50 mM ammonium bicarbonate, pH 8.0 (50 μL). After that, gel pieces were dried 3 times with 50 μL acetonitrile for 10 min. Once the gel pieces shrank and turned opaque, 15 μL of 12.5 ng/μL sequencing grade modified trypsin (Promega, Madison, USA) dissolved in 50 mM ammonium bicarbonate, pH 8.0, were added (Sigma–Aldrich Co., St. Louis, MO, USA). An additional 15 μL of 50 mM ammonium bicarbonate was added to each tube in order to cover the gel pieces. The tubes were incubated at 37 °C overnight and, then, the supernatant was transferred to a clean Eppendorf tube. The gel pieces were washed with 25 μL of ACN/0.1% TFA (50:50), sonicated for 10 min and the supernatant combined with the previous one. This solvent was evaporated using a Savant SPD121P Speed Vac concentrator equipped with a RVT400 refrigerated vapor trap (Thermo Scientific, San Jose, CA); then, generated peptides were further redissolved in 40 μL of 0.1% v/v trifluoroacetic acid (TFA).

2.6. Peptide sequence identification by liquid chromatograpy-tandem mass spectrometry

From each peptide solution obtained as described in Section 2.5, 25 μL were injected on a Surveyor LC system equipped with an autosampler and coupled to a LCQ Advantage ion trap MS instrument through an electrospray ion source (Thermo Scientific). Separation of peptides was carried out on a Jupiter Proteo reversed-phase column (15.0 cm × 0.5 mm; Phenomenex, Torrance, CA, USA). Mobile phases consisted of solvent A containing 0.1% formic acid (v/v) in bidistilled water and solvent B containing 0.1% formic acid (v/v) in acetonitrile. The separation conditions consisted of a stepwise isocratic gradient including 15 min of 0% B, 5 min of 20% B, 10 min of 40% B and finally 10 min of 100% B at a flow rate of 30 μL/min, after which it was returned to its original gradient having 0% of solvent B. Operating parameters of the ion trap detector were the following: electrospray ionization in the positive mode, capillary temperature 250 °C, collision energy normalized to 35%, spray voltage 4.0 kV, and capillary voltage 15.0 V. First scan event was full MS detection for m/z values in the range 400–2000. The second event was a dependent MS/MS scan of the most intense ions having charges from +2 to +4, enabling dynamic exclusion after three scans of the most intense ion for a period of 10 min. The minimum ion intensity for triggering a MS/MS scan was 10^5 intensity units. Data acquisition was done using the Xcalibur v2.0 software. Peptide identification was performed from the information contained in the generated MS/MS spectral data using an in-house version of the Mascot search engine v2.3 (www.matrixscience.com) against Uniprot KB v2017_11 (www.uniprot.org) and NCBInr v20170428 protein databases. The following search parameters were defined: Enzyme: "trypsin"; no fixed or variable modifications, but the "Error tolerant" option enabled. Mass accuracy was set to 1.2 and 0.6 Da for MS and MS/MS mode, respectively. The option "Mammalia" was selected as taxonomy restriction parameter. For protein family identifications obtained from Mascot, only those having a protein score derived from individual ion scores indicating identity or extensive homology ($p < 0.05$) were considered as true protein identifications.

2.7. Statistical and protein functional analysis

For statistical analysis, the SPSS 22.0 software (IBM, Armonk, NY, USA) was used. Statistically significant differences ($p < 0.05$) between the two groups considered, "Tender" and "Tough", were determined by the Welch variant of the *t*-test.

Each identified protein was classified according to its biological process using the descriptions of Gene Ontology (GO) terms retrieved from the AmiGO website (http://amigo.geneontology.org/amigo/). Taking into account the species under investigation (*Bos taurus*), a protein-protein interaction map of protein species showing significantly different abundances between tender and tough sample groups was elaborated by the String 10.5 freeware software (ELIXIR, Wellcome Genome Campus, Hinxton, Cambridgeshire, UK, https://string-db.org).

3. Results and discussion

Samples analyzed in this work corresponded to meat cuts from *Rouge des Près* bovine animals that had been previously classified as tender or tough meats according to instrumental tenderness. Mean values obtained for WB measurements of samples corresponding to each quality group were 27.9 N and 69.6 N for tender and tough groups, respectively. Four biological replicates were analyzed per group, and duplicate gels were made per sample. Fig. 1 summarizes the developed procedure for the analysis of myofibrillar muscle sub-proteome using liquid isoelectric focusing.

After the OFFGEL fractionation, 12 fractions were obtained for each sample, being easily recovered by pipetting and subsequently analyzed by SDS-PAGE. Fig. 2 shows representative examples of the distribution of myofibrillar sub-proteome along the pH range (pH 4–7) of tender and tough meats. The obtained profiles were highly reproducible in all assayed samples, which allowed the identification and analysis of the most significant variations between tender and tough sample groups.

According to the densitometry analysis, the most notable changes in abundances between studied sample groups were observed in bands A to G (Fig. 3) and the statistical analysis (Welch variation of the *t*-test) confirmed the significant differences between tender and tough meat samples ($p < 0.05$; Table 1). Bands A to G were, then, trypsin digested and the generated peptides further analyzed by LC-ESI-MS/MS. Protein identifications obtained for each band are summarized in Table 1. Of the seven bands showing differences between meat qualities, five of them (bands C to G) comprised a pool of two proteins that co-migrate in the gel. These were proteins with a higher pI, whose separation is less efficient under the employed OFFGEL conditions. In fact, cathodic drift phenomena is observed when resolving proteins having alkaline pIs, obtaining a worse resolution, as reviewed in Moreda-Piñeiro et al. [17].

The pH range of fractions where bands A-G were focused is shown in Table 1 (pH value in the OFFGEL fraction). The lack of match between the isoelectric point of the protein and the interval pH value of each fraction it is normally associated to changes in protein structure due to post translational modifications such as phosphorilation, oxidative modification or glycosilation that may result in changes in the pI. As it can be observed, myozenin-1 has a considerably higher pI (9.17) than the pH values of fractions 11 (6.33–6.54) and 12 (6.54–6.75). This is explained by the fact that proteins having pI values higher than the limit of the employed pH gradient (4–7) will necessarily be placed in fractions having the higher pH values. Bands C and F, and D and G were attributed to the same pair of proteins respectively, which revealed that proteins had distributed in two different lanes during the fractionation, as it can be noted in their migration pattern. Identification in multiple fractions has been reported in previous studies [22]. However, they have been considered separately in order to evaluate their behavior as independent markers.

Band E comprises two protein species of the myosin heavy chain (Table 1). Indeed, myosin-1 and myosin-2 are also known as myosin heavy chain isoforms 2× and 2a, respectively (http://www.uniprot.

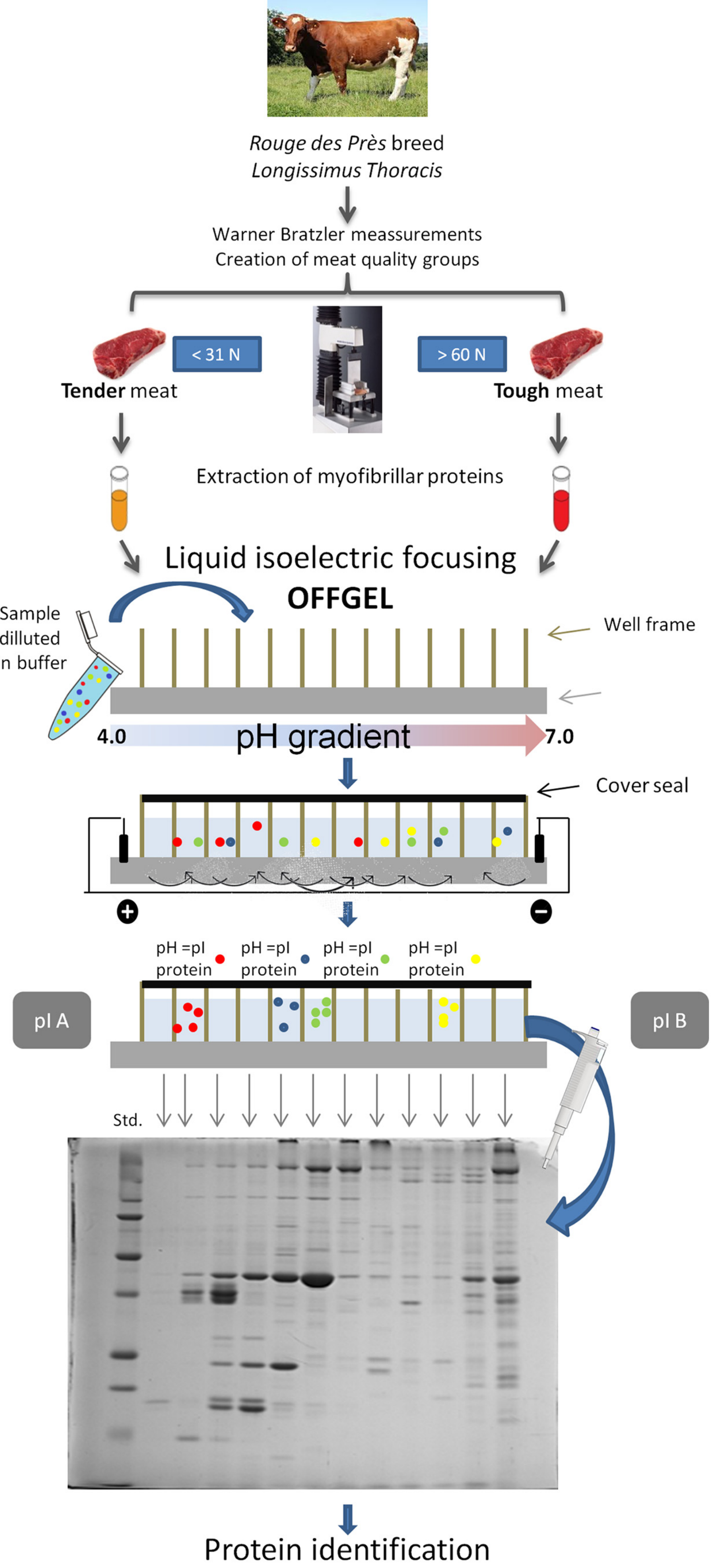

Fig. 1. Scheme of the different steps of the developed hybrid approach using liquid isoelectric focusing: Tenderness of meat samples was assessed by Warner Bratzler shear force measurements in order to establish meat quality groups. Then, myofibrillar proteins were extracted and protein concentration determined prior to OFFGEL fractionation. Each muscle extract was loaded onto the 12 wells of the IPG strip covering the pH range 4–7. Fractionation was carried out at a constant electric current of 50 mA. Proteins were forced to migrate through the strips, as there is no fluidic connection among wells. Focusing occurs through the IPG strips where each protein migrates until it is positioned at the right well at the pH value corresponding to its pI. Proteins were then recovered by pippeting and further analyzed by SDS-PAGE. Differentially accumulated protein bands were finally identified by LC-ESI-MS-MS.

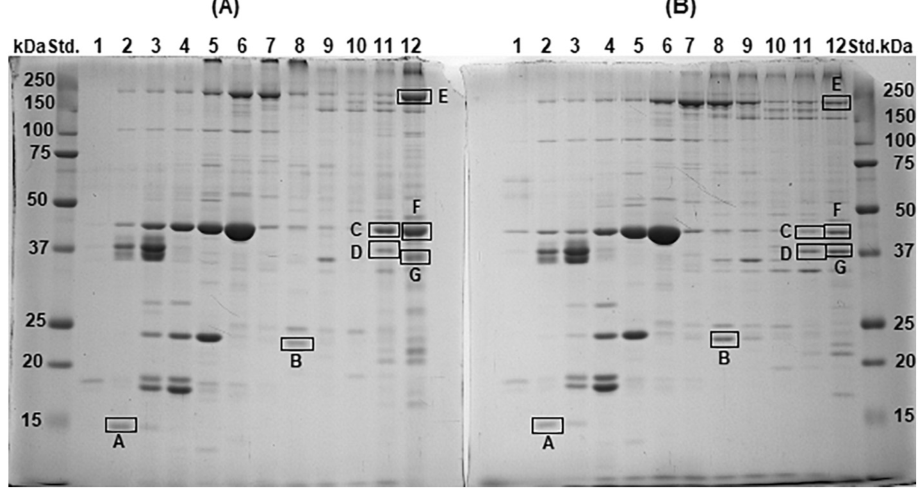

Fig. 2. 12% SDS-PAGE of the 12 fractions obtained after protein separation with OFFGEL isoelectric focusing of two representative myofibrillar extracts of (A) tender and (B) tough bovine meat along the pH range 4–7. Std: Molecular standard.

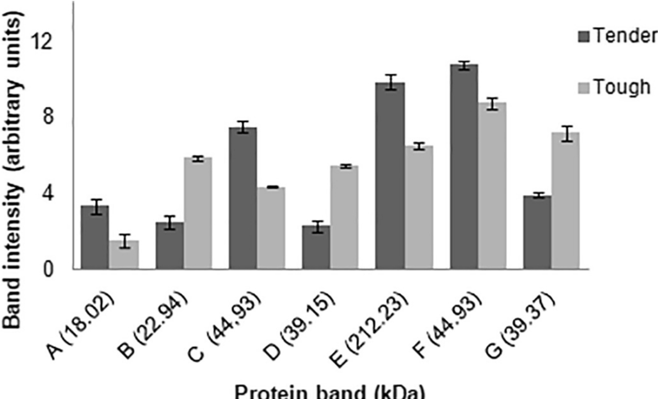

Fig. 3. Abundance means and standard deviations of the seven selected protein bands (summarized in Table 1) in tender (dark) and tough (light) groups, averaging the values of the four individuals and the corresponding two technical replicates for each group. Spots were quantified in arbitrary units and normalized with respect to the total band volume per gel. Error bars indicate the standar error of the mean for each group.

org). In adult skeletal muscles, isoforms 1, 2a, 2× and 2b are the predominant. Myosin species are generally considered the molecular markers of the muscle fiber type, and fiber types are often indicated by the name of the myosin isoform that is accumulated [23]. Isoform 1 is present in slow-twitch fibers (type I fibers), and the other three isoforms are accumulated in fast twitch fibers (types IIA, IIB and IIX). Although the gene of isoform 2b has been identified in several studies [24], this isoform is generally absent in cattle [25]. The muscle under study is considered a mixed fast twitch oxido-glycolytic muscle, so the presence of both 2a and 2× isoforms is in agreement with the literature [26]. Due to the similar characteristics (molecular mass and pI) of the identified myosin isoforms (Table 2), their separation was not possible to achieve under standard electrophoretic conditions employed in this study. Instead, a gradient gel electrophoresis would be necessary [27]. However, LC-ESI-MS/MS allowed the identification of isoform specific peptides [28], confirming the identification of a mixture of isoforms 2× and 2a in band E. Table 2 summarizes the unique peptides identified for each of the myosin species in band E. In addition, fragmentation analyses of these peptides obtained from MS/MS data are included as supplemental material.

Bands A, C, E and F showed an increased level in tender samples

Table 1
Summary of the main band differences found between tender and tough bovine meats as analyzed by liquid isoelectric focusing and SDS-PAGE, together with their identification by LC-ESI-MS/MS, using the MASCOT search engine and NCBInr and Uniprot KB protein databases. All identified proteins belong to *Bos Taurus* species.

Band	Identification[a]	SWISS-PROT accession number[a]	Gene[a]	Protein sequence coverage (%)	Theoretical/apparent MM (kDa)[b]	Theoretical pI,[b] estimated/(pH value in the OFFGEL fraction)		p-Value[c]
A	Troponin C	P63315	TNNC1	15	18.41/18.02	4.03	(4.46–4.67)	0.01
B	Heat shock protein beta-1	Q3T149	HSPB1	28	22.39/22.94	5.98	(5.71–5.92)	0.02
C	Creatine kinase M-type	Q9XSC6	CKM ACTA1	28	42.99/44.93	6.68	(6.33–6.54)	< 0.01
	Actin	P68138		12	42.05/44.93	5.23		
D	Troponin T	Q8MKI3	TNNT3	17	32.13/39.15	5.99	(6.33–6.54)	0.02
	Myozenin-1	Q8SQ24	MYOZ1	15	31.67/39.15	9.17		
E	Myosin-2	Q9BE41	MYH2 MYH1	13	223.30/212.23	5.62	(6.54–6.75)	< 0.01
	Myosin-1	Q9BE39		16	223.30/212.23	5.58		
F	Creatine kinase M-type	Q9XSC6	CKM ACTA1	26	42.99/44.93	6.68	(6.54–6.75)	0.01
	Actin	P68138		18	42.05/44.93	5.23		
G	Troponin T	Q8MKI3	TNNT3	13	32.13/39.37	5.99	(6.54–6.75)	0.01
	Myozenin-1	Q8SQ24	MYOZ1	17	31.67/39.37	9.17		

[a] Protein and gene names and accession numbers were derived from Uniprot KB database.

[b] Theoretical molecular mass and isoelectric points were retrieved from SWISS Prot database, and observed molecular mass was calculated from the band position of the gel,

[c] Statistically significant differences between groups were found by the Welch variation of the *t*-test, averaging intensities across replicates from the same biological sample.

Table 2
Myosin heavy chain isoforms of bovine *longissimus thoracis* muscle identified by the detection of their isoform-specific peptides. Coloured amino acids correspond to those that are specific of either isoform 2× (blue) or isoform 2a (red).

Identified isoform	Mascot protein score (% sequence coverage)	Protein Mr (Da)/(pI)	Isoform specific sequences identified	Mr sequences (Da) calc.
Myosin heavy chain isoform 2x	1372 (15%)	2228514 (5.57)	AAYLQGLNSADLLK + Deamidated (NQ)	1476.67
			GQTVEQVYNAVGALAK	1647.83
			VLNASAIPEGQFIDSK	1688.87
			IVESMQSTLDAEIR	1591.78
Myosin heavy chain isoform 2a	1188 (13%)	223180 (5.63)	AAYLQSLNSADLLK + Deamidated (NQ)	1500.70
			GQTVEQVTNAVGALAK	1585.76
			VLNASAIPEGQYIDSK	1704.88
			IEDEQALGIQLQK	1484.65
			MEIDDLASNVETISK	1664.83
			TLEDQVNELK	1188.28

with respect to those classified as tough, whereas bands B, D and G showed the opposite trend, as depicted in Fig. 3.

3.1. Functional analysis of identified proteins

Differentially accumulated proteins identified in bands A to G were classified according to their main known function: oxidative stress related proteins (Heat Shock protein beta-1), proteins involved in metabolism and, in particular, glycolysis (creatine kinase), cell structure (actin, myozenin-1) and contractile apparatus (troponin T and C, plus myosins 1 and 2). As it can be noted, the soluble enzyme creatine kinase appeared in the myofibrilar fraction. The effect of early *post-mortem* pH fall, together with high muscle temperatures, can denature proteins giving rise to aggregates precipitating into myofibrils as reported in the literature [29]. Further information relative to the selected proteins can be obtained by protein-protein interaction analysis by means of String 10.5 as depicted in Fig. 4. This provides insights to biological functions of proteins present in different abundances. The established interactions were built up according to experimental evidence, databases, literature results and co-expression in *Bos taurus*.

Actin and myosin (myosins 1 and 2) comprise the central node as they are the main components of thin and thick filaments, respectively. These two proteins form a strongly bonded rigor actomyosin complex *post-mortem,* and this rigor bond undergoes protein degradation [30]. The interaction between Heat Shock protein beta-1 and actin can be explained by the important chaperone role of the small Heat Shock proteins. They show an immediate redistribution from the cytosol to the insoluble fraction in response to several stressors [31], and they participate in multiple processes such as protein assembly, protein folding and unfolding, refolding of damaged proteins and preservation of cellular proteins against denaturation and degradation [32], as it befalls in the *post-mortem* period.

Troponin C and T belong to the heterotrimeric troponin complex, which is the central regulatory protein of striated muscle contraction process. As evidenced in the interactome, troponin complex is associated with the actin thin filament. From the three subunits of troponin (C,I and T), only troponin T and C have been found to be differentially abundant in the two meat quality groups of this study. During the muscle contraction process, calcium binds to the triggering sites of the troponin C globular NH_2-terminal domain leading to the interaction

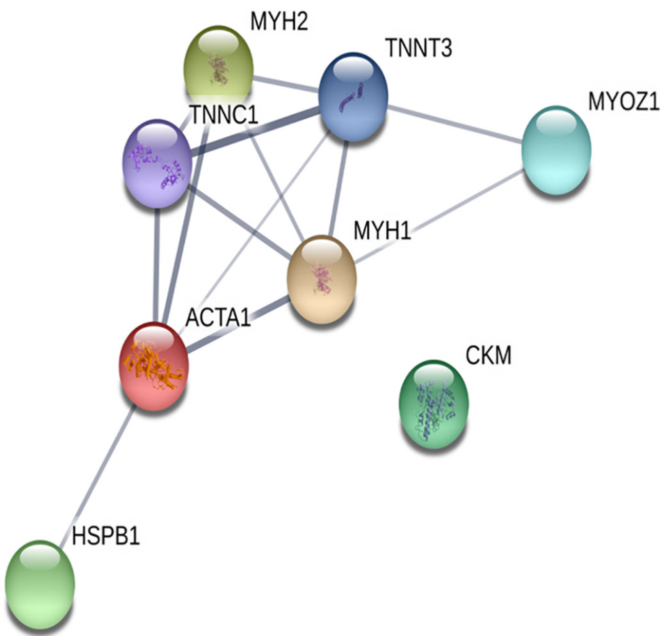

Fig. 4. Protein-protein interaction analysis of differentially accumulated protein bands in tender and tough meat groups. Data have been elaborated and graphed through String 10.5. The network nodes (circles) represent proteins, the edges represent known or predicted functional associations and line thickness is an indicator for the strength of the association (threshold: 0.4, medium confidence level). ACTA1 = Actin; CKM = Creatine kinase M-type; HSPB1 = Heat shock protein beta-1; MYH1 = Myosin-1; MYH2 = Myosin-2; MYOZ1 = Myosin-1; TNNC1 = Troponin C; TNNT3 = Troponin T.

with troponin I, and resulting in the dissociation of its main inhibitory region from actin. Finally, the interaction of troponin I with troponin T, which anchors the troponin complex to tropomyosin-actin filament, enhances the actomyosin bond [33,34]. This fact explains the close interaction between both troponin T and C and myosin and actin (Fig. 4).

Myozenin, which is part of the complex Z line structure, is a poorly studied protein in cattle [35]. It directly interacts with actin binding proteins α-actinin and γ-filamin, which are in charge of F-actin binding and crosslinking. Myozenin-1 is exclusively accumulated in fast twitch fibers of skeletal muscle and it is a calcineurin binding protein. When active, calcineurin promotes slow-fiber-specific myosin heavy chain abundance conversion. However, the exact function of myozenin remains unclear [36] although the interactome shows a correlation with troponin T, as they interact in the periphery of the Z line [37]. In this study, these two proteins (myozenin and troponin T) co-elute in bands D and G.

Creatine kinase remains out of the interaction network (Fig. 4). Unlike other proteins that form or are related to the skeletal muscle structure, creatine kinase is a glycolytic enzyme playing a central role in energy transduction. Its main function is to catalyse a reversible phosphotransferase reaction between creatine and ATPwhich is required when high ATP regereration is demanded, for instance, in situations of prolonged muscle contraction. The muscle isoform of creatine kinase has been associated with the M line [38], interacting with structural myomesin [39].

3.2. Relationship between meat quality and changes in band intensities

The abundance of myofibrillar sub-proteome was measured in the LT muscle. This muscle, as part of the loin, is known to be a tender muscle [40]. Several studies have confirmed that the energy metabolism responsible of the rate and extent of *post-mortem* pH decrease varies within muscle types leading to differences in the tenderization process [41]. LT muscle contains a higher proportion of slow-twitch fibers and thus higher oxidative metabolism compared to other muscles in the carcass such as the *Semitendinosus* (ST) muscle. This could be related to the presence of metabolic proteins such as stress-related Heat Shock Proteins (HSP) in LT muscle. According to the muscle and animal type (breed, sex, age), tenderness may be explained by different multifactorial processes and this can be the reason of many contradictions found in the literature between biochemical and sensory quality data [42]. Therefore, in order to understand the mechanisms underlying tenderness each animal should be evaluated separately [43].

In the present study, subunits C and T of the heterotrimeric complex troponin (present in bands A, D and G) were quantitatively different (Fig. 3). Troponin is considered to play a major role in the tenderization process as this phenomenon affects all subunits. In the case of troponin C (band A), a shift in the abundance ratio over the aging period was reported by Laville et al. [29]. The concentration in tough meat samples was higher immediately after slaughter while a shift in this trend was observed along the aging process [29]. Although these authors detected the aforementioned change after the fifth day *post-mortem*, our results showed a significantly higher concentration of troponin C in tender samples at 24 h *post-mortem* compared to tough samples.

The fast isoform of troponin T co-eluted with myozenin-1 (bands D and G), being the intensities of both bands higher in tough samples. Despite the co-existence of the two proteins hinders the study of the independent behavior of troponin T, the bands successfully worked as quality markers in all analyzed samples. Troponin T has already been used as a marker of ongoing proteolysis in beef and pork [44,45] due to the appearance of polypeptides derived from this protein that migrate at approximately 30 kDa and whose progress runs simultaneously to the tenderization process. It has been reported that this protein is cleaved at its glutamic acid-rich N-terminal region, generating basic fragments that are good predictive markers for beef tenderness [46]. Troponin T has also been suggested to be a substrate for μ-calpain [47]. Although troponin T fragments have not been detected in this study, a higher content of intact troponin T has been identified in tough meat samples. This result is in agreement with previous studies such as the one performed in *Blonde d'Aquitaine* and Charolais young bulls concluding that troponin T could be considered a toughness marker [48].

Myozenin-1 has not been identified as a tenderness biomarker before. Morzel et al. [49] reported a quantitative *post-mortem* decrease of myozenin in pork suggesting that this protein could be a target for proteolysis. D'Alessandro et al. [50] studied myozenin-1 in tender and tough meat samples from *Maremmana* beef cattle breed. Although myozenin abundance was not reported to change between the two sample groups, a different phosphorylation was described for tough samples. This phosphorylation was suggested to increase interactions of myozenin 1, troponin and myotilin at the *Z*-line, and consequently increasing sarcomere cohesion and reducing the accessibility of proteases in the tenderization process [50]. In line with this statement, in our study, a higher amount of myozenin was found in tough meat samples (Fig. 3).

Although the role of HSPs in living animals has been extensively described, the action of these proteins in *post-mortem* muscle is still unclear, although several studies have reported correlations between Heat Shock protein beta-1 concentration and tenderness [10]. This stress-related protein and molecular chaperone (band B) had a lower presence in tender meat samples in our study (Fig. 3). In previous studies, however, Morzel et al. [51] described a positive correlation between Heat Shock protein beta-1 isoforms and beef tenderness in LT muscle, suggesting its relation with a higher actin fragmentation during storage time and possibly preventing actin aggregation while facilitating the action of proteases. In contrast, Kim et al. [52] reported two fold lower abundance of Heat Shock protein beta-1 in tender loins, in accordance with our data. In another study, Picard et al. [42] demonstrated that the relationship between this protein and tenderness is dependent of muscle type and breed. Also in agreement with our results, Ouali et al. [53] suggested that Heat Shock proteins, induced by stress, may delay the process of cellular death due to their diverse antiapoptotic actions including the hindering of caspase activity. As reviewed by Ouali et al. [4], according to most studies the increase in Heat Shock protein abundance results in meat toughening. Several antiapoptotic actions are suggested for this protein family such as the complexation with active caspases, the protection of substrates from effector caspases and the restoration of active structural proteins.

Proteins identified in bands C and F were creatine kinase and actin. Both proteins have been reported to be targets of *post-mortem* proteolysis. The *post-mortem* degradation of creatine kinase and the appearance of creatine kinase fragments have been previously described. These fragments have been found to increase during the aging of meat [51]. In addition, creatine kinase protein dimmers were also reported to be more abundant in tender samples [50]. In agreement with this trend, our study showed a higher content of intact creatine kinase in tender samples (Fig. 3). The same trend has been observed for actin, in agreement with Kim et al. [52] and previous studies that detected a correlation between the amount of actin and meat tenderness, being this higher in tender groups.

Band E comprises myosin heavy chains 1 and 2, which are primary proteins in the myofibril. Therefore, their contribution to the structure and tenderization of meat should not be ignored. Although myosin and its components have been reported not to change much during *post-mortem* storage [30], different abundances in tender and tough meats have been reported. Picard et al. reported a positive correlation between myosin 1 and tenderness in Angus cattle, and negative correlations have been also reported in young bulls of *Blonde D'Aquitaine* and Charolais, being one of the most explicative proteins in the tenderness model [43]. This study suggested that these relationships may depend on the contractile and metabolic properties of a particular muscle. In breeds having muscles with fast glycolytic properties, those animals

with relatively high proportions of oxidative fibers were more tender. Our results indicate a positive correlation between myosin heavy chain and tenderness in LT muscle from *Rouge des Près* cattle breed which is in good agreement with the high proportion of oxidative fibers observed for this muscle in this breed, similar to Angus breed [54].

3.3. Advantages and constraints of OFFGEL based methodology

An important advantage of the presented approach consists on the ease of use of the liquid isoelectric focusing equipment. Within the framework of animal sciences where fast and affordable results are required, simplification and automation are important features regarding proposed methodologies. Unlike widely used 2-DE, which is a more skill-demanding method difficult to automate, OFFGEL electrophoresis method requires less manual work as the equipment performs the fractionation automatically.

Several alternative methods to classical 2-DE have been proposed in the last years. In terms of gel based methodologies, 2-DE was refined into differential gel electrophoresis (DIGE), which is based on fluorescent dyes [55]. This methodology has been proved to resolve complex protein mixtures with a high resolution, although high cost of the dyes, difficulties in quantification and low reproducibility have been reported [56], thus not being the best option for animal based research,

Also, extremely efficient non-gel based methodologies have been introduced, such as isobaric tag for relative and absolute quantitation (iTRAQ) or isotope-coded affinity tags (ICAT). These techniques have been reported to be adequate for quantitative analysis [57,58] although unsuitable for large experimental setups as they generate a great amount of information.

In fact, variability is an important issue in animal studies because animals are subjected to external factors that are often hard to identify and control. Proteomic tools implemented in animal sciences should be able to accommodate large sample sizes and have high reproducibility. For this reason, innovative methods need to be adapted to the specific research scenario of meat research [59].

In this line, contrary to most pre-fractionation methods that are time consuming, complicated to handle or do not allow to process more than a few samples in parallel [60], OFFGEL electrophoresis offers the important benefit of occurring in solution, providing the direct recovery of the compounds for further analysis, then minimizing the losses of specific proteins [61]. Moreover, pre-fractionation of the sample prior to SDS-PAGE enhances the sample loading capacity, and it is possible to analyze several samples in a single run.

However, as the last stage of the proposed approach consists on 1-DE, examples of co-migrating proteins were identified in this study (Table 1), which complicates the comparative pattern analysis. Because of the lower resolution of 1-DE compared to 2-DE, the presence of comigrating proteins in a given band is typical [16]. As we have observed here, co-migration of muscle proteins can occur despite the OFFGEL fractionation step previous to 1-DE. The absence of overlapping is important for the detection of variations between the two conditions analyzed but the high reproducibility and throughput of 1-DE allowed to analyze the behavior of the composed bands as a single biomarker and obtain acceptable results. On the other hand, same as in 2-DE, the resolution of basic proteins continues to be problematic.

4. Conclusion

The goal of the present work was to develop a new approach in the study of bovine myofibrilar muscle sub-proteome based on the use of liquid isoelectric focusing (OFFGEL) in order to discriminate meat samples of different quality. In this particular case, beef samples with different instrumental tenderness (tender vs tough) have been studied. The comparison of myofibrillar sample sub-proteomes was successfully achieved as profiles were highly reproducible, allowing the identification of differentially abundant bands between the two meat quality groups. Seven bands comprising proteins with different functions were identified as their abundancies changed between tender and tough meat sample groups. Identified marker proteins (troponin C, Heat Shock protein beta 1, creatine kinase, actin, troponin T, myozenin 1 and myosins 1 and 2) were consistent with the ones previously proposed in the literature. As a novelty, myozenin-1 has been identified in one of the selected bands, being the relationship between the abundance of this protein and meat tenderness not been previously reported, therefore deserving further investigation.

The methodology proposed can be used to investigate the proteome in a simple and reliable way in order to differentiate beef samples with different qualities. Understanding the mechanisms behind meat quality has the potential of increasing the economic value of meat animals in beef production. The capacity to predict the quality of the final product would allow the stakeholders to take early choices in order to provide the product with the greatest added value and quality in relation to market price.

To this extent, the developed approach constitutes a promising alternative to methodologies based on traditional two-dimensional gel electrophoresis since protein fractions are recovered in liquid state, reducing the risk of sample contamination and manual work. Also, a lower presence of artifacts and/or missing spots, typical of 2-DE analyses, would facilitate further data treatment and interpretation. Moreover, once the target proteome has been well characterized, the direct analysis of the 12 fractions by means of liquid chromatography coupled to mass spectrometry could be achieved through gel-free approaches. Also, this methodology could be transferred to study muscle proteome of other species.

Conflict of interest

The authors declare no conflict of interest to disclosure.

Acknowledgements

The Department of Economic Development & Competitiveness of the Basque Government is acknowledged for the fellowship of L. R. Beldarrain. This work was funded by the Spanish Ministry of Economy and Competitiveness (project AGL2012-32146).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.05.005.

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APPENDIX III

Publication III

Beldarrain, L.R., Morán, L., Sentandreu, M.A., Insausti, K., Barron, L.J.R., Aldai, N. (2021). Muscle and Subcutaneous Fatty Acid Composition and the Evaluation of Ageing Time on Meat Quality Parameters of Hispano-Bretón Horse Breed . *Animals*, 11(5), 1421.

https://doi.org/10.3390/ani11051421

ISSN: 2076-2615

IF (2020): 2.752

Category: Veterinary Sciences (Q1), Agriculture, Dairy & Animal Science (Q1)

Article

Muscle and Subcutaneous Fatty Acid Composition and the Evaluation of Ageing Time on Meat Quality Parameters of Hispano-Bretón Horse Breed

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Simple Summary: Horse meat; even though is still not popular in most countries; its consumption is slowly increasing and has the potential to become an alternative future red meat. However; research is still insufficient and a deeper understanding of its nutritional and physicochemical characteristics would be beneficial for the horse meat industry. The capacity of horses to efficiently uptake polyunsaturated fatty acids into their tissues has been reported; but detailed knowledge about horse meat fatty acid composition is limited. The present work provides a comprehensive fatty acid composition analysis of subcutaneous and muscle tissues from semiextensively reared Hispano-Breton horses; results indicated that finishing on a high-grain diet limited muscle n-3 accumulation. In addition; the evolution of physicochemical quality parameters such as pH, instrumental color, texture and cook loss were thoroughly studied during vacuum ageing (0, 7, 14 and 21 days), and the conclusion was that an ageing period between 7 and 14 days would be recommended for an optimum horse meat quality. The reasons for this recommendation were that tenderness increased during the first two weeks and then stayed stable and that visual properties deteriorated after 14 days. Overall; these results will help to standardize *post mortem* practices to obtain a homogeneous final horse meat quality.

Abstract: A full-randomized block design was used for the study of the FA composition and meat quality parameters, considering ageing time as a split-plot factor. Chemical and fatty acid composition of steaks (longissimus thoracis and lumborum muscle) from 15 month old semiextensively reared Hispano-Bretón horses were characterized (day 0), and the effect of vacuum ageing (0, 7, 14 and 21 days) on several meat quality parameters (pH, instrumental color and texture and cook loss) was determined. The average fat content of horse loin was 3.31%, and the n-3 polyunsaturated fatty acid content, although higher than in ruminant meats, suggested that the finishing on a high-grain diet limited muscle n-3 accumulation. Results revealed that ageing affected all meat quality measurements; color started to turn brownish at 14 days of ageing, with a decrease in redness but not in yellowness. Tenderness improved during the first two weeks, and the Warner-Bratzler shear force scores showed that meat aged for 7 days could be considered as 'intermediate tender'. Under the present study conditions, an ageing period between 7 and 14 days is recommended for an optimum horse meat quality.

Keywords: ageing; color; equine; foal; intramuscular fat; texture

Citation: Beldarrain, L.R.; Morán, L.; Sentandreu, M.Á.; Insausti, K.; R. Barron, L.J.; Aldai, N. Muscle and Subcutaneous Fatty Acid Composition and the Evaluation of Ageing Time on Meat Quality Parameters of Hispano-Bretón Horse Breed. *Animals* **2021**, *11*, 1421. https://doi.org/10.3390/ani11051421

Academic Editor: Aristide Maggiolino

Received: 6 March 2021
Accepted: 12 May 2021
Published: 15 May 2021

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1. Introduction

Horse meat, due to religious and/or cultural reasons, is still not popular [1,2]. Nevertheless, its consumption is slowly increasing in several countries due to its recognized nutritional value, mainly related to grazing systems [3]. Even though the effects of horse meat consumption on human health have not been widely studied [4], several investigations have addressed the interest of its nutritional properties with special attention on its lipid composition, which is a direct consequence of the equine digestive physiology [5–8]. Horses are non-ruminant herbivores and hindgut fermenters that efficiently digest and absorb the major part of dietary lipids in the foregut, before reaching microbial metabolism in the hindgut [9,10]. Thereupon, lipid composition of horse muscle tissue is generally characterized by high levels of polyunsaturated fatty acids (PUFAs) [11] and low levels of branched-chain fatty acids (BCFAs), *trans*-fatty acids and conjugated linolenic acids (CLA); that are all associated with rumen metabolism and are, therefore, more abundant in ruminant derived products [12–14]. Recent investigations have opened an interesting research field describing plasmalogenic lipids in horse tissues [5,15] that deserve further research due to their implications in human health [16,17]. Regarding horse meat quality, a number of studies have been performed in several breeds slaughtered at different ages [18–22] but there is still no general agreement concerning the *post mortem* ageing of horse meat and its effect on the final meat quality [23].

In this context, meat ageing has been commonly applied in the meat industry for decades [24]. In particular, vacuum packaging of subprimals or cuts for a certain period of cooling storage, namely wet ageing, is the most extended type of ageing [25]. This process is widely known to have a pivotal role in the improvement of meat palatability, especially tenderness [26], and variation in color [27,28], while avoiding contamination and weight loss.

The role of ageing and biochemical mechanisms involved in the improvement of meat tenderness have been widely studied [29,30], and it is well known that the level of tenderization will majorly depend on duration and temperature of the ageing process, together with other factors such as muscle type, breed and animal species [31]. Regarding color, when meat is vacuum packaged, the oxygen depleted atmosphere causes meat surface turn purple color (deoxymyoglobin) and when the package is opened and exposed to air (oxygenated), purple turns back to red color (oxymyoglobin). As the ageing process progresses, oxygen consumption of respiratory enzymes within mitochondria decreases, resulting in more oxygen available to bind myoglobin and form a thicker layer of oxymyoglobin under the surface; this fact translates into a redder meat after oxygenation [26]. However, in extended ageing, the residual oxygen can oxidize oxymyoglobin and deoxymyoglobin, leading to the formation of a brownish layer (metmyoglobin). In essence, as *post mortem* time increases, the reduction ability of muscle decreases and, consequently, the formed metmyoglobin cannot be converted back, giving rise to meat discoloration and brownish color [27,32]. It is known that myoglobin content and reduction activity are muscle and species specific [33].

The implications of ageing process in meat quality have been extensively studied in beef [34], in pork [35] and in sheep meat [36], but only few studies have been performed in horse meat in which, again, differences in animal age and breed are considerable [23,37–41].

Considering the variability of the results obtained and the numerous sources of variation, specific conditions to maximize ageing positive impacts in horse meat are far from being understood and, therefore, further studies are needed. In this regard, the aim of the present work is to study the effect of ageing time (0, 7, 14 and 21 days) in meat quality parameters. This knowledge is necessary to contribute to the recommendation of a certain ageing period, based on quality parameters, for the horse meat industry. Moreover, a thorough characterization of fatty acid (FA) composition of two horse fat tissues, muscle and subcutaneous (SC), has been reported.

2. Materials and Methods

2.1. Animal Handling

Ten Hispano-Bretón horses (five females and five males) were reared in a commercial farm under grazing conditions while suckling their mothers from birth (May–June 2017) until weaning (6–8 months of age). Then, they continued grazing until 11–13 months of age, when they were moved to a commercial feedlot and finished for 100–120 days on a high-grain diet and straw ad libitum. Concentrate was composed by barley, soybean hulls, molasses, palm oil and salts (13.3% protein, 2.70% fat and 7.60% fiber).

Horses were slaughtered in a commercial abattoir, following the specifications in the European legislation [42], at 15–17 months of age. More details about the experimental design have been previously described [43].

The average carcass weight was of 246 ± 14.0 kg (250 ± 15.0 kg for females and 242 ± 12.5 kg for males). All carcasses were classified as U (conformation) and 2 (fat cover) according to the community scale for the classification of carcasses of adult bovine animals [44] as there is no specific classification system for horses at the EU level.

2.2. Experimental Design and Sampling

A full-randomized block design was used considering slaughter day as a blocking factor. Fat tissue was the main factor in the study of the variability of the FA composition of non-aged horse meat samples, and ageing time when assessing the variability of meat quality parameters. In both studies, the loin was the experimental unit. Animal sex, carcass side and carcass weight were distorting variation sources controlled by the experimental design in order to minimize the residual variation. For assessing the effect of ageing time on meat quality parameters a split plot design was used where ageing time levels were randomly allocated to different individual steaks obtained from the loin of each carcass. In this case, the experimental unit (loin) was considered as a plot and the steaks were the subplots (sampling units) in which ageing time effect was assessed.

Two horses (female and male) were slaughtered per week during five consecutive weeks. After 48 h *post mortem* at 4 °C (day 0), both right and left rib sections were removed from carcasses and transported to the laboratory under refrigerated conditions (total number of loins = 20). The loin, longissimus thoracis and lumborum (LTL) muscle, was excised, trimmed of visible adipose and connective tissues and cut into 10 consecutive steaks (1.5 cm thick) from the thoracic side. The first two steaks were trimmed, divided in three similar portions, vacuum packed and frozen (−80 °C) for chemical and FA composition and myoglobin content determinations, respectively. A portion of SC fat (20–30 mg) taken from the thoracic region of the loin was also vacuum packed and frozen at −80 °C for FA profile determination. The following four steaks were vacuum packed and randomly assigned to an ageing time: 0, 7, 14 or 21 d for instrumental color, cooked loss and instrumental texture determinations. The same procedure was used for the last four steaks used for pH measurements. Ageing was performed in a walk-in cooler (4 ± 1 °C) and without illumination. After instrumental color was determined (unpacked and bloomed samples), the steaks were vacuum packed again and frozen at −80 °C for further cook loss and instrumental texture determinations.

2.3. Chemical and Fatty Acid Composition

Chemical and FA compositions were determined only in non-aged steaks as not relevant compositional changes were expected within 21 d of ageing. Standard procedures were used for dry matter [45], crude protein [46], ether extract [47] and ash [48] determinations.

For SC fat analysis, 50 ± 1 mg of middle layer adipose tissue were freeze-dried and directly methylated with sodium methoxide (Methanolic-Base, 0.5 N; Supelco Inc., Bellefonte, PA, USA) using 1 mL of internal standard (4 mg/mL of methyl ester (ME) 23:0; Nu-Check Prep. Inc., Elysian, MN, USA). For muscle tissue, total lipids were extracted from 1 g of freeze-dried horse meat with chloroform-methanol (2:1, v/v) [49]. Lipid aliquots of 10 mg were methylated using anhydrous methanol containing 2% H_2SO_4 [16]. Prior to

methylation, 1 mL of internal standard (1 mg/mL of 23:0 ME) was added. For both tissues, muscle and SC fat tissues, FAMEs were analyzed using a 7890A gas chromatograph (GC) with flame ionization detector (Agilent Technologies, Madrid, Spain) coupled to a 7693 automatic injector (Agilent Technologies). Separation was carried out using a SP-2560 column (100 m, 0.25 mm i.d. and 0.20 µm film thickness; Supelco. Bellefonte, PA, USA) and following the conditions described by Kramer et al. [50]. Peak identification was performed using commercial reference standards (#463 and #603 mixtures, individual 21:0, 23:0 FAMEs and a CLA mixture #UC-59 M composed by 9*c*,11*t*-/8*t*,10*c*-/11*c*,13*t*-/10*t*,12*c*-/8*c*,10*c*-/9*c*,11*c*-/10*c*,12*c*-/11*c*,13*c*-/11*t*,13*t*-/10*t*,12*t*-/9*t*,11*t*-/8*t*,10*t*-18:2 obtained from Nu-Chek Prep Inc., Elysian, MN, USA; BCFA containing bacterial mixture purchased from Matreya, Pleasant Gap, PA, USA), confirmed using FAME fractions obtained from silver-ion solid phase extraction glass cartridges [51,52], and following retention times and elution orders reported in the literature [5,50,53]. Chromatographic areas were corrected according to theoretical response factors [54] and internal standard was used to calculate quantitative data (mg per g of SC fat tissue and mg per 100 g fresh meat). Then, FAME contents were expressed as percentages (%). In general, FAMEs representing below 0.05% were excluded to reduce the size of tables, except for minor FAMEs of particular nutritional interest.

2.4. pH Measurements

Triplicate pH measurements were taken in each steak at each ageing time (0, 7, 14 and 21 d) using a portable pH meter (HI99163, Hanna Instruments, Smithfield, RI, USA) equipped with a penetrating glass electrode (FC232D, Hanna Instruments, USA).

2.5. Myoglobin Content Determinations

Total myoglobin content was measured in non-aged steaks as not relevant changes were expected over ageing time. The method by Faustman and Philips [55], with minor modifications was followed. Previously thawed (4 °C, overnight) and minced (domestic grinder) 5 g of meat were homogenized in iced cold sodium phosphate buffer and set aside for 1 h (4 °C, darkness). Samples were then centrifuged (32,000× *g*) for 45 min and filtered through a Whatman filter paper (n °1, Whatman PLC, UK). Absorbance was read at 525 nm in a UV 1280 spectrophotometer (Shimadzu Corporation, Japan) and the total myoglobin concentration was estimated applying the extinction coefficient of 7.6 mM^{-1} cm^{-1} as proposed by Bowen [56].

2.6. Instrumental Color Measurements

For instrumental color measurements, color in non-aged steaks was measured after they were cut, covered with an oxygen-permeable polyvinylchloride film (oxygen permeability of 580 $mL/m^2/h$) and exposed to air for 1 h (4 °C). The rest of the color measurements were taken when the corresponding ageing period was reached. As for 0 d, steaks were unpacked, covered with film and exposed to air for 1 h (4 °C). A Minolta CR-200 colorimeter (Konica Minolta, Japan) with a D65 illuminant and a 10 ° visual angle was used to measure L^* (lightness), a^* (redness) and b^* (yellowness) values according to CIELAB color space [57]. Five spectral readings per sample were taken in different parts of the steaks. From these measurements, two additional color parameters were calculated: hue angle (h^*), which defines color = arctangent (b^*/a^*) and chroma or saturation index (C^*) = $(a^{*2} + b^{*2})^{1/2}$ (AMSA, 2012).

2.7. Cook Loss Determinations

Steaks were thawed overnight (4 °C) and after 1 h at room temperature, they were weighted, individually introduced in open plastic bags and cooked in a water bath at 80 °C (SV Thermo, Orved, Italy) until they reached an internal temperature of 71 ± 0.1 °C, monitored by temperature probes (TFA-301040, TFA, Germany). Cooking was performed in batches of 8 steaks, which were randomly chosen (2 sets of 4 ageing times). Then, steaks were removed from the water bath and cooled at room temperature for 1 h. Excess moisture

was eliminated and samples were reweighted. Cook loss was determined calculating the weight difference between raw and cooked steaks [58].

2.8. Instrumental Texture Measurements

For instrumental texture measurements, 8 cuboids of approximately $1 \times 1.5 \times 1$ cm^3 were cut from each cooked steak and parallel to the muscle fibers. Maximum Warner-Bratzler shear force (WBSF) of each cuboid was measured using a TA-XT2i texture analyzer (Stable Micro Systems, UK) connected to an IBM-compatible Foxen computer, with an AutenticAMD-K6 ™ 3D microprocessor. The 'Texture Expert' software, version 1.22 for Windows (Stable Micro Systems, Surrey, UK) was used. The texture analyzer was equipped with a WBSF device (cutting blade at a constant speed of 1.70 mm/s and a load cell of 300 N).

2.9. Statistical Analysis

Analyses were conducted using IBM-SPSS Statistics Software (Version 25.0, IBM Corporation, Chicago, IL, USA). Normality and homoscedasticity of the variables were checked and pH values were log transformed.

The general linear model (GLM) of ANOVA was used to determine, separately, the significant differences in the FA composition between fat tissues (muscle and subcutaneous fat) of non-aged horse meat samples, and in the instrumental color (L^*, a^*, b^*, C^*, h^*), pH, cook loss and WBSF measurements of horse meat samples among ageing times (0, 7, 14 and 21 d). The GLMs included the corresponding main factor and the controlled distorting variation factors (animal sex and carcass side) as fixed effects, and carcass weight as a covariate. Slaughter day was also included as a random effect because this blocking factor was a simultaneous distorting factor of uncontrolled variation coming from at least individual animal, feeding, transport or slaughter conditions. Moreover, GLMs included binary interactions between all factors.

Eta square (η^2) was used for effect size estimation and Fisher's least significance difference test of estimated marginal means was used for pairwise comparisons among ageing time levels. Three significant figures were used to express data and significance was declared at $p \leq 0.05$.

3. Results and Discussion

3.1. Chemical and Fatty Acid Composition

Chemical composition (%) and muscle FA composition in absolute basis (mg/100 g of raw meat) are reported in Table 1. Mean moisture (75.3%) was comparable to values described in the literature for 15–16 month old horse loins [7,59,60]. As expected, mean protein content (20.4%) was also in the range of values reported in the literature for different horse breeds, ages and production systems (19–22%; [7,19,21,23,59–63]). The muscle fat content obtained in the present study (3.31%) was comparable to other studies where horses were extensively reared, finished on concentrates and slaughtered at similar ages [7,19,60]. Ash content in horse meat has been reported to vary due to the age of animals [64] while the mineral content of feedstuffs may also have an influence [64]. The results obtained in the present study (1.03%) were in line with meat from horses slaughtered at similar age [7,19,59].

In terms of FA composition, in absolute basis (Table 1), wide ranges were observed while these differences were substantially reduced in percentage basis (Tables 2–4). Variable lipid content in horse meat has been previously reported, depending primarily on animal-related (i.e., breed, age, feeding) but also methodology-related (i.e., fat extraction procedure) factors [3].

Table 1. Chemical (%) and fatty acid (absolute basis: mg/100 g of meat) composition of horse longissimus thoracis and lumborum muscle (n = 20).

	Mean	Min	Max	SEM
Chemical composition				
Moisture	75.3	72.9	78.1	0.3
Crude protein	20.4	19.1	22.3	0.2
Fat (ether extract)	3.31	1.87	5.13	0.19
Ash	1.03	0.750	1.17	0.02
Fatty acid composition				
Total FAME	2427	893	4238	199
SFA	956	333	1743	85
BCFA	4.98	2.32	7.97	0.35
MUFA	1054	380	1877	93
cis-MUFA	1051	378	1872	92
trans-MUFA	3.32	1.07	5.42	0.29
CLA (18:2)	2.75	1.42	4.36	0.21
NC-dienes (18:2)	1.40	0.814	2.91	0.11
Trienes (18:3)	0.522	0.105	1.14	0.05
PUFA	345	147	546	22
n-6	266	113	390	15
18:2n-6	231	96	350	14
n-3	78	25	177	8
18:3n-3	56.1	15.6	154	8
DMA	60.3	26.6	84.7	3.3

Min, minimum value; Max, maximum value; SEM, standard error of the mean. FAME, fatty acid (FA) methyl esters; SFA, saturated FA; BCFA, branched-chain FA; MUFA, monounsaturated FA; CLA, conjugated linoleic acids; NC, non-conjugated; PUFA, polyunsaturated FA; DMA, dimethylacetals (sum of DMA, AME (alk-1-enyl methyl ethers) and ALD (fatty aldehydes).

Table 2. Effect of fat tissue (muscle and subcutaneous fat) on the saturated and branched-chain fatty acid composition of horse meat in percentage basis (%) (n = 20).

	Muscle				Subcutaneous		p-Value
	Mean	Min	Max	SEM	Mean	SEM	
SFA	38.9	36.8	43.8	0.3	39.5	0.5	0.383
12:0	0.206	0.151	0.256	0.006	0.224	0.008	0.079
14:0	3.65	3.00	4.58	0.09	4.33	0.09	<0.001
15:0	0.274	0.186	0.429	0.015	0.410	0.017	<0.001
16:0	29.3	26.8	31.3	0.3	29.9	0.4	0.256
17:0	0.357	0.238	0.489	0.015	0.505	0.020	<0.001
18:0	4.79	4.38	5.50	0.09	3.85	0.14	<0.001
20:0	0.0590	0.0450	0.0700	0.0020	0.0496	0.0017	<0.001
22:0	0.0665	0.0370	0.106	0.0039	0.0138	0.0018	<0.001
BCFA	0.211	0.176	0.260	0.006	0.285	0.012	<0.001
i-16:0	0.0890	0.068	0.107	0.0023	0.0844	0.0037	0.297

Min, minimum value; Max, maximum value; SEM, standard error of the mean. SFA, saturated fatty acids (FA); BCFA, branched-chain FA.

Table 3. Effect of fat tissue (muscle and subcutaneous fat) on the monounsaturated fatty acid composition of horse meat in percentage basis (%) (n = 20).

	Muscle				Subcutaneous		*p*-Value
	Mean	Min	Max	SEM	Mean	SEM	
MUFA	42.9	37.6	47.7	0.6	44.3	0.4	0.074
cis-MUFA	42.8	37.4	47.5	0.6	44.2	0.4	0.076
9*c*-14:1	0.412	0.297	0.511	0.013	0.389	0.016	0.295
7*c*-16:1	0.182	0.138	0.272	0.008	0.328	0.014	<0.001
9*c*-16:1	8.09	5.602	9.693	0.27	7.58	0.23	0.174
9*c*-18:1	31.4	27.4	34.1	0.4	32.7	0.4	0.025
11*c*-18:1	1.93	1.57	2.22	0.04	1.51	0.03	<0.001
13*c*-18:1	0.0965	0.0740	0.119	0.0027	0.0531	0.0017	<0.001
11*c*-19:1	0.0675	0.0491	0.0880	0.0027	0.0738	0.0042	0.201
11*c*-20:1	0.335	0.283	0.416	0.008	0.470	0.016	<0.001
trans-MUFA	0.136	0.101	0.237	0.006	0.149	0.005	0.143
9*t*-18:1	0.0970	0.0680	0.117	0.0026	0.0625	0.0026	<0.001

Min, minimum value; Max, maximum value; SEM, standard error of the mean. MUFA, monounsaturated fatty acids. (FA).

Table 4. Effect of fat tissue (muscle and subcutaneous fat) on the conjugated and non-conjugated diene, triene, polyunsaturated fatty acid and dimethylacetal composition of horse meat in percentage basis (%) (n = 20).

	Muscle				Subcutaneous		*p*-Value
	Mean	Min	Max	SEM	Mean	SEM	
CLA (18:2)	0.118	0.0884	0.198	0.007	0.0931	0.0050	0.008
9*c*,11*t*-	0.0535	0.0394	0.0712	0.0022	0.0581	0.0043	0.318
NC-dienes (18:2)	0.0605	0.0372	0.1002	0.0033	0.0475	0.0030	0.008
Trienes (18:3)	0.0220	0.0073	0.0371	0.0016	0.0263	0.0020	0.099
PUFA	15.0	11.1	21.6	0.7	15.6	0.5	0.455
20:3n-9	0.0263	0.0174	0.0441	0.0019	0.0125	0.0014	<0.001
n-6	11.7	8.76	19.38	0.7	12.5	0.6	0.424
18:2n-6	10.1	7.55	16.6	0.6	12.0	0.5	0.026
20:2n-6	0.206	0.141	0.335	0.012	0.281	0.012	<0.001
20:3n-6	0.240	0.124	0.387	0.015	0.0338	0.0022	<0.001
20:4n-6	0.946	0.491	1.74	0.071	0.0631	0.0053	<0.001
22:4n-6	0.0705	0.0381	0.160	0.0067	0.0194	0.0021	<0.001
22:5n-6	0.0470	0.266	0.793	0.0054	ND	-	-
n-3	3.19	2.05	5.31	0.21	3.18	0.23	0.975
18:3n-3	2.26	1.11	4.14	0.19	2.98	0.21	0.018
20:3n-3	0.110	0.0720	0.188	0.007	0.106	0.008	0.714
20:5n-3	0.125	0.0480	0.296	0.0134	0.0119	0.0010	<0.001
22:5n-3	0.503	0.266	0.793	0.027	0.0561	0.0034	<0.001
22:6n-3	0.115	0.0460	0.187	0.0084	0.0120	0.0010	<0.001
n-6/n-3	3.97	2.06	8.77	0.38	4.30	0.45	0.565
P/S	0.386	0.284	0.587	0.020	0.399	0.016	0.609
DMA	2.63	1.531	3.472	0.11	ND	-	-

Min, minimum value; Max, maximum value; SEM, standard error of the mean. CLA, conjugated linoleic acids; NC, non-conjugated; PUFA, polyunsaturated fatty acids; DMA, dimethylacetals (sum of DMA, AME (alk-1-enyl methyl ethers) and ALD (fatty aldehydes)); ND, not detected.

Considering the saturated fraction of muscle lipids (Tables 1 and 2), the obtained results (333–1743 mg/100 g of meat; 38.9%) were in accordance with those reported in horse meat [3]. Palmitic acid (16:0) was the major FA (29.3%) followed far behind by stearic (18:0) and myristic (14:0) acids (4.79% and 3.65%, respectively). The rest of minor SFA altogether constituted approximately only 1% of the total SFA.

Traditionally, BCFAs have not been discussed in horse meat studies since they are primarily associated to ruminant derived products due to their microbial origin [14].

However, they were described in tissues of other herbivores, such as rabbits, with microbial fermentation in the hindgut [65], which is also the case of horses. In this sense, recent studies have reported low contents of BCFAs in horse meat (6.25 mg/100 g of meat) [5,61], in line with the present study (4.98 mg/100 g of meat). The major BCFA in these studies was *iso*-16:0 and this is also supported by the present results (42% of total BCFA) (Tables 1 and 2).

Regarding monounsaturated FAs (MUFA), the absolute content ranged between 380 and 1877 mg/100 g meat, comprising 42.9% of total FAMEs (Tables 1 and 3). This value locates among those reported in the literature for extensively reared (14.6%) and concentrate fed (50.2%) horses slaughtered at different ages [3]. *Cis(c)*-MUFAs represented 99.7% of MUFAs, with oleic (9*c*-18:1), palmitoleic (9*c*-16:1) and ascleptic (11*c*-18:1) acids being the most abundant. *Trans (t)*-MUFAs have not been generally reported in horse meat studies [6,8,62,66,67] as it is known that their content is low compared to ruminant products. The low accumulation of these FAs in equids is related to their digestive physiology [12,13]. In the present study, *t*-MUFA accounted only for 0.32% of total MUFA, with elaidic acid (9*t*-18:1) being the major *t*-MUFA. CLAs are also minor in horse meat compared to ruminants, as they are primarily produced from PUFA by the action of rumen microbiota. However, as previously discussed, a low accumulation of these compounds is possible. In the present study, the total CLA represented 0.12% and similar percentages have been reported by He et al. [6] in 3 year old Breton horses fattened for 12 months (0.15%) and by Belaunzaran et al. [61] in fattened and suckling crossbred horses (0.11%). In contrast, higher proportions have reported Juarez et al. [62] in 24 month old Hispano-Bretón and Burguete horses (0.42%) and by Lanza et al. [7] in 18 month old Sanfratello and Haflinger horses (0.46%), which could well be related to the higher muscle fat content but also to the overestimation of CLA, taking into account that 21:0 elutes in the CLA region (GC technique). Rumenic acid (9*c*,11*t*-18:2) was the major CLA isomer and in relation to non-conjugated (NC) dienes and trienes, their contribution was minor (<0.1%) as reported by Belaunzaran et al. [61].

In general, horse meat has been described as rich in PUFAs (specially in essential linoleic (LA, 18:2n-6) and linolenic (LNA, 18:3n-3) acids) because, as discussed earlier, the FA absorption in the equine digestive system happens before the fermentative chambers, allowing an efficient absorption and deposition of dietary PUFAs in horse products with little transformation [3]. An average PUFA content of 345 mg/100 g of meat was found, accounting for 15.0% (Tables 1 and 4). This percentage was low compared to other studies reporting values from 15.6% in 3 year old concentrate-fed Breton horses [6] to 46% in 2 year old grass fed Galician Mountain horses [66]. The low PUFA content related to the low n-6 content, especially LA that is the major n-6 PUFA (86% of total n-6 PUFA). On average, LA accounted for 10.1% of total FAMEs, a percentage that is below those usually reported in horses slaughtered at similar ages and managed under semiextensive (20.9–21%, [7]; 16.6–18.9%, [63]) systems. However, similar values to the present study were obtained by Lorenzo et al. [68], in a study performed with semi-extensively reared 18 month old Galician Mountain × Hispano-Bretón horses (10.1%). Thus, the high-grain finishing period of the animals probably caused a remarkable decrease in LA percentage in favor of MUFAs. Regarding the rest of n-6 PUFAs, arachidonic acid (20:4n-6) was the second most abundant, in good agreement with previous works [5,7,18,66–68].

The capacity of horses to absorb dietary PUFAs before microbial hydrogenation has been related to the high accumulation of n-3 PUFAs from pastures (rich in LNA, 18:3n-3) [3]. More recently, as reviewed by Sahaka et al. [9], a specific pancreatic enzyme (pancreatic lipase related protein 2) hydrolyzing the LNA esterified in plant galactolipids has been described to be uniquely present in horse, contributing to LNA deposition in horse tissues. In the scientific literature, n-3 values from 1.53% in 36 month old Hispano-Bretón horses finished on concentrate for 12 months [18] up to 24.4% in 9 month old extensively reared Galician Mountain horses [69] have been reported, being diet the main source of variation and LNA the main responsible for those elevated n-3 contents. In the present study, an average value of 3.19% of n-3 PUFA (78 mg/100 g of meat) was observed, evidencing that the high-grain finishing limited the n-3 PUFA deposition. On average, LNA accounted

for 2.26% and the next major n-3 PUFAs were 22:5n-3 (docosapentaenoic acid, DPA), 20:5n-3 (eicosapentaenoic acid, EPA), 22:6n-3 (docosahexaenoic acid, DHA) and 20:3n-3. These four FAs, although being cell membrane components, are seldom reported in horse meat studies.

Overall, obtained n-3 percentage was similar to the ones typically described in non n-3 enriched chicken (3.03%, [70]) or rabbit (3.40%, [71]) and higher than non n-3 enriched pork (1.22% vs. 8.94% in 10% flaxseed enriched pork [72]).

Alkenyl moieties from plasmalogenic lipids in horse meat have not received much attention, although they have been studied in other meats [73,74]. The explanation for this may lie in the technical difficulties to resolve these compounds [15]. Under the conditions of the present study (acid-catalyzed methanolysis), these were detected as the sum of dimethylacetals, alk-1-enyl methyl ethers and fatty aldehydes. An average content of 60.3 mg/100 g of meat was found, in line with other horse meat studies [15,61]. These findings should not be diminished and deserve further research due to the increasing reports concerning the biological activities of plasmalogenic lipids and their relationship with human health [16,17].

The results of the statistical analysis showed several significant differences in the FA content between muscle and SC fat (Tables 2–4). SC tissue showed significantly higher SFA content than muscle fat as the former is mainly composed of triacylglycerols, major components of neutral lipids that, in general, exhibit low PUFA and high SFA and MUFA depositions [61]. Conversely, 18:0 showed significantly higher content in muscle than in SC fat as observed by Belaunzaran et al. [5], which justified this phenomenon proving that this FA is mainly esterified in polar lipids [61]. Regarding MUFAs, significant differences were observed in several individual FAs and oleic acid (9*c*-18:1) was found in higher percentages in SC fat. In the literature, higher MUFA contents have been reported in SC compared to muscle fat [5,62,63]. Finally, no difference was observed in total PUFA content between the tissues although most long-chain n-6 and n-3 PUFAs were significantly higher in muscle compared to SC fat. In line with this, Belaunzaran et al. [61] described FA deposition preferences in horse muscle, indicating that all PUFAs were more abundant in polar lipids (phospholipids) except LNA that was preferentially deposited in neutral lipids (triacylglycerols).

3.2. Effect of Ageing on Horse Meat Quality Parameters

Ageing time significantly affected all the quality parameters (pH, instrumental color, cook loss, WBSF; Table 5). Additionally, the statistical analysis revealed that slaughter day (five consecutive weeks) and its interaction with ageing time were also significant for some of the quality parameters studied, which could be attributed to other preslaughter factors affecting the oxidation state and color [75–77], texture and other quality attributes of meat [78]. However, when the interaction term was statistically significant, it was ordinal for all parameters and its effect size (measured by η^2) was smaller than the main effect (ageing time), except for pH measurements. In this case, the interaction between ageing time and slaughter day evidenced the effect of other uncontrolled preslaughter factors (Figure 1).

Table 5. Statistical parameters on the effect of ageing time (0, 7, 14 and 21 days), slaughter day (5 consecutive weeks) and ageing time*slaughter day on pH, instrumental color (*L**, *a**, *b**, *C**, *h**), cook loss (%) and Warner-Bratzler shear force (N) measurements in horse longissimus thoracis and lumborum muscle (n = 20).

	AT		D		AT*D	
	p-Value	η^2	*p*-Value	η^2	*p*-Value	η^2
pH	< 0.001	0.583	< 0.001	0.758	< 0.001	0.796
*L**	< 0.001	0.884	0.001	0.348	< 0.001	0.586
*a**	< 0.001	0.665	0.015	0.251	0.319	0.254
*b**	< 0.001	0.858	0.067	0.185	0.025	0.394
*C**	< 0.001	0.718	0.024	0.230	0.230	0.278
*h**	< 0.001	0.923	0.041	0.207	< 0.001	0.558
Cook loss	0.007	0.246	0.004	0.297	0.577	0.200
WBSF	< 0.001	0.926	< 0.001	0.684	0.114	0.321

T, ageing time; D, slaughter day; η^2, eta square; WBSF, Warner-Bratzler shear force.

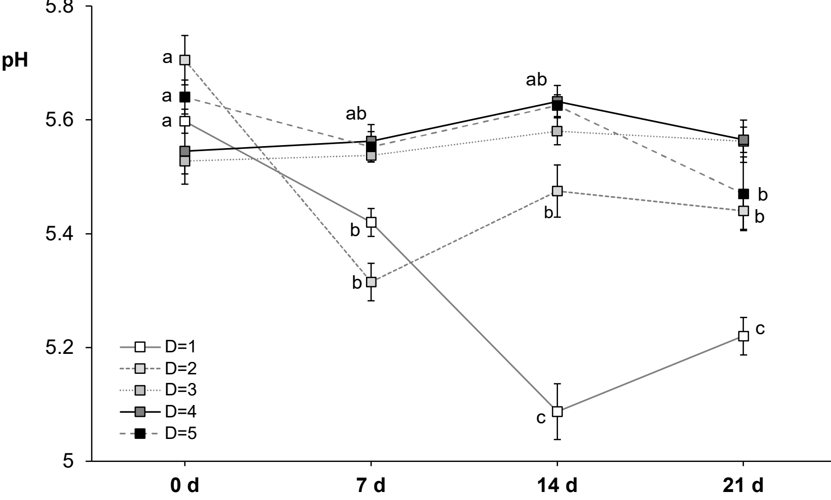

Figure 1. Interaction between ageing time (0, 7, 14 and 21 days) and slaughter day (D; 5 consecutive weeks) for pH measurements in horse longissimus thoracis and lumborum muscle (n = 20). Mean values and standard error of the means have been represented. Different letters indicate significant differences ($p \leq 0.05$) among ageing times.

3.2.1. Measurement of pH

Initial pH value measured at 48 h *post mortem* (5.60 ± 0.09) was similar to that reported by others [23,59,60,62] in horse loins of Hispano-Bretón, Galician Mountain, Burguete and Jeju breeds, respectively. This would mean that a normal acidification was achieved during *post mortem* metabolism. In the literature, defects coming from abnormal *post mortem* metabolism have not been reported in horse meat [22,79]. The reason of pH value being slightly lower than the one typically registered in beef [80,81] could be related to the higher glycogen content reported in horse meat compared to beef [79], after rigor completion it would store more residual glycogen and give lower pH values. During the ageing period, in general, pH values decreased from 0 to 7 d (mean value of 5.48) and, then, stayed stable until the end of the experiment (21 d), except for the first slaughter day that values continued decreasing until 14 d (Figure 1). The reason for this behavior is not immediately apparent but it is likely attributed to other non-controlled factors. Related to pH decrease under vacuum, growth of lactic acid bacteria (LAB) has been reported to be the main responsible for pH decrease in refrigerated meats (pH < 5.8; [32]). Gomez and Lorenzo [38] reported a slight increase of LAB in vacuum packed horse meat during the first 7 d of ageing, without developing spoilage odors that could be the reason for the pH decrease

during vacuum ageing. In contrast, Seong et al. [23] observed an increase of pH until 20 d of ageing, whereas others reported pH increases during the first 2 weeks of ageing [38,40].

3.2.2. Myoglobin Content and Instrumental Color

Myoglobin content in meat is highly relevant as it determines meat color via its four chemical forms [82]. In particular, horse meat has been described as rich in myoglobin compared with other meats [83]. The chemistry of myoglobin is species specific [33]. In this sense, a high ability of oxygen to combine with the red oxymyoglobin and convert to brown metmyoglobin has been described in horse meat, affecting to the shelf life of meat [84]. The results obtained in the present study (3.47 mg/100 g of meat) were comparable with values reported in extensively reared horses slaughtered at similar age [63,84], but higher than those found in animals slaughtered at earlier ages [21], which is in accordance with what Badiani and Manfredini [83] indicated, that myoglobin content in horses increases during the first two years of life.

Regarding instrumental color parameters (Figure 2), lightness (L^*) values of the loin (40.8–45.1) were consistent with the literature [21,40]. However, others have reported lower (28–35 [62]; 35–37 [23]; 32–37 [85]) and higher (49–52 [84]) L^* values in horses slaughtered at a similar age. In the present study, lightness significantly increased during the ageing period ($p \leq 0.001$) with no changes among 7 and 14 d (Figure 2A). Lightness values reported during vacuum ageing in horse meat studies showed diverse patterns. For instance, Lorenzo and Gomez [40] and Seong et al., [23] did not observe any difference during the first 14 d and 30 d of ageing, respectively, while Ruiz [41] reported increased loin L^* values when 13 month old horses were fed linseed enriched concentrate compared to regular concentrate. Overall, according to literature, L^* values are not supposed to vary much during *post mortem* storage, showing only slight increases [86], which is due to changes in light scattering associated to muscle fibers shrinkage and fluids expulsion to the extracellular space [87].

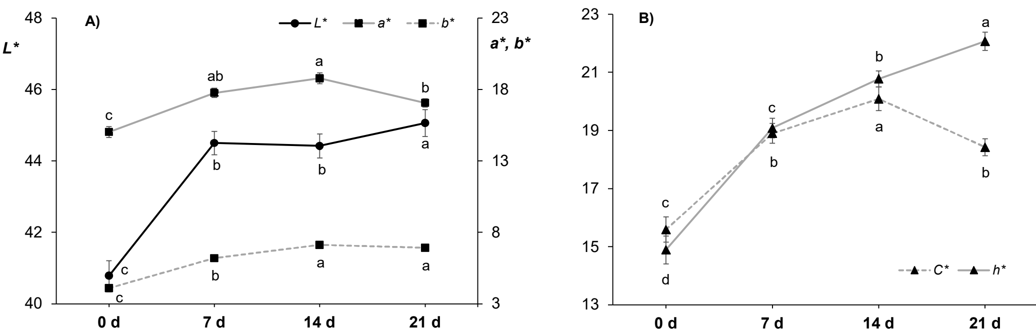

Figure 2. Effect of ageing time (0, 7, 14 and 21 days) on instrumental color measurements in horse longissimus thoracis and lumborum muscle (n = 20): (**A**) L^* (left axis), a^* and b^* (right axis), and (**B**) C^* and h^*. Different letters indicate statistically significant differences ($p \leq 0.05$).

Redness (a^*) values (15.0–18.8; Figure 2A) were in good agreement with those reported by others in horse loins [23,38,40,62], although others reported lower values [21,84]. These variations in redness could be attributed to the total myoglobin content [63] that depends primarily on animal age [83]. Regarding the ageing time, redness was the highest at 14 d (18.8) and the lowest (15.0) in non-aged loins, whereas at 7 d and 21 d intermediate values were observed. A significant increase in redness from 0 to 14 d in vacuum aged meat was also observed by Lorenzo and Gomez [40] while Seong et al. [23] reported a continuous increasing trend up to 30 d.

Yellowness (b^*) values (4.09–7.12; Figure 2A) were similar to some [20,21,23] but lower compared to other horse meat studies (12–14 [38]; 10–14 [40]; 8–15 [84]) and these variations could be attributed to differences in muscle fat content and slaughter age of horses [63]. In the present study, during ageing time, b^* values increased from 4.09 (0 d) to 7.12 (14 d)

while no difference was found between 14 and 21 d. Gomez and Lorenzo [38] and Lorenzo and Gomez [40] also observed an increase in yellowness during vacuum storage that plateaued at 7 and 10 d, respectively, while Seong et al. [23] reported an increase in b^* values over during 30 d of ageing.

In terms of calculated chroma (C^*) and hue (h^*) values, the decrease observed in redness but not in yellowness between 14 and 21 d resulted in a significant increase of hue value from 20.8 to 22.1 (Figure 2B) in the yellow ($+b^*$) direction of the CIELAB color space. This happened together with a decrease in chroma (C^*) from 20.1 to 18.4, being recognized as a more grey or dull color (dull yellow) perceived as brown. This would indicate that horse meat started to turn brownish between 14 and 21 d of ageing due to metmyoglobin formation [28,86]. The color changes during ageing period are well illustrated in Figure 3, where a representative photograph of each ageing time is shown. It is worth commenting that samples aged for 21 d were visually evaluated by 120 consumers and these samples obtained the worst acceptability scores [43]. After 14 d of ageing, the visually apparent color shift to brownish could be the reason why consumers rejected the meat aged for 21 d, as the cherry-red color that they associate with freshness was not present [88].

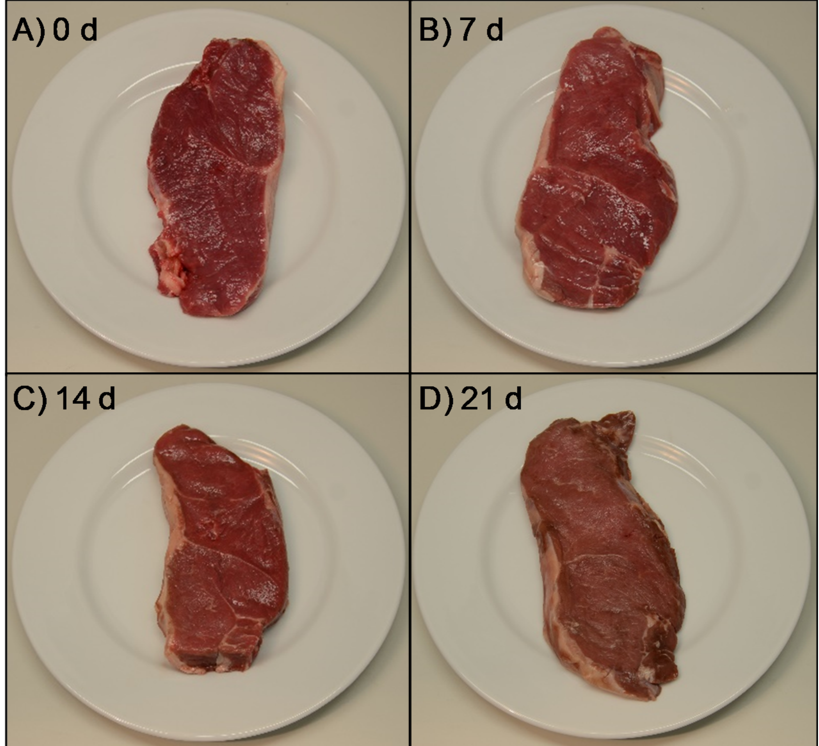

Figure 3. Representative photographs of non-aged (**A**) and aged horse longissimus thoracis and lumborum steaks ((**B**) 7 days, (**C**) 14 days and (**D**) 21 days).

3.2.3. Cook Loss and Instrumental Texture

It is known that cooking contributes to meat texture changes, while moisture and cook losses happen in a time and temperature dependent manner [89]. Cook loss values ranged between 20.3 and 23.0% (Figure 4). The value obtained in non-aged horse meat (23%) was similar to those values reported by others in cooked horse loins [23,39]. Values decreased from non-aged to 7 d aged meat (20.3%) and then stayed constant. It is known that ageing affects water distribution and mobility in meat [90] but contradictory results have been reported in the literature regarding cook loss data. In some studies, an increase in cook loss with ageing has been observed as a result of a weakened muscle structure unable to trap or retain water [87] while in others a decrease has been reported as a result of a lower initial water content related to a pH drop [91,92]. The second statement seem to better explain the results of the present study as a decrease in both pH and cook loss happened between

0 and 7 d of ageing (Figures 1 and 4). In the scientific literature, Seong et al. [23] did not observe any differences in cook loss during 30 d ageing whereas Kaic et al. [39] reported an increase in cook loss from 14 to 28 d of horse meat ageing. Overall, only few studies have reported cook loss data in horse meat and the variability of results is considerable.

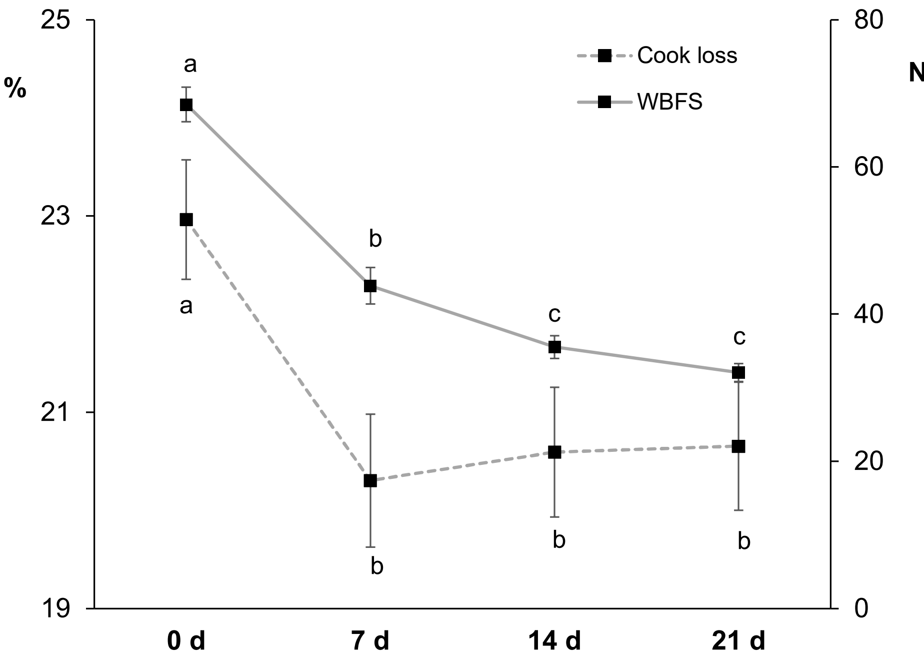

Figure 4. Effect of ageing time (0, 7, 14 and 21 days) on cook loss (left axis) and Warner-Bratzler shear force (WBSF; right axis) in horse longissimus thoracis and lumborum muscle (n = 20). Different letters indicate statistically significant differences ($p \leq 0.05$).

Regarding instrumental texture measured in cooked horse meat, values ranged between 32.1 and 68.5 N (Figure 4). The WBSF value in non-aged horse loin (68.5 N) was higher than values reported in the literature (40 N [23]; 31 N [37]) but close to values reported in 4–6 d aged meat from 18 month old Sanfratellano and Haflinger horses (56.5 N [7]), in 2 d aged meat from 10 year old horses (63 N [22]), and in non-aged meat from 13 and 26 month old Galician Mountain × Burguete horses (52.5 N [41]). In the present study, as expected, WBSF values decreased significantly from 0 (68.5 N) to 14 d (35.5 N), with no additional significant changes (32.1 N at 21 d). Considering the WBSF-dependent classification proposed by Shackelford, Morgan, Cross and Savell [93] for beef loin as there is no homologous classification for horse meat, non-aged meat would be classified as 'tough', 7 d aged meat as 'intermediate', and 14 d and 21 d aged meat as 'tender'. In essence, due to expected *post mortem* proteolysis [30], ageing period improved meat tenderness, especially during the first two weeks. Similarly, Seong et al. [23] observed a decrease in WBSF values during the first 10 d of ageing (getting classified as 'very tender') in meat from 28 month old Jeju horses. Some other horse meat studies have reported earlier tenderization; for instance, Della Malva et al. [37] reported the most tender horse meat after only 3 d of ageing and they did not perceive any additional changes thereafter. Others have reported significant improvement of WBSF values after 4 and 8 d of ageing depending on animal feeding and age [41].

4. Conclusions

The fatty acid composition of Hispano-Bretón horse meat characterized in the present study indicated that the high-grain finishing period may have limited the deposition of polyunsaturated fatty acids in tissues, especially of n-3, compared to values typically described in grazing horses. However, obtained n-3 polyunsaturated fatty acid contents were still higher than those generally reported in ruminant derived products although LNA

was preferentially deposited in backfat compared to muscle fat, confirming the potential of horse as an interesting red meat alternative in terms of its nutritional quality. The present study highlights that the ageing process affects horse meat quality parameters that are very relevant for the consumer. Visual properties started to deteriorate after 14 days of ageing and the improvement in tenderness also happened during the first 14 days, although meat could be considered as 'intermediate tender' after the first week of ageing. From the results obtained, the optimum ageing time can be established between 7 and 14 days for Hispano-Bretón horse meat; longer periods would not be justified from the quality point of view nor from the economic point of view. All in all, further research is required in order to standardize *post mortem* practices so as to obtain homogeneous final horse meat quality.

Author Contributions: Conceptualization, N.A., L.R.B. and L.M.; methodology, L.R.B., L.M. and K.I.; software, L.R.B. and L.J.R.B.; validation, L.R.B., L.M. and N.A.; formal analysis, L.R.B., L.M., K.I. and N.A.; investigation, L.R.B. and N.A.; resources, L.J.R.B.; data curation, L.R.B. and L.J.R.B.; writing—original draft preparation, L.R.B. and L.M.; writing—review and editing, N.A., M.Á.S., L.J.R.B. and K.I.; visualization, L.M. and N.A.; supervision, N.A. and M.Á.S.; project administration, N.A.; funding acquisition, L.J.R.B. and N.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Basque Government (grant IT944-16) that also funded the postdoctoral grant of L.M. Department of Economic Development and Infrastructures of the Basque Government is acknowledged for the fellowship of L.R.B.

Institutional Review Board Statement: Not applicable, as commercial samples were studied.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the writing of the manuscript.

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APPENDIX IV

Publication IV

Beldarrain, L.R., Morán, L., Sentandreu, M.A., Barron, L.J.R., Aldai, N. (2022). Effect of ageing time on the volatile compounds from cooked horse meat. *Meat Sci.*, 184, 108692.

https://doi.org/10.1016/j.meatsci.2021.108692

ISSN: 0309-1740

IF (2020): 5.209

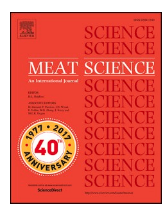
Category: Food Science & Technology (Q1)

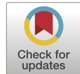
Meat Science 184 (2022) 108692

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci





Effect of ageing time on the volatile compounds from cooked horse meat

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ARTICLE INFO

Keywords:
Foal meat
Equine
Volatile composition
Cooked meat aroma
Lipid oxidation
Vacuum ageing

ABSTRACT

Volatile compounds from cooked and aged (0, 7, 14, 21 days) Hispano-Bretón horse meat (loin) were analyzed by solid-phase microextraction coupled to gas chromatography–mass spectrometry. A total of 77 volatile compounds were found, from which aldehydes were the predominant family. Most of the identified compounds had their origin in the degradation of lipids, with a negligible contribution of Maillard derived products. Odour impact ratios were calculated and used as indicators of the contribution of each compound to the total aroma and aldehydes were, in general, the major contributors to cooked horse meat aroma. Results revealed that ageing affected 15 of the volatile compounds detected. From them, hexadecanal and 2- and 3-methylbutanal significantly increased during ageing, presumably affecting the cooked meat odour as these have considerable odorant impact. Under the present study conditions, periods longer than 14 days would be necessary for significant changes in the volatile profile of cooked horse meat.

1. Introduction

The characteristic aroma of cooked meat is an important quality attribute for consumer acceptability (Mottram, 1998) which is developed during cooking by the generation of odour active volatile compounds (Calkins & Hodgen, 2007). These compounds result from different complex reactions such as Maillard reaction between amino acids and sugars, lipid (mainly oxidation) and vitamin degradation, and other reactions among intermediate and final compounds derived from these pathways (Mottram, 1998). The formation of these compounds is conditioned by the cooking process and conditions (Domínguez, Gómez, Fonseca, & Lorenzo, 2014a; Kerth, 2016; Morán, Aldai, & Barron, 2021; Wall, Kerth, Miller, & Alvarado, 2019). Indeed, lower cooking temperatures and shorter cooking times are known to favour lipid degradation (thermal oxidation) products, while higher temperatures (especially direct heat sources as grilling) and longer times enhance Maillard reaction compounds (Kerth, 2016; Mottram, 1985). The contribution of volatile compounds to cooked meat aroma depends on their concentration and odour threshold (Zellner, Dugo, Dugo, & Mondello, 2008), and in this regard, Maillard reaction products (pyrazines, pyrroles, oxazoles, thiophenes and other heterocyclic compounds) are in general described as potent odorous compounds (Mottram, 1998). In addition, other factors including animal species (Calkins & Hodgen, 2007; Gasser & Grosch, 1988), breed (Insausti, Beriain, Gorraiz, & Purroy, 2002), sex (Gorraiz, Beriain, Chasco, & Insausti, 2002) muscle (Van Ba, Park, Dashmaa, & Hwang, 2014) and ageing time (Koutsidis et al., 2008) have been reported to influence the volatile profile of cooked meat.

Among all the main precursors of volatiles in cooked meat (amino acids, sugars, lipids and vitamins), a special attention has been placed on lipids (content and composition) (Estevez, Morcuende, Ventanas, & Cava, 2003; Mottram, 1998), the most important contributors to the characteristic aroma of different animal species (Calkins & Hodgen, 2007; Mottram, 1998). Relationships between meat lipids and generated volatile compounds have been found in cooked meat from different species (Elmore, Mottram, Enser, & Wood, 1999; Rivas-Cañedo et al., 2013).

Overall, extensive research has been conducted in the identification of volatile compounds in meat and to elucidate their contribution to cooked meat aroma (Gasser & Grosch, 1988; Van Ba, Hwang, Jeong, & Touseef, 2012). However, despite the recent increase in its consumption (Belaunzaran et al., 2015), very few works have studied the volatile profile of cooked horse meat (Domínguez et al., 2014a; Domínguez, Gómez, Fonseca, & Lorenzo, 2014b; Maggiolino et al., 2019; Tateo et al., 2020).

Meat ageing, practice especially applied for meat tenderness improvement (Koohmaraie, 1994) can also affect meat aroma due to

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https://doi.org/10.1016/j.meatsci.2021.108692
Received 25 June 2021; Received in revised form 27 September 2021; Accepted 4 October 2021
Available online 6 October 2021
0309-1740/© 2021 Published by Elsevier Ltd.

changes in the concentration of precursors and to the development of odorous compounds in raw aged meat by enzymatic reactions (Koutsidis et al., 2008), bacterial action (Casaburi, Piombino, Nychas, & Villani, 2015) and lipid oxidation (Resconi et al., 2012; Rivas-Cañedo et al., 2013). Volatile composition of aged meat (different periods) has been widely explored in cooked meat of the most consumed species such as beef (Gorraiz et al., 2002; Van Ba et al., 2014; Watanabe et al., 2015), lamb (Rivas-Cañedo et al., 2013) and pork (Estevez et al., 2003). In addition, sensory studies addressing the implication of ageing in the odour of cooked meat reported that long ageing periods can result in unpleasant aromas (Spanier, Flores, McMillin, & Bidner, 1997). These results have been attributed to decreased levels of volatile compounds with pleasant odour notes (Van Ba et al., 2014) or to an excess concentration of hexanal, one of the major volatile compound in cooked meat (Melton, 1983).

In this sense, to our knowledge, only two studies have reported the ageing time effect on the volatile compounds of cooked meat from Italian Heavy Draught Horses (several muscles aged up to 14 days; Maggiolino et al., 2019; Tateo et al., 2020). To this extent, considering the crucial role of meat aroma in the acceptance and preference of the consumer (Beldarrain et al., 2020), the aim of the present work was to study the changes in the volatile profile of cooked horse meat previously vacuum aged for 0, 7, 14 and 21 days.

2. Materials and methods

2.1. Animal handling, experimental design and sampling

Ten Hispano-Bretón horses (five females and five males) were reared in a commercial farm under grazing conditions while suckling their mothers from birth (May–June 2017) until weaning (6–8 months of age). Then, they continued grazing until 11–13 months of age, when they were moved to a commercial feedlot and finished for 100–120 days (d) on a high-grain diet and straw *ad libitum*. Concentrate was composed by barley, soybean hulls, molasses, palm oil and salts (13.3% protein, 2.70% fat, 7.60% fibre).

Horses were slaughtered in a commercial abattoir, following the specifications in the European legislation (Council Regulation, 2009), at 15–17 months of age. The average carcass weight was of 246 ± 14.0 kg (250 ± 15.0 kg for females and 242 ± 12.5 kg for males). All carcasses were classified as U (conformation) and 2 (fat cover) according to the Community scale for the classification of carcasses of adult bovine animals (Council Regulation, 1981) as there is no specific classification system for horses at EU level.

Two horses (female and male) were slaughtered per week during five consecutive weeks. After 48 h (h) *post mortem* at 4 °C (day 0), both right and left rib sections were removed from carcasses and transported to the laboratory under refrigerated conditions ($n = 20$). The loin, *Longissimus thoracis et lumborum* (LTL) muscle ($n = 20$), was excised, trimmed of visible adipose and connective tissues and cut into 1.5 cm thick steaks.

From each loin, four consecutive steaks, starting from the 2nd rib, were obtained. Steaks were vacuum packaged and randomly assigned to an ageing time of 0, 7, 14 and 21 d. Ageing was performed under refrigeration conditions (4 ± 1 °C), without illumination. When each ageing day was reached, steaks were frozen (−80 °C) until volatile compound analysis was performed.

The aforementioned procedure was considered as a full-randomized block design with slaughter day as a blocking factor and ageing time as split plot factor where ageing levels were randomly allocated to different individual steaks obtained from each loin. The experimental unit (loin) was considered as a plot and the steaks were the subplots (sampling units) in which ageing time (factor) was assessed. Animal sex, carcass side and carcass weight were distorting variation sources controlled by the experimental design in order to minimize the residual variation.

2.2. Volatile compound analysis

2.2.1. Meat cooking procedure

Cooking was carried out according to AMSA (2015) recommendations. Before cooking, horse steaks were thawed (4 °C) overnight and kept at room temperature for 2 h covered with an oxygen permeable polyvinylchloride film (580 mL/m^2/h of permeability). Eight steaks from two loins coming from different animals (4 ageing times from female and 4 ageing times from male) were cooked at the same time in four plane double clamp electric grills (Dalyko MB-30, Sogo, Spain). Same grill was used for the steaks belonging to the same ageing time (0, 7, 14 and 21 d), grills were set at 200 °C and meat cooked for 9–13 min until an internal temperature, monitored individually with a multichannel (Lutron electronic, Pennsylvania, USA), of 71 ± 1 °C was reached. Cooked steaks were allowed to cool at room temperature, minced, vacuum packed and frozen (−80 °C) until volatile analysis.

2.2.2. Solid-phase microextraction-gas chromatography–mass spectrometry (SPME-GC–MS) analysis

Minced meat samples were thawed for 2 h at room temperature. Then, approximately 10 g of cooked meat, to which anhydrous sodium sulphate (Sigma Aldrich) was added in a ratio of 4:1 (by weight), were homogenized in a blender. From this mixture, 2.5 ± 0.001 g were weighted on a 10 mL amber vial and 20 μL of a deionized water solution of methyl isobutyl ketone (1 g/L, 99.8% purity, Sigma Aldrich) were added as the internal standard (IS). Vials were sealed with PTFE septa and steel magnetic cap (Agilent Technologies).

Volatile compounds were extracted by SPME on a 30/50 μm DVB/Carboxen/PDMS fibre (Supelco) using a PAL RSI 85 autosampler (CTC Analytics AG, Zwingen, Switzerland). Extraction was done over 50 min at 80 °C after 15 min of pre-equilibration time at extraction temperature. Volatiles trapped onto the fibre were desorbed in the injection port during 25 min at 240 °C in splitless mode (split valve was opened at 200 mL/min after 30 min of the injection). Analyses were performed using a 7820A GC equipment coupled to a 5975E MS detector (Agilent Technologies). Volatile compounds were separated on a Supelcowax-10 capillary column (60 m, 250 μm i.d., and 0.25 μm film thickness; Supelco) using the following temperature gradient: oven temperature was held at 40 °C for 10 min, then increased at a rate of 5 °C/min until 110 °C, increased again at 10 °C/min until 240 °C, and finally held at 240 °C for 15 min. Helium (99.999% purity, Air liquid, Madrid, Spain) was used as the carrier gas (constant pressure of 30 psi) and volatiles were transferred to MS detector throughout a transfer line at 280 °C. Chromatographic data were registered with the MSD ChemStation Data Analysis software (version 5.52, Agilent Technologies). MS detector operated at 150 °C in full scan mode (1.4 scan/s; m/z range 26–350) using 70 eV as total ion current. Two replicates were done for each cooked meat sample.

Volatile compounds in the cooked horse meat samples were quantified using the chromatographic peak area. The limit of detection (LOD) was set as two times the average baseline noise calculated in three different chromatographic regions from the analysis of 10 blanks (empty vial). Peak areas (arbitrary units) of volatile compounds were used to calculate the abundance relative to the IS area according to the following equation:

$$\text{Relative abundance} = \frac{\text{peak area}}{\text{IS area}} \bullet \frac{2.5\ \text{g}}{\text{mixture weight (g)}} \bullet 100$$

Tentative identifications of volatile compounds were performed by comparing the mass spectra of the peaks with those of NIST 2.0 (National Institute of Standards and Technology, Gaithersburg, USA) library, using a matching factor > 700. Mixtures of C7-C24 alkanes (Sigma Aldrich) were used for the calculation of the experimental linear retention indices (LRI) for cooked horse meat and commercial standard peaks. Experimental LRI values were also compared to values obtained in the literature under similar chromatographic conditions.

Additionally, when available, several high purity commercial standard compounds were used for positive identification of cooked meat volatiles: 1-penten-3-ol, 1-pentanol, 1-octen-3-ol, 1-heptanol, 6-methyl-2-heptanone, 1-octanol, (*E*)-2-octen-1-ol, 1-tetradecanol, 1-tridecanol, acetaldehyde, hexanal, 1-hexanol, (*E*)-2-hexenal, heptanal, (*E*)-2-octenal, (*E*)-2-nonenal, (*E*)-2-decenal, 2-butyl-2-octenal, toluene, benzaldehyde and 1-hexadecene were purchased from Sigma Aldrich (Madrid, Spain), and 1-propanal, 2-methylbutanal, 3-methylbutanal, octanal and 2-heptanone from Honeywell-Fluka Research Chemical-Fisher Scientific (Madrid, Spain).

2.2.3. Odour impact ratio calculation

In order to obtain an indicator of the odour intensity of each volatile compound detected by SPME-GC–MS in cooked horse meat samples, odour impact ratio (OIR) was calculated as previously described (Abilleira, Schilichtherle-Cerny, Virto, de Renobales, & Barron, 2010) with minor modifications. Available odour threshold (OT) values measured in water were obtained from Van Gemert (2011) unless otherwise indicated in the table (Table 1), and subsequently used to calculate OIR for each of the volatile compound:

$$\mathrm{OIR} = \frac{\text{mean relative abundance}}{\mathrm{OT}\ (\mu g/kg)}$$

The mean relative abundance of each volatile compound detected in cooked horse meat samples (all 4 ageing times) was used for OIR calculations, not quantitative values. Moreover, it is noteworthy to point out that the present meat protein content might cause a matrix effect and alter the OT values of volatile compounds, compared to literature values obtained in water. However, OIR values allowed the comparison of odour impacts of the different volatile compounds detected in cooked horse meat samples.

2.3. Data treatment and statistical analysis

Peak areas of individual volatile compounds present in both replicates (> LOD) and in over 70% of the samples from each ageing time were used to calculate the mean relative abundance in cooked horse meat samples. The general linear model of analysis of variance (GLM-ANOVA) (IBM-SPSS statistics software (version 25.0, IBM, New York, USA) was used to investigate the ageing effect on the volatile composition of cooked horse meat. The GLM included ageing time and the controlled distorting variation factors (animal sex and carcass side) as fixed effects, and carcass weight as covariate. Slaughter day was also included as a random effect in the model because this blocking factor was a simultaneous distorting factor of uncontrolled variation coming from at least individual animal, feeding, transport or slaughter conditions. Moreover, GLM included binary interactions among all factors. Fisher's Least Significance Difference test of estimated marginal means was used for pairwise comparisons among ageing time levels.

Partial least squares for discrimination analysis (PLS-DA) (Unscrambler X software version 10.3, CAMO ASA, Oslo, Norway) was applied to the volatile composition of cooked horse meat samples to discriminate among ageing times. Ageing day was used as categorical *Y*-variable and weighed volatile relative abundances (used as the inverse of standard deviation) as *X*-variable. Full (leave-one-out) cross-validation with an uncertainty test was applied to extract the optimal number of model factors. Variable importance in projection scores were used to estimate the *X*-variables contribution to PLS-DA model and uncertainty limits were used to estimate the significance of the weighted coefficients that correlated *X*-variables to categorical *Y*-variables (Martens & Martens, 2000).

Statistical significance was declared at $P \leq 0.05$ and numerical values of volatile relative abundances were log transformed to assure data normality. Three significant figures were used to express the data.

3. Results and discussion

3.1. Volatile composition of cooked horse meat

A total of 77 individual volatile compounds were found (peak area > LOD) in the headspace of cooked horse meat samples with aldehydes constituting most of compounds (30 compounds), followed by alcohols (13), non-aromatic hydrocarbons (11), ketones (8), benzenoic compounds (4), furans (2) and a sulphur compound (Table 1). In addition, 8 volatile compounds were detected but their chemical nature could not be established as their MS signal was not clear enough, and they have been labelled as unknowns.

From the compounds identified, the predominant chemical family in terms of mean relative abundance were the aldehydes (around 75% of the total abundance) as observed in cooked beef (Wall et al., 2019), pork (Estevez et al., 2003) and in horse meat (Domínguez et al., 2014a). Among them, hexanal was the dominating aldehyde followed by nonanal (15% and 8% of the total abundance, respectively). The next major chemical family was that of alcohols (10%), and the remaining families together accounted for around 13% of the total volatile abundance.

It is noteworthy that some of the chemical families reported in relatively high concentrations in cooked horse meat and other species meats were absent in the present study, namely short-chain fatty acids (C4-C10) (horse meat, Tateo et al., 2020), esters (horse meat, Domínguez et al., 2014a) or nitrogen and sulphur compounds (beef, Wall et al., 2019) (only dimethyl trisulphide was detected). Short-chain fatty acid results are controversial, as several studies have not reported them in the volatile profile of cooked beef (Gorraiz et al., 2002). The reason of the high variability usually found in the literature has been attributed to several reasons such as the complexity in the formation of these compounds and their possible further degradation into other aroma compounds as observed in different types of meat (Casaburi et al., 2015), their dissociation equilibrium and pH of the matrix analyzed, or different sensitivities on extraction methods as observed in beef (Song et al., 2011). Certainly, the extraction conditions used in the present study do not allow the detection of > C3 carboxylic acids and, consequently, they are not reported.

The absence of esters and nitrogen and sulphur compounds (except for dimethyl trisulphide) is likely related to the cooking process of the meat. In fact, widely differing cooking methods applied in studies performed in meat from different species (considering sample preparation, type of heat treatment, temperature and cooking time) may lead to diverse concentrations of these compounds; thus, comparisons need to be carefully done (deer meat, Morán et al., 2021; pork, Mottram, 1985). In the present study, steaks were grilled at 200 °C for 9–13 min until an internal temperature of 71 °C was reached (medium degree of doneness according to beef steak colour guide; AMSA, 1995). It has been reported that only when the meat is grilled under severe conditions the Maillard reaction derived nitrogen and sulphur compounds are major components of cooked meat (Mottram, 1998). In this line, Mottram (1985) proved that when beef steaks were grilled at the same temperature but for a longer time (15 min *per* side, medium degree of doneness *versus* 30 min *per* side, well-done degree of doneness), the concentration of nitrogen and sulphur heterocyclic compounds increased and pyrazines dominated the volatile profile. Moreover, Wall et al. (2019) proved that the concentration of these compounds also increased significantly when grilling temperature was increased (232 °C *versus* 177 °C) even if all beef steaks were cooked until a medium degree of doneness (internal temperature of 71 °C). Kerth (2016) reported the influence of steak thickness on the type of volatile compound generated in cooked beef and concluded that the volatile profile of thinner steaks (~ 1.27 cm) was dominated by volatiles originating from lipid degradation, while thicker steaks (~ 2.81 cm) presented higher Maillard reaction derived products under the same cooking conditions. In the present study, a combination of the aforementioned factors related to the cooking process could have been partially responsible for the low contribution of Maillard reaction

Table 1
Mean relative abundance and estimated mean odour impact ratio (OIR), odour threshold (OT) (Van Gemert, 2011) and odour description of volatile compounds of cooked horse meat analyzed by solid-phase microextraction coupled to gas chromatography–mass spectrometry. Mean relative abundance values were calculated from volatile compounds detected in aged (0, 7, 14 and 21 days) and cooked horse meat samples ($n = 80$).

Volatile compound	IM	LRI	Relative abundance	OT[a]	OIR	SEM	Odour description	Ref.
Aldehydes								
Acetaldehyde	P	704	7.07	25	0.283	0.009	Fruity	1
Propanal	P	796	5.78	145	0.0399	0.0025	Nut like	2
2- Methylbutanal	P	911	2.88	4.40	0.655	0.115	Cinnamon, toast	3
3- Methylbutanal	P	915	7.04	1.20	5.87	1.18	Chocolate, caramel, green, nutty	4
Pentanal	T	981	46.5	12.0	3.88	0.09	Almond, malt, pungent, acrid	5
Hexanal	P	1078	501	1.00	500	32	Grassy, tea, vegetable, lemony, sour, beefy	5
2-Methyl-2-butenal	T	1085	6.72	458	0.0147	$8.02{\cdot}10^{-4}$	Coffee like	6
Heptanal	P	1183	95.2	2.80	34.0	0.9	Fruity, nutty	4
(*E*)-2-Hexenal	P	1223	5.20	110	0.0473	0.0045	Eucalyptus, fruit/flower, potato, toast	3
Octanal	P	1282	134	0.700	191	6	Soap/orange	3
(*E*)-2-Heptenal	T	1333	31.8	13	2.44	0.08	Fishy	3
2-Methyl-2-heptenal	T	1363	5.60					–
Nonanal	T	1398	278	2.80	99.2	0.6	Grassy, tea, vegetable, lemony, sour, beefy	7
(*E*)-2-Octenal	P	1441	44.2	3.00	14.7	0.017	Green, nut, fat	5
Decanal	T	1505	13.3	0.150	88.6	0.12	Powerful, waxy, aldehydic, orange, citrus peel	5
(*E,E*)-2,4-Heptadienal	T	1509	8.65	15.4	0.562	0.036	Roast meat, fried potatoe	8
(*E*)-2-Nonenal	P	1550	69.5	0.0800	868	21	Earthy, fermented, burnt	5
(*E*)-2-Decenal	P	1662	171	0.350	487	11	Tallow, orange	5
2-Butyl-2-octenal	P	1683	36.8	20.0	1.84	0.22		–
Dodecanal	T	1722	53.6	55	0.974	0.026	Onion, green, yeast,vomit	9
(*E,E*)-2,4-Nonadienal	T	1726	43.1	0.100	430	1	Meaty, burnt, chocolate	4
(*E*)-2-Undecenal	T	1774	217	1.40	155	2		–
(*E,Z*)-2,4-Decadienal	T	1786	11.1	0.070	158	7	Fried onion, lemon	10
Tridecanal	T	1831	108	70	1.54	0.05	Nutty	11
(*E,E*)-2,4-Decadienal	T	1839	60.1	0.0270	2226	58	Fatty, fried potatoe	12
Tetradecanal	T	1940	219	53	4.14	0.07	Roasted, fried meat	13
(*E,E*)-2,4-Undecadienal	T	1955	14.1	0.01	1410	0.4		–
Pentadecanal	T	2049	222	1000	0.222	0.004	Hot timber	9
Hexadecanal	T	2156	96.9	0.910 (14)	106	17	Sweet	9
cis-11-Hexadecenal	T	2189	17.3				Waxy	15
Ketones								
2-Heptanone	P	1181	8.74	140	0.0624	0.0061	Rancid, flower, vinegar, soap/orange	3
6-Methyl-2-heptanone	P	1242	9.71	8.10	1.20	0.09	Cloves, menthol, eugenol	5
3-Octanone	T	1259	4.17	28	0.149	0.024	Herbal	6
5-Methyl-3-hepten-2-one	T	1342	35.8					–
(*E*)-3-Octen-2-one	T	1415	7.18	250	0.0287	0.0008	Nut, crushed bug, earthy, spicy, herbal, sweet, mushroom, hay	5
(*E,E*)-3,5-Octadien-2-one	T	1586	13.5	125	0.108	0.008	Fruity, green, grassy	15
6,10-Dimethyl-(*E,E*)-5,9-undecadien-2-one	T	1870	12.0	60	0.200	0.011	Fresh, green, fruity, waxy, rose, woody, magnolia tropical	15
2- Pentadecanone	T	2038	8.65				Fresh, jasmin, celery, fatty, oily, waxy, burnt	–
Alcohols								
1-Penten-3-ol	P	1055	0.674	400	$1.69{\cdot}10^{-3}$	$6.50{\cdot}10^{-5}$	Flower, burnt, meaty	3
1-Pentanol	P	1251	36.5	$4.00{\cdot}10^{3}$	$9.12{\cdot}10^{-3}$	$4.78{\cdot}10^{-4}$	Mild odour, fusel oil, fruit, balsamic	5
1-Hexanol	P	1352	14.8	5.60	2.64	0.11	Woody, cut grass, chemical-winey, fatty, fruity, weak metallic	5
1-Octen-3-ol	P	1446	83.3	1.50	55.5	1.8	Fishy, fatty, mushroom, grassy	16
Heptanol	P	1453	42.6	5.40	7.88	0.11	Fragant, woody, oily, green, fatty, winey, sap, herb	5
2-Ethyl-hexan-1-ol	T	1488	2.79	$2.54{\cdot}10^{4}$	$1.09{\cdot}10^{-4}$	$4.74{\cdot}10^{-6}$	Resin, flower, green	5
1-Octanol	P	1555	76.8	190	0.404	0.010	Fatty, waxy, citrus, oily, walnut, moss, chemical, metal, burnt	5
3,5-Octadien-2-ol	T	1581	6.47					–
(*E*)-2-Octen-1-ol	P	1613	24.7	20.0	1.24	0.05	Green, citrus	5
9-Decen-2-ol	T	1781	8.04					–
1-Dodecanol	T	1966	18.8	158	0.119	0.005	Earthy, soapy, waxy, fatty, honey, coconut	15
1-Tridecanol	P	2083	10.8				Musty	15
1-Tetradecanol	P	2174	27.4				Fruity, waxy, coconut	15
Non-aromatic hydrocarbons								
2,2,4,6,6-Pentamethyl-heptane	T	958	5.12					–
Butyl-cyclopentane	T	1038	2.24					–
Tridecane	P	1297	14.5	$2.14{\cdot}10^{3}$(17)	$6.75{\cdot}10^{-3}$	$2.13{\cdot}10^{-4}$	Alkane	18
3-Ethyl-2methyl-1,3-hexadiene	T	1430	23.0					–
Pentadecane	P	1498	23.7	$1.30{\cdot}10^{7}$*	$1.82{\cdot}10^{-6}$	$3.10{\cdot}10^{-8}$	Waxy	15
1- Pentadecene	T	1525	8.14	$3.60{\cdot}10^{3}$	$2.26{\cdot}10^{-3}$	$1.08{\cdot}10^{-4}$		
5,5-Dimethyl-1,3-heptadiene	T	1591	7.58	$1.30{\cdot}10^{7}$*	$5.87{\cdot}10^{-7}$	$5.98{\cdot}10^{-8}$		–
Hexadecane	P	1598	12.1	500*	0.0242	0.011	Mild waxy	15
1-Hexadecene	P	1622	8.60	$3.20{\cdot}10^{3}$*	$2.69{\cdot}10^{-3}$	$1.45{\cdot}10^{-4}$		–
1-Heptadecene	T	1647	11.3	$8.00{\cdot}10^{3}$	$1.42{\cdot}10^{-3}$	$4.65{\cdot}10^{-5}$		–

(*continued on next page*)

Table 1 (*continued*)

Volatile compound	IM	LRI	Relative abundance	OT[a]	OIR	SEM	Odour description	Ref.
1,15-Hexadecadiene	T	2270						–
Benzenoic compounds								
Toluene	P	1035	3.78	24.0	0.158	0.0032	Chemical solvent aroma	19
Benzaldehyde	P	1545	75.9	350	0.217	$4.92{\cdot}10^{3}$	Almond oil, bitter almond, burning aromatic taste	5
3-Ethyl-benzaldehyde	T	1743	109					–
4-Pentyl-benzaldehyde	T	2057	23.3					–
Furans								
2-Ethyl-furan	T	950	3.14	8000	$3.92{\cdot}10^{-4}$	$5.59{\cdot}10^{-5}$	Sweet corn	20
2-Pentyl-furan	T	1231	50.2	5.80	8.65	0.19	Green, vean, butter	5
Miscelaneous								
Dimethyl trisulphide	T	1394	17.4	0.100	173	53	Sulfury, burnt,onion	4
Unknown$^{m/z: 97, 55, 41, 71, 84}$		1471	80.5					
Unknown$^{m/z: 67, 95, 41, 81, 12}$		1630	7.70					
Unknown$^{m/z: 43, 84, 71, 57, 128}$		1696	16.5					
Unknwon$^{m/z: 121, 91, 77, 150, 65}$		1889	70.5					
Unknwon$^{m/z: 95, 81, 43, 55, 67}$		1918	24.9					
Unknwon$^{m/z: 43, 41 57, 83, 69}$		1997	13.2					
Unknwon$^{m/z: 55, 43, 69, 83, 97}$		2070	27.3					
Unknown$^{m/z: 45, 55, 67, 73, 41}$		2295	22.5					

IM, identification method; LRI, linear retention index; SEM, standard error of the mean; P, positive identification; T, tentative identification; [a]OT expressed as μg/kg of water; *OT expressed as μg/kg of oil; Ref, reference.
References: (1) Sollner and Schieberle (2009); (2) Frankel (1993); (3) Resconi et al. (2012); (4) Machiels et al. (2004); (5) Calkins and Hodgen (2007); (6) Giri, Osako, and Ohsima (2010); (7) Moon, Cliff, and Li-Chan (2006); (8) Frank et al. (2017); (9) Gkarane et al. (2018); (10) Resconi et al. (2010); (11) Sutherland and Ames (1995); (12) Gasser and Grosch (1988); (13) Xie, Sun, Zheng, and Wang (2008); (14) Xie, He, Lv, Zhang, and Li (2016); (15) The Good Scents Company Information System (2021); (16) Tao, Wu, Zhou, Gu, and Wu (2014); (17) Kataoka, Lord, and Pawliszyn (2000); (18) Van Ba et al. (2012); (19) Moio, Dekimpe, Etievant, and Addeo (1993); (20) Evans, Moser, and List (1971)

derived compounds to cooked horse meat volatile profile.

Mechanisms of lipid oxidation in foodstuff are still an area of controversy and remain as a subject of active research. Indeed, several researchers have highlighted that lipid oxidation should be considered as multiple interrelated pathways rather than a single radical chain based on hydrogen abstraction (Schaich, 2013). Moreover, the interrelation between lipid and protein oxidation has been proposed to affect the odour of cooked meat from different species (Estevez, 2011), and the complexity of the issue becomes evident.

In the present study, most volatiles detected in cooked horse meat samples derived from the thermal degradation of lipids (Table 1). In this regard, high proportions of polyunsaturated FA (PUFA) are known to increase the susceptibility of meat to undergo lipid oxidation and the subsequent formation of volatile compounds during cooking. This has already been proved in other species in which cooked meat from animals with differing PUFA/saturated FA (SFA) ratios were studied (beef, Insausti et al., 2002; pork, Estevez et al., 2003; lamb, Gkarane et al., 2018). Moreover, high PUFA concentrations seem to form free radicals that promote the oxidation of other FA and inhibit the formation of Maillard reaction derived products, as confirmed in model system experiments (Elmore, Campo, Enser, & Mottram, 2002).

The unsaturated FA content of raw meat is relevant to understand the volatile profile of cooked horse meat, as the unsaturation degree of FA determines the overall concentration of volatiles coming from lipid thermo-oxidation (lamb, Rivas-Cañedo et al., 2013). More concretely, heptanal, octanal, nonanal, decanal, (*E*)-2-decenal and (*E*)-2-undecenal which are relevant contributors of cooked horse meat aroma have been reported as major oxidation products derived from oleic acid (Van Ba et al., 2012), which was the predominant FA in raw horse meat (31%; Beldarrain et al., 2021). 1-Octanol, the second most abundant alcohol, and 1-heptanol also originate from oleic acid (Schaich, 2013). Although this monounsaturated FA is less susceptible to lipid oxidation than PUFA, Elmore et al. (1999) hypothesized that a promoting effect of heating on the degradation of PUFA increased the amount of free radicals capable of attacking other less susceptible FA in beef.

The most abundant alcohol in the cooked horse meat samples, 1-octen-3-ol (Table 1), has been reported to be an oxidation product derived from linoleic acid (Rivas-Cañedo et al., 2013), and this is also true for 1- pentanol, 1-hexanol and some aldehydes such as pentanal, hexanal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*)-2-nonenal and (*E,E*) and (*E,Z*)-2,4-decadienal (Elmore et al., 2002; Van Ba et al., 2012). On the other hand, (*E,E*)-2,4-heptadienal and benzaldehyde have been reported to arise from the oxidation of linolenic acid (Van Ba et al., 2012; Van Ba et al., 2014).

It becomes evident that most relevant volatile compounds detected in the present study have been reported to be thermal oxidation/degradation products of oleic, linoleic and linolenic acids. But, some compounds (mean relative abundance <8) formed *via* Strecker amino acid degradation namely acetaldehyde and 2- and 3-methylbutanal were also identified. Regarding dimethyl trisulphide, it has been suggested that this volatile is formed from dimethyl disulphide, which is a consequence of the degradation of methionine or methanethiol (Baines & Mlotkiewicz, 1983). In contrast, Golovjna and Rothe (1980) attributed the origin of dimethyl trisulphide in beef to the reaction between hydrogen sulphide and ethanol. Other compounds such as methyl alcohols and ketones can be originated from the thermal degradation of branched-chain amino acids, although their presence in the volatile profile of cooked horse meat samples was not relevant, and benzaldehyde has also been suggested to derive from Strecker amino acid degradation according to studies performed in beef (Watanabe et al., 2015).

3.2. OIR values and aroma of cooked horse meat

In order to estimate the contribution of each volatile compound to the aroma of cooked horse meat, OIR values were calculated and odour descriptors reported by other authors or gathered in specialized flavour data bases were considered (Table 1). Overall, aldehydes showed the highest OIR values in cooked horse meat samples and they were, in consequence, the most odour-active compounds. Low detection thresholds have been reported for aldehydes, thus they contribute to cooked beef aroma even when present in low concentration (Elmore et al., 1999). In addition, aldehydes have been reported as the main compounds responsible for species-specific odours in cooked meat due to

aldehydes are essentially derived from lipid oxidation, and the diverse FA composition of different species constitutes a big source of variation in generated volatile aldehydes (Calkins & Hodgen, 2007).

Among linear saturated aldehydes, C6-C10 aldehydes and hexadecanal showed the highest OIR values (> 34). C6-C10 aldehydes are in general related to grassy and fruity odours, and of them, hexanal should be highlighted with the highest OIR value (500) (Table 1). Grassy, tea, vegetable, lemony, sour and beefy are odour notes that have been attributed to hexanal in cooked beef, so this aldehyde is considered to positively contribute to beef aroma (Calkins & Hodgen, 2007). In contrast, Kerler and Grosch (1997) reported that C6-C10 linear saturated aldehydes contribute to unpleasant odour notes in poultry and Melton (1983) concluded that an excessive concentration of hexanal might produce off-flavours in beef. Hexadecanal presented a high OIR value (106) and has been related to sweet odour notes. This fact, together with other taste precursors, may influence in the sweet perception of cooked horse meat, as recently reported by Beldarrain et al. (2020).

The contribution of unsaturated aldehydes to cooked horse meat aroma should not be underestimated, especially that of C9-C11 2-alkenals and 2,4-alkedienals, which have been described as aroma contributors in other cooked meats (Calkins & Hodgen, 2007). Even though seldom detected or reported in very low concentrations in cooked horse meat (Domínguez et al., 2014a; Domínguez et al., 2014b; Maggiolino et al., 2019; Tateo et al., 2020), (*E*)-2-nonenal, −decenal and -undecenal showed high OIR values (> 155) in the present study, and so did (*E,E*)-2,4-nonadienal, −decadienal, (*E*)-2-octenal and (*E,Z*)-2,4-decadienal (OIR > 14). These unsaturated aldehydes have been described to generate fatty/burnt odour notes in beef (Machiels, Istasse, & Van Ruth, 2004).

OIR values calculated for alcohols in studied cooked horse meat samples were in general low (≤ 1), with the exception of two linear saturated alcohols (1-hexanol and 1-heptanol) and 1-octen-3-ol. These three compounds have already been described as active odorants in other cooked meats (Calkins & Hodgen, 2007) and the 1-octen-3-ol presented a relatively high OIR value in the present cooked horse meat samples (55.5) which has been agreed to contribute with a mushroom-like odour in beef (Van Ba et al., 2012). Some studies did not report this compound in grilled horse meat (Domínguez et al., 2014a; Domínguez et al., 2014b), whereas others, under similar meat cooking and volatile extraction conditions, did (Maggiolino et al., 2019; Tateo et al., 2020). The rest of the alcohols found in the cooked horse meat samples (Table 1) are generally related to pleasant odours, but as previously stated, they did not seem to significantly contribute to cooked horse meat aroma.

Detected ketones showed low OIR values (≤ 1), poorly contributing to cooked horse meat aroma (Table 1). 2,3-Butanedione, a typical odour active volatile compound reported to contribute to cooked beef aroma with buttery odour notes (Gorraiz et al., 2002; Machiels et al., 2004; Van Ba et al., 2014) was not detected in the present study. Again, this diketone was not detected by Domínguez et al. (2014a and 2014b) in cooked horse meat, although Maggiolino et al. (2019) and Tateo et al. (2020) did detect.

Benzaldehyde has been related to unpleasant odours in cooked meat from different species (Calkins & Hodgen, 2007) and some studies have suggested that a high abundance of benzaldehyde might cause off-flavour in lamb (Elmore et al., 2002), however, this compound showed a low OIR value (0.217) in cooked horse meat. OT values of 3-ethyl- and 4-pentyl-benzaldehyde were not available in the scientific literature although these volatiles have been reported in cooked meat and meat products (Maggiolino et al., 2019).

From the two detected furans, 2-pentylfuran showed a relevant OIR value (8.65) (Table 1). This compound has been described as characteristic of grilled meat aroma in several meats (Shibamoto, 1980) and has been previously reported in cooked pork (Estevez et al., 2003), lamb (Rivas-Cañedo et al., 2013), beef (Calkins & Hodgen, 2007) and horse meat (Maggiolino et al., 2019; Tateo et al., 2020).

On the other hand, dimethyl trisulphide, generally related to unpleasant odours in beef (Machiels et al., 2004), has been reported as an important contributor of cooked beef aroma (Van Ba et al., 2014). This sulphur compound showed the highest OIR value (173) after that of alkenals and alkedienals, due to its moderate abundance in the cooked horse meat samples but low OT (Table 1). Thus, it appears feasible that this compound contributes significantly to the cooked horse meat aroma.

Non-aromatic hydrocarbons, which show high odour detection thresholds, poorly contribute to the aroma of cooked meats (Ho & Chen, 1994), with OIR values below 1 (Table 1).

3.3. Effect of ageing time on the volatile profile of cooked horse meat

Changes in volatile composition of cooked meat caused by the ageing process may indicate chemical, enzymatic or microbial degradation of volatile precursors such as peptides, amino acids, sugars and lipids of raw meat happened during cooling storage of meat from different species (Casaburi et al., 2015; Estevez et al., 2003; Insausti et al., 2002; Koutsidis et al., 2008). Lipid oxidation can occur in raw meat during storage, before lipid thermal oxidation related to the cooking process (Jelen & Wąsowicz, 2012), altering the volatile profile of the aged and cooked meat. These events also depend on the type of packaging used during the ageing process. In the present study, steaks were aged in an anoxic environment (vacuum), thereby limiting oxidative processes as observed by Spanier et al. (1997) in beef.

From the 77 individual volatile compounds detected in the cooked horse meat samples, the relative abundance of 15 changed significantly with ageing time ($P \leq 0.05$; Table 2). The relative abundance of some volatile compounds, including three aldehydes (hexadecanal and 2- and 3-methylbutanal), benzaldehyde and 2-pentadecanone increased with ageing time. Interestingly, both 2- and 3-methylbutanal were not detected in the cooked unaged meat samples but their relative abundance increased from 7 to 14 days of ageing. These branched aldehydes were previously found in higher amounts in cooked beef aged for 28 d than for 7 d (Van Ba et al., 2014), and the reason may be an increase in the content of free leucine and isoleucine due to proteolysis during beef ageing process (Koutsidis et al., 2008). Relative abundance of benzaldehyde also increased during ageing as observed in previous horse meat studies (Maggiolino et al., 2019; Tateo et al., 2020). As aforementioned, hexadecanal has been related to sweet odour notes, and in consequence, its higher relative abundance in the aged and cooked meat samples could be translated in a sweeter aroma in comparison to the unaged and cooked meats. This increase is likely related to oxidative degradation of lipids although autoxidation did not seem to be relevant during the ageing process as other volatile compounds normally derived from lipid oxidation did not change, or even, decreased their relative abundance with ageing time (Table 2) as observed by others in vacuum aged beef (Watanabe et al., 2015). In this sense, some other volatile compounds related to lipid oxidation such as hexanal, 2-methyl-2-heptenal, 2-butyl-2-octenal, hexanal, dodecanol, 5-methyl-3-hepten-2-one, (*E,E*)-3,5-octadien-2-one, and 1-pentadecene decreased significantly over time in the cooked horse meat samples ($P \leq 0.05$). The reported decrease in abundance of these compounds is in contrast with the published literature about vacuum aged and cooked meat, where an increase in lipid oxidation derived compounds have been described (Maggiolino et al., 2019). In agreement to our observations, Rivas-Cañedo et al. (2013) reported a decrease in the content of some oxidation compounds (aldehydes and ketones) of cooked meat over ageing time in n-3 enriched grazed lambs which was attributed to the presence of antioxidants from pasture plants (Resconi et al., 2010). Furthermore, reactions or interactions among products of proteolysis and lipid oxidation yielding non-volatile compounds could also explain the decrease in the abundance of lipid oxidation derived volatile compounds as observed in pork and lamb (Estevez et al., 2003; Rivas-Cañedo et al., 2013). In this regard,

Table 2
Mean relative abundance of volatile compounds analyzed by solid-phase microextraction coupled to gas chromatography in aged (0, 7, 14 and 21 days) and cooked horse meat samples (n = 80).

Volatile compound	0 d	7 d	14 d	21 d	SEM	*P*-value
Aldehydes						
Acetaldehyde	7.71	6.66	6.80	7.09	0.30	0.791
Propanal	5.60	5.25	6.49	ND	0.26	0.085
2- Methylbutanal	ND	1.88^{b}	3.28^{a}	3.49^{a}	0.17	≤0.001
3- Methylbutanal	ND	4.23^{b}	8.17^{a}	8.73^{a}	0.40	≤0.001
Pentanal	48.4	46.2	48.0	43.5	1.7	0.187
Hexanal	564^{a}	527^{a}	497^{ab}	415^{b}	21	0.005
2-Methyl-2-butenal	7.44	6.12	7.27	6.06	0.38	0.294
Heptanal	98.6	95.4	98.5	88.1	4.0	0.527
(*E*)-2-Hexenal	ND	4.70	5.70	ND	1.91	0.357
Octanal	122	139	143	133	6	0.094
(*E*)-2-Heptenal	34.6	30.2	32.3	29.9	1.5	0.165
2-Methyl-2-heptenal	6.40^{a}	6.12^{a}	6.05^{a}	3.82^{b}	0.39	0.013
Nonanal	277	274	280	280	11	0.866
(*E*)-2-Octenal	46.1	44.0	46.0	40.8	2.0	0.480
Decanal	13.8	13.0	13.2	13.3	0.5	0.765
(*E,E*)-2,4-Heptadienal	8.22	8.04	10.3	8.04	0.43	0.147
(*E*)-2-Nonenal	72.8	67.5	71.9	65.7	2.9	0.364
(*E*)-2-Decenal	177	166	177	162	8	0.179
2-Butyl-2-octenal	45.9^{a}	37.6^{a}	39.1^{a}	24.4^{b}	3.4	0.003
Dodecanal	51.7	57.6	53.5	51.4	2.8	0.357
(*E,E*)-2,4-Nonadienal	43.0	ND	43.2	ND	2.8	0.594
(*E*)-2-Undecenal	225	213	221	210	9	0.549
(*E,Z*)-2,4-Decadienal	12.0	10.0	12.0	10.4	0.5	0.203
Tridecanal	115	108	108	98.9	4.5	0.327
(*E,E*)-2,4-Decadienal	59.8	57.1	64.5	59.0	2.8	0.624
Tetradecanal	224	217	227	210	10	0.730
(E,E)-2,4-Undecadienal	15.0	13.8	14.4	13.0	0.6	0.907
Pentadecanal	230	218	226	214	10	0.715
Hexadecanal	71.2^{c}	74.5^{c}	102^{b}	140^{a}	5.5	≤0.001
cis-11-Hexdecenal	16.6	16.2	18.1	18.2	1.0	0.719
Ketones						
2-Heptanone	ND	9.59	7.88	ND	1.79	0.695
6-Methyl-2-heptanone	11.6	9.69	9.43	8.11	0.51	0.021
3-Octanone	5.78	4.14	3.49	3.27	0.46	0.130
5-Methyl-3-hepten-2-one	45.1^{a}	37.7^{ab}	33.7^{bc}	26.6^{c}	2.1	≤0.001
(*E*)-3-Octen-2-one	6.73	7.33	7.70	6.98	0.35	0.895
(*E,E*)-3,5-Octadien-2-one	12.2^{b}	13.5^{b}	16.4^{a}	11.8^{b}	0.7	0.013
6,10-Dimethyl-(*E,E*)-5,9-undecadien-2-one	12.7	11.3	ND	ND	0.54	0.166
2- Pentadecanone	ND	7.63^{b}	8.92^{ab}	9.41^{a}	0.42	0.001
Alcohols						
1-Penten-3-ol	0.708	0.631	0.730	0.629	0.026	0.239
1-Pentanol	40.5	38.3	35.3	31.7	1.6	0.052
1-Hexanol	15.2^{a}	15.2^{a}	15.6^{a}	13.0^{b}	0.6	0.035
1-Octen-3-ol	88.4	85.4	83.8	75.6	3.2	0.249
1-Heptanol	42.9	42.0	44.0	41.4	1.6	0.766
2-Ethyl-1-hexanol	2.60	2.75	2.66	3.14	0.11	0.191
1-Octanol	77.2	78.6	80.0	71.4	2.9	0.894
3,5-Octadien-2-ol	7.16	ND	6.03	6.23	0.65	0.499
(E)-2-Octen-1-ol	26.9	24.7	24.1	23.1	0.9	0.075
9-Decen-2-ol	9.75	6.32	ND	ND	0.59	0.016
1-Dodecanol	20.5^{a}	19.3^{ab}	18.7^{ab}	16.9^{b}	0.8	0.037
1-Tridecanol	11.9	9.87	10.6	10.6	0.55	0.595
1-Tetradecanol	28.1	27.7	28.7	25.2	1.5	0.345
Non-aromatic hydrocarbons						
2,2,4,6,6-Pentamethyl-heptane	ND	5.1	ND	ND	0.4	
Butyl-cyclopentane	ND	2.2	ND	ND	0.1	
Tridecane	14.6	13.3	15.6	14.3	1.0	0.829
3-Ethyl-2-methyl-1,3-hexadiene	22.2	23.0	23.7	22.9	1.1	0.697
Pentadecane	24.6	22.7	23.5	23.9	1.2	0.946
1- Pentadecene	8.84^{a}	8.60^{a}	8.05^{ab}	7.09^{b}	0.36	0.012
5,5-Dimethyl-1,3-heptadiene	9.14	7.95	7.81	5.43	0.64	0.126
Hexadecane	13.1	11.4	13.0	11.0	0.7	0.187

Table 2 (*continued*)

Volatile compound	0 d	7 d	14 d	21 d	SEM	*P*-value
1-Hexadecene	9.20	7.51	9.52	8.16	0.50	0.468
1-Heptadecene	11.6	10.3	11.5	12.0	0.4	0.099
1,15-Hexadecadiene	23.2	22.0	23.6	23.6	1.0	0.937
Bencenoic compounds						
Toluene	ND	3.54	4.01	3.79	0.14	0.472
Benzaldehyde	67.6^{c}	72.1^{bc}	79.8^{ba}	83.9^{a}	2.5	≤0.001
3-Ethyl-benzaldehyde	115	108	111	102	3	0.387
4-Pentyl-benzaldehyde	25.0	23.4	23.0	21.8	1.0	0.442
Furans						
2-Ethyl-furan	ND	2.69	3.58	ND	0.18	0.022
2-Pentyl-furan	ND	52.3	49.6	48.7	2.5	0.174
Miscelaneous						
Dimethyl trisulphide	32.0	8.90	18.3	10.3	4.73	0.488
Unknown$^{m/z: 97, 55, 41, 71, 84}$	61.5^{b}	87.1^{a}	85.2^{a}	88.2^{a}	3.9	≤0.001
Unknown$^{m/z: 67, 95, 41, 81, 12}$	7.86	7.68	8.00	7.26	0.35	0.736
Unknown$^{m/z: 43, 84, 71, 57, 128}$	16.8	16.2	16.7	16.1	0.8	0.979
Unknwon$^{m/z: 121, 91, 77, 150, 65}$	69.0	67.7	75.6	69.6	3.0	0.560
Unknwon$^{m/z: 95, 81, 43, 55, 67}$	27.0^{a}	25.8^{a}	27.0^{a}	19.6^{b}	1.2	0.032
Unknwon$^{m/z: 43, 41 57, 83, 69}$	15.6	12.7	12.1	12.4	0.7	0.051
Unknown$^{m/z: 55, 43, 69, 83, 97}$	28.6	28.1	27.5	25.2	1.3	0.357
Unknown$^{m/z: 45, 55, 67, 73, 41}$	21.1	21.9	25.4	21.5	1.3	0.172
Total alcohols	372	351	350	319	12	0.218
Total aldehydes	2600	2468	2590	2357	95	0.741
Total benzenoic compounds	207	207	218	211	6	0.646
Total furans	ND	55.0	53.2	48.7	3.0	0.124
Total hydrocarbons	136	134	136	128	6	0.810
Total ketones	94.1^{a}	101^{a}	87.5^{ab}	66.2^{b}	4.1	0.004
Total sulphur compounds	32.0	8.90	18.3	10.3	4.73	0.488
Total volatiles	3442	3325	3453	3141	131	0.702

SEM, standard error of the mean; ND, not detected; ^{a,b,c} Means with different superscripts indicate statistically significant (P ≤ 0.05) differences among ageing days.

Goodridge, Beaudry, Pestka, and Smith (2003) reported that hexanal may bind to chicken meat proteins and, in consequence, reduce its volatility.

The PLS-DA methodology was used to assess from a multivariate approach the effect of the ageing time on the volatile profile of the cooked horse meat. The results of PLS-DA confirmed that the volatile profile of the cooked horse meat was affected by ageing time. Fig. 1 shows the scores calculated for the cooked horse meat samples in the two-dimensional plot formed by the factors of the PLS-DA model with the greatest variance explained for volatile relative abundances and ageing times. Unaged samples were clearly separated from the 14 and 21 d aged samples showing, in general, negative scores on factor 1. Likewise, 21 d aged samples were clearly differentiated from the 7 d aged and unaged samples showing positive score values on factor 1. However, horse meat samples aged for 7 and 14 d were very close to each other and both had positive and negative scores on the horizontal axis (factor 2). Therefore, for the vacuum ageing period studied (21 d), a difference of at least two weeks (14 d) in the ageing time resulted in a differentiated volatile profile in the cooked horse meat samples.

4. Conclusions

For the first time, the volatile profile of up to 21 days vacuum aged and cooked horse meat was reported. Aldehydes, which primarily originate from lipid oxidation, were in general the major contributors of

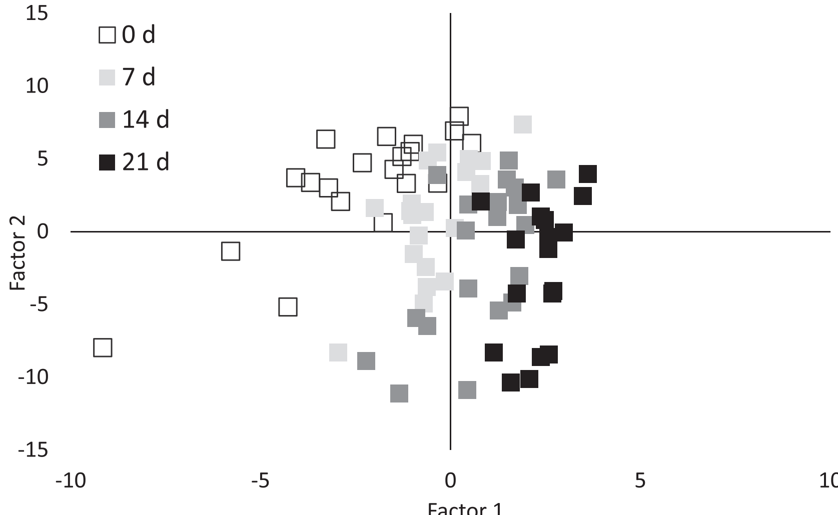

Fig. 1. Partial Least Squares-Discriminant Analysis factor scores depicting cooked horse meat sample distribution according to ageing time (0, 7, 14 and 21 d). *Y*-variable: ageing time; *X*-variable: weighed relative abundance of volatile compounds. Factor 1: explained *X*-variance 18%; explained *Y*-variance 19%. Factor 2: explained *X*-variance 36%; explained *Y*-variance 7%.

cooked horse meat odour in terms of relative abundance and odour impact. In contrast, the contribution of Maillard derived compounds was negligible probably due to cooking conditions utilized.

With ageing time from 0 to 21 days, the abundance of several aldehydes such as hexadecanal, and 2- and 3-methylbutanal increased in the studied cooked horse meat samples, presumably affecting the cooked meat odour as these have considerable odorant impact. Overall, ageing periods longer than 14 days are necessary for significant changes in the volatile profile of cooked horse meat.

The sensory implications that could derive from the odorant impact of volatile compounds should be further studied in order to elucidate their practical repercussion in cooked horse meat quality.

Acknowledgements

Department of Economic Development and Infrastructures of the Basque Government is acknowledged for the doctoral fellowship of L.R. B. Lactiker Research Group (Basque Government IT944-16) funded this work as well as the contract of L.M.

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APPENDIX V

Publication V

Beldarrain, L.R., Sentandreu, E., Aldai, N., Sentandreu, M.A. (2022). Horse meat tenderization in relation to *post-mortem* evolution of the myofibrillar sub-proteome. *Meat Sci.*, 188, 108804.

https://doi.org/10.1016/j.meatsci.2022.108804

ISSN: 0309-1740

IF (2020): 5.209

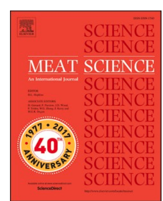
Category: Food Science & Technology (Q1)

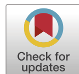
Meat Science 188 (2022) 108804

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci





Horse meat tenderization in relation to *post-mortem* evolution of the myofibrillar sub-proteome

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ARTICLE INFO

Keywords:
Foal meat
Equine
Tenderness
Proteolysis
OFFGEL
Mass spectrometry

ABSTRACT

The ageing process after animal slaughter enhances tenderness and influences the value of meat. Horse meat is becoming more popular but lacks standardized ageing practices that should be supported by a better understanding of *post-mortem* muscle biochemistry. Steaks from *Longissimus Thoracis et Lumborum* (LTL) of eight Hispano-Bretón horses were aged for 0, 7, 14 and 21 days and myofibrillar proteins were resolved by one dimensional gel electrophoresis (1-DE). Ten protein bands were found to change ($p \leq 0.05$) among ageing periods. Most changes were observed between days 0 and 14, suggesting that tenderization occurred primary during the first two weeks. Liquid isoelectric focusing (OFFGEL) technology was applied to better resolve myofibrillar sub-proteome and evidenced fourteen protein bands that changed ($p \leq 0.05$) between 0 and 21 days. Three of them were protein fragments coming from troponins T and I and from creatine kinase. Identified molecules could be further studied as potential markers for horse meat tenderness.

1. Introduction

Over the last decades, the meat industry has applied *post-mortem* ageing practices to improve overall quality of traded meat and meat products (Warren & Kastner, 1992) as it is known that ageing enhances tenderness, aroma/flavour and colour properties of meat (Faustman & Cassens, 1990; Watanabe et al., 2015). Since these attributes exert great influence on the consumer acceptability of meat (Font-i-Furnols & Guerrero, 2014), the study of the ageing process became a major topic for researchers that mainly focused their efforts on texture and sensory evaluations of horse (Ruiz, 2018) and bovine (Moran et al., 2020) meats, as well as the study of the protein degradation of horse (Della Malva et al., 2019), bovine (Laville et al., 2009) and pig (Morzel et al., 2004) meats. Among existing practices, wet ageing is the most popular ageing alternative where meat portions (primals, sub-primals or steaks) are vacuum packaged and stored under refrigeration conditions (Kim et al., 2018). Independently of the strategy considered, the importance of this practice becomes evident since inconsistencies in meat texture ocurring during ageing are known to entail major economic losses (Ramanathan et al., 2020). However, it is necessary to point out that several other factors need to be considered when analysing meat texture. Indeed, irrespective of the ageing process, meat takes a "background toughness" as starting point. The background toughness is determined by both intrinsic (species-specific, breed, sex, age, muscle fibre characteristics, collagen amount) and extrinsic (animal feeding, handling, transport) animal factors (Ferguson & Warner, 2008; Koohmaraie & Geesink, 2006; Lepetit, 2007; Monson, Sañudo, & Sierra, 2005).

Both species-specific (Montowska & Pospiech, 2013) and muscle-specific (Moran et al., 2020) tenderization processes take place during ageing, which happen at different rates depending on genetic traits and individual experience of animals (Lana & Zolla, 2016; Lonergan, Zhang, & Lonergan, 2010). According to some authors, tenderization starts after animal death, sparking apoptosis followed by a multi-enzymatic cascade reactions causing degradation of proteins (Ouali et al., 2013). According to this, meat would be tenderized by the action of different proteases, mainly caspases, calpains, cathepsins and proteasome (Lana & Zolla, 2016; Sentandreu, Coulis, & Ouali, 2002). Understanding of protein degradation occurring in meat during ageing is conventionally approached through the study of the myofibrillar sub-proteome merging two dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) analysis (horse, Della Malva et al., 2019; bovine, Laville et al., 2009; pig, Morzel et al., 2004). Recently, liquid isoelectric focusing (OFFGEL) coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis has been successfully implemented as a reliable alternative

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https://doi.org/10.1016/j.meatsci.2022.108804
Received 13 January 2022; Received in revised form 7 March 2022; Accepted 8 March 2022
Available online 13 March 2022
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to study the myofibrillar sub-proteome in meat research (Beldarrain et al., 2018; Fuente-Garcia, Sentandreu, Aldai, Oliván, & Sentandreu, 2020). In any case, understanding of *post-mortem* myofibrillar protein degradation is essential to develop efficient ageing strategies (Kim et al., 2018). In this line, the search of protein biomarkers related to meat tenderness at early *post-mortem* period can help to predict meat quality as demonstrated in beef studies (Gagaoua et al., 2021; Ouali et al., 2013).

In the particular case of horse meat, there are no standardized ageing practices established yet. However, several studies addressed the effect of ageing time on instrumental texture (Beldarrain et al., 2021; Gomez & Lorenzo, 2012; Lorenzo & Gomez, 2012; Ruiz, 2018; Seong et al., 2016) and sensory properties (Beldarrain et al., 2020; Beldarrain, Moran, Sentandreu, Barron, & Aldai, 2022; Ruiz et al., 2019) of horse meat. Up to date only the studies of Della Malva et al. (2019, 2021) have approached this topic from a proteomic perspective. They studied meat from Heavy Draft horses aged up to two weeks using 2-DE/MS analysis. In this sense, the implementation of innovative proteomic methodologies to other breeds could imply further advancement in horse meat quality research, responding to current market demands in terms of increased horse meat consumption (Belaunzaran et al., 2015, 2017) and associated environmental benefits (Insausti et al., 2021).

The objective of this work was to study the evolution of myofibrillar sub-proteome of Hispano-Bretón horse meat during three weeks of ageing process. OFFGEL was used, for the first time, as an alternative to traditional 2-DE for protein fractionation of horse meat samples collected at different ageing times. Protein bands were further characterized by LC-MS/MS analysis to unveil changes in the myofibrillar proteome over time. The proposed proteomic approach could contribute to the development of optimized ageing practices by the horse meat industry and may favour the creation of new insights into the biochemistry of *post-mortem* horse muscle.

2. Materials and methods

2.1. Solvents and chemicals

Tris (tris-hydroxymethyl]-aminomethane), glycerol, urea, thiourea, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), bromophenol blue and LC-MS grade solvents acetonitrile (ACN) and formic acid (FoA) were from Scharlab (Scharlab S.L., Barcelona, Spain). Water was of ultrapure grade from Millipore (EMD Millipore Co., Billerica, MA, USA). Sucrose, CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) hydrate, protease inhibitor cocktail (P8340), sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), Coomassie Brilliant Blue G-250 and ammonium bicarbonate were from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Bradford Protein Assay Kit and 30% Acrylamide/Bis solution were from Bio-Rad (Bio-Rad, Hercules, CA, USA). Modified trypsin was from Promega (Promega, Madison, WI, USA).

2.2. Experimental design: Animal handling and sampling

Eight Hispano-Bretón horses (four females and four males), a well-stablished breed in northern Spain, were reared commercially under grazing conditions while suckling their mothers from birth until weaning (6–8 months). This breed is part of the genetic heritage and its conservation is key to maintain mountain ecosystems. Animals continued grazing (11–13 months of age) until they were moved to a feedlot and were finished on concentrate and straw *ad libitum*. Horses were slaughtered at 15–17 months of age in a commercial abattoir (average carcass weight of 246 ± 14.0 kg) following European Union regulations (Council regulation (EU), 2009). Two horses (female and male) were slaughtered per week during four consecutive weeks. After 48 h *post-mortem* at 4 °C (day 0), the whole right rib joint was removed from carcasses (n = 8) and transported to the laboratory under refrigerated conditions. *Longissimus thoracis et lumborum* (LTL) muscle was excised. After trimming adipose and connective tissues of the muscle, four 1.5 cm thick steaks (n = 32) were cut from it, starting from the 5th rib. Average pH value at day 0, measured using a portable pH meter (HI99163, Hanna Instruments, Smithfield, RI, USA) equipped with a penetrating glass electrode (FC232D, Hanna Instruments, USA), was 5.60 ± 0.09, meaning that a normal pH drop was assessed for all samples. Samples were vacuum packed (99%) using a EVTGI-450 vacuum packing machine (Irimar, Navarre, Spain) in polyethylene bags (120 μm and and oxygen permeability of 1 cc/m^2/day at 23 °C; Merkapack, Vitoria, Spain), randomly assigned to an ageing time of 0, 7, 14 and 21 days (d), and kept in a refrigerated room (4.0 ± 1.0 °C) without illumination. After reaching the corresponding ageing period, steaks were stored at −80 °C until analyzed.

The aforementioned procedure was considered as a full-randomized block design with slaughter day as a blocking factor and ageing time as split plot factor, where ageing levels were randomly allocated to different individual steaks obtained from each LTL. The experimental unit (LTL muscle) was considered as a plot and the steaks were the subplots (sampling units) in which ageing time (factor) was assessed. Animal sex and carcass weight were distorting variation sources controlled by the experimental design in order to minimize the residual variation.

2.3. Extraction of myofibrillar proteins

Protein extraction was carried out as described in Fuente-Garcia et al. (2020). Briefly, a representative sample of 10 g of meat was retrieved from the center of the steak, from which half a gram was cut in small cubes and weighted; then homogenized in 5 mL extraction buffer (10 mM Tris pH 7.6, 1 mM EDTA, 0.25 M sucrose) containing 25 μL of protease inhibitors cocktail using an Ultra-Turrax Yellow Line Di 25 (IKA-Werke, Staufen, Germany). The homogenate was centrifuged at 20000*g* for 20 min at 4 °C, the supernatant discarded and the precipitate was washed and centrifuged again. The resultant pellet was dissolved in 10 mM Tris buffer, pH 7.6, containing 7 M urea, 2 M thiourea and 2% CHAPS and centrifuged again. The supernatant (myofibrillar extract) was filtered through glass wool and total protein content was determined in triplicate according to Bradford assay using a Bio Rad commercial kit.

2.4. Protein OFFGEL fractionation

Myofibrillar extracts from non-aged and 21 d aged meat samples (n = 16) were fractionated according to their isoelectric point (pI) along 12 liquid fractions using 13 cm IPG strips with a linear gradient in the pH range 3–10 (GE healthcare, Uppsala, Sweden). A total of 3 mg protein per sample were fractionated in a Agilent 3100 OFFGEL fractionator (Agilent Technologies, Palo Alto, CA, USA) at a constant electric current of 50 mA for about 20 h. OFFGEL fractions were individually collected and stored at −20 °C until further analysis.

2.5. SDS-PAGE analysis

Sodium Dodecyl Sulphate-Polyacrilamide Gel Electrophoresis (SDS-PAGE) was used to resolve total myofibrillar extracts from 0, 7, 14 and 21 d aged meats (n = 32) in 12% polyacrylamide gels. In addition, OFFGEL fractions from non-aged and 21 d aged samples were resolved in 5–16% polyacrilamide gradient gels, previously cast using a Hoefer SG-100 gradient maker (Hoefer, San Francisco, CA, USA).

For both, total myofibrillar extracts and individual OFFGEL fractions, protein samples were gel loaded after being mixed (50:50, *v*/*v*) with a sample buffer solution (0.88 M Tris-HCl pH 6.8, 10% v/v glycerol, 2% *w*/*v* SDS, 0.2 M DTT and 0.04% bromophenol blue) and heated at 95 °C for 4 min to denature proteins. Two gels (1.5 mm × 8 cm × 9 cm) were simultaneously run in a Mighty Small II SE260 electrophoresis unit

(Hoefer, San Francisco, CA, USA) at a constant current of 50 mA. Subsequently, gels were fixed into 12% TCA for 1 h, washed twice with bidistilled water and stained overnight with colloidal Blue Coomassie (Candiano et al., 2015). Gels were destained with bidistilled water and digitalized using an Amersham ImageQuant 800 (GE healthcare, Uppsala, Sweden) biomolecular imager. Analyses were carried out in duplicate and samples were randomly assayed.

2.6. Image analysis

Gel images were processed and analyzed using ImageQuant TL 8.2 software (GE healthcare, Uppsala, Sweden). Intensity of bands was quantitatively determined using 1-DE gel analysis after rolling ball background subtraction with the radius set at 200 pixels. For an accurate correction of images, individual band intensities were normalized with respect to the total band volume per lane (for total myofibrillar extracts) or per gel (for individual OFFGEL fractions). Molecular weight of protein bands was determined by the software comparing migration results of sample proteins with those from the commercial Bio-Rad 1610317 broad-range molecular mass protein standards (Bio-Rad, Hercules, CA, USA).

2.7. Statistical analysis

Analyses were conducted using IBM-SPSS Statistics Software (v. 26.0, IBM, Armonk, NY, USA). Relative band intensities from image analysis were log transformed after checking normality and homoscedasticity. The General Linear Model (GLM) of ANOVA was considered to determine significant differences in the relative abundance of selected bands among ageing periods assayed: 0, 7, 14 and 21 d (total myofibrillar extracts) or between 0 and 21 d of ageing (individual OFFGEL fractions). The GLM included ageing time and the controlled distorting variation factor animal sex as fixed effects, and carcass weight as covariate. Slaughter day was also included as a random effect in the model (it was a simultaneous distorting factor of uncontrolled variation coming from at least individual animal, feeding, transport or slaughter conditions). Moreover, GLM included binary interactions ageing time*sex and ageing time*slaughter day. Fisher's Least Significance Difference test of estimated marginal means was used for pairwise comparisons among ageing time levels 0, 7, 14 and 21 d. Three significant figures were used to express the data and significance was declared at $p \leq 0.05$.

2.8. In-gel trypsin digestion of proteins bands

Protein bands showing significant differences among ageing times were excised from gels and digested with trypsin for further protein identification. Excised bands were cut into pieces and washed with 50 mM ammonium bicarbonate, then dehydrated with ACN and the remaining liquid removed using a Speed-Vac concentrator. Gel pieces were digested overnight at 37 °C with 15 μL of a 12.5 μg/mL trypsin solution in 50 mM ammonium bicarbonate (pH 8.5). After incubation, the liquid was transferred into a clean Eppendorf tube and the remaining peptides were recovered by adding ACN/0.1% TFA (50:50). This supernatant was combined with the previous one and the liquid phase was evaporated in a Speed-Vac. Samples were then acidified with 0.1% FoA and transferred into glass vials.

2.9. Peptide sequence identification by LC-MS/MS analysis

Tryptic digests were analyzed by liquid chromatography featured by a Thermo Surveyor Plus system (Thermo Sci., San Jose, CA, USA) with a quaternary pump, vacuum degasser and refrigerated autosampler coupled to a Thermo LCQ Advantage (Thermo Sci., San Jose, CA, USA) ion trap mass analyzer loading an electrospray ionization (ESI) probe operating in positive mode. Separation of peptides was performed through a 150 mm × 2.1 mm, 3 μm particle-size Luna Omega PS C18 column (Phenomenex Inc., Torrance, CA; USA) under the following separation conditions: solvent A, water/FoA (99.9:0.1); solvent B, ACN/FoA (99.9:0.1); separation gradient, initially 0% B, held for 2 min, linear 0–80% B in 23 min, 95% B in 0.1 min, held for 4.9 min for washing, 0% B in 0.1 min, and column equilibration for 14.9 min; total run time, 45 min; flow rate, 200 μL/min; injection volume, 25 μL. Column flow was conducted into the MS system during the 1–40 min time range diverting the rest of running time to waste. Autosampler and column temperatures were set at 10 and 23 °C, respectively.

Instrumental MS settings considered were: capillary temperature, 300 °C; normalized collision-induced dissociation (CID) energy for MS/MS analysis, 35%; spray voltage, 4.0 kV; capillary voltage, 42.0 V; seath gas, 50 (arbitrary units); auxiliary gas, 20 (arbitrary units). The analysis combined two scan events: Full-MS analysis (scan event 1) of intact peptide masses in the 400–2000 m/z range followed by a data-dependent MS/MS (dd-MS^2, scan event 2) experiment of most intense ions from event 1 considering +1 to +4 charges with the following dynamic parameters: minimum MS/MS ion intensity threshold, 3×10^5; exclusion list, 25 masses including those from background provided by a blank injection; exclusion time, 3 min; exclusion mass width, 3 amu; repeat count for MS/MS of most intense ion, 2; repeat count duration, 0.3 min. Number of micro scans-maximum injection time was 1–200 ms for both scan events assayed. Control of the LC-MS system was featured by a PC loading Thermo Xcalibur v2.04 software (Thermo Scientific, San Jose, CA, USA).

Protein identification was carried out interrogating dd-MS^2 data against NCBIprot protein database using Mascot v2.7 search engine with the following settings: enzyme, trypsin; no fixed, or variable modifications but "Error tolerant" option enabled; mass accuracy set to 1.2 and 0.8 Da for MS^1 and MS/MS analyses, respectively; the option "Mammalia" was selected as taxonomy restriction parameter. Estimation of false positive rates by means of false discovery rate threshold 1% was achieved through the activation of the "Decoy" option. Only those assignments with high individual ion scores indicating identity on extensive homology were considered as reliable results ($p < 0.05$).

2.10. Functional and interaction analysis of differentially abundant proteins between non-aged and 21 d aged meats revealed by OFFGEL fractionation

A functional annotation of differential proteins found in OFFGEL fractions was performed using Gene Ontology (GO) slim terms through the AmiGO website (http://amigo.geneontology.org/amigo/). Protein networks were explored using STRING v.11.5 database (ELIXIR, Cambridgeshire, UK, https://string-db.org), selecting "*Equus caballus*" as target organism for interrogations, and they were constructed with a minimum interaction score confidence of 0.4 and four criteria for possible linkage: co-ocurrence, experimental evidences, existing databases and text mining.

3. Results and discussion

3.1. SDS-PAGE of myofibrillar extracts from 0, 7, 14 and 21 d aged horse meat

The myofibrillar protein distribution achieved in 12% SDS-PAGE gels from 0, 7, 14 and 21 d aged horse meat samples is illustrated in Fig. 1. Ten protein bands (A-J) were significantly affected by ageing time, and their LC-MS/MS identifications and relative image abundances are shown in Table 1. It should be noted that most of selected bands had more than one identified protein as consequence of limitations of 1-DE for resolving complex protein mixtures (Rabilloud et al., 2009). Still, tandem-MS allowed the simultaneous identification of those co-eluting proteins. Therefore, although in most cases there was not enough evidence to associate the observed patterns to a single protein, 1-DE enabled a visual exploration of changes occurring during the ageing

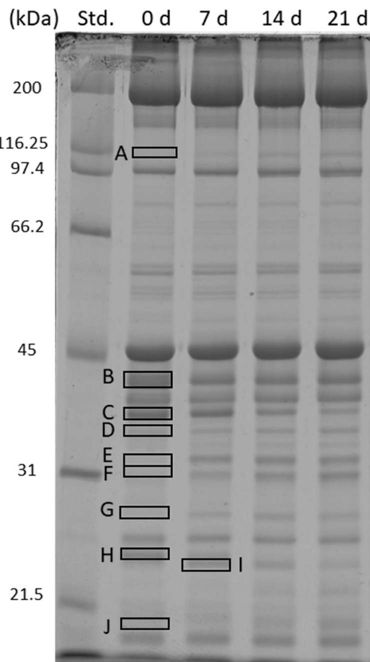

Fig. 1. 12% SDS-PAGE of myofibrillar extracts from 0, 7, 14 and 21 days aged horse meat samples. Std: Commercial molecular mass protein standard.

period, highlighting those proteins that were potentially involved. Furthermore, no significant effect of the interactions ageing time*sex or ageing time*slaughter day were found.

Theoretically, only structural proteins should be present in the myofibrillar sub-proteome, but previous studies performed in beef have demonstrated that changes in protein solubility may result in their different extractability patterns (Feng et al., 2020). This means that highly soluble proteins may appear in the myofibrillar fraction specially during the first days (0–5) of ageing, being this phenomenom attributed to *post-mortem* pH alterations, among others (Laville et al., 2009). In this regard, proteins related to energy metabolism, cellular processes or stress were also identified and included in the discussion, since although they are not targets in muscle architecture degradation, they could act as sentinels of horse meat ageing.

Most of changes in protein abundance were found in proteins with molecular weights below 45 kDa (Bands B-J, Fig. 1), as already observed by Della Malva et al. (2019) and Laville et al. (2009) in *post-mortem* proteomes of horse and bovine meats, respectively. The only exception was myosin-binding protein 1 fragment (band A; 115 kDa), its abundance increased by ageing (Table 1). This protein is one of the accessory components that regulates the rate, force and timing of muscle contraction in the cyclic interaction of actin and myosin (McNamara & Sadayappan, 2018) and achieving a high level of phosphorylation in *post-mortem* muscle compared to other structural proteins (Ren et al., 2019). The latter authors also reported that phosphorylation could be translated into a slower protein degradation as a way to protect muscle structure. To our knowledge, the increasing abundance of this protein fragment over *post-mortem* time has not been reported before. Indeed, detailed action mechanism and properties of this protein remain poorly characterized (McNamara & Sadayappan, 2018).

A single protein was identified in band I: myosin light chain 1/3. Its abundance decreased after two weeks of ageing and, then, remained constant. This is in accordance with what Anderson, Lonergan, and Lonergan (2012) hypothesized - myosin light chain 1/3 is released from the myofibrillar to the soluble fraction as consequence of *post-mortem* proteolysis, thus acting as an indicator of such a phenomenon. The same conclusion was reported by Della Malva et al. (2019) in meat from Italian Heavy Draft Horses after 14 d of ageing, clearly evidencing the usefulness of myosin light chain 1/3 as an indicator of horse meat proteolysis. Moreover, it could be also considered as a potential protein biomarker of meat tenderness as suggested by Gagaoua et al. (2021) in beef.

Among the remaining eight bands, some with increased abundance throughout ageing time (bands E, F and G) consisted of protein fragments generated by proteolytic activity. In such cases, proteins were correctly identified but showed a remarkable inconsistency between the theoretical and apparent molecular weights. Overall, from the selected bands (A-J, Fig. 1), five showed statistically significant differences between 0 and 7 days (bands B, E, F, G and H) and four of them between 7 and 14 days (bands C, H, I and J). However, none of the protein bands considered showed statistically significant differences between 14 and 21 days (Table 1). This is in line with changes of instrumental texture of horse meat recently observed by Beldarrain et al. (2021) in samples from the same study. Authors evidenced that 7 d aged steaks were significantly more tender than non-aged meats considering Warner-Bratzler shear force results achieved, while similar results were observed for 7 and 14 d aged steaks. In this sense, it was reported that the third week of ageing (21 d) did not significantly enhance meat tenderness, suggesting that horse meat tenderization mainly occurs during the first two weeks of ageing. Those findings were corroborated by the results of the present study considering the behaviour exhibited by bands A-J between 14 and 21 d of ageing.

3.2. SDS-PAGE of individual OFFGEL fractions from non-aged and 21 d aged horse meat

To overcome drawbacks caused by the limited resolving power of 1-DE and with the aim to analyse individual protein changes, OFFGEL fractionation was incorporated as a preliminary protein fractionation step coupled to SDS-PAGE in the analysis of non-aged and 21 d aged samples. These extreme ageing periods were chosen since the highest differences in protein abundance were observed when analysing total myofibrillar extracts by 1-DE. Since most protein bands showing significant changes in 1-DE analysis had molecular weights below 45 kDa, 5–16% polyacrylamide gradient gels were employed to analyse OFFGEL fractions, as they were the gels showing the highest resolving power for the aforementioned molecular weight range (data not shown).

Fig. 2 illustrates SDS-PAGE gels from OFFGEL fractions of non-aged (**2.a**) and 21 (**2.b**) d aged horse meat whereas Fig. 3 reports the relative quantification of signals, revealing the presence of 14 protein bands (A-N, **Figs. 2** and **3**) with significant abundance differences. No significant effect of the interactions ageing time*sex or ageing time*slaughter day were found. Table 2 shows LC-MS/MS identification of proteins from A-N bands, distributed through the 12 lanes depending on their pIs. It must be highlighted the feasibility of proteins to be isolated in two adjacent OFFGEL fractions since they were initially focused according to their pI, thus appearing at the same position in contiguous channels of SDS-PAGE gels (bands A, B, C and M, Fig. 2). In such cases, quantitative analysis merged individual measures from different bands corresponding to the same protein in a one single value (*i.e.*, A_1 and A_2 from Fig. 2**.a** and Table 2 is A in Fig. 3).

As expected, results from hyphenation of OFFGEL with SDS-PAGE fractionation favored the obtaining of protein bands with isolated species in most cases (Table 1 *vs* Table 2). Thus, discussion about functional analysis and protein interaction network of horse meat proteome elucidated will be mainly carried out according to qualitative (Table 2) and quantitative (Fig. 3) results.

3.3. Functional analysis of proteins

3.3.1. Structural and muscle contraction related proteins

Abundance of myosin light chain 1/3 (bands A_1 and A_2) was lower in meat aged for 21 d than in non-aged samples (Fig. 3) because of its *post-mortem* release from the myofibrillar to the soluble fraction (Anderson

Table 1
LC-MS/MS identification and relative image abundance (in arbitrary units) of proteins found in A-J bands depicted in Fig. 1 from total myofibrillar extracts of 0, 7, 14 and 21 days aged horse meat samples.

Band[1]	Protein identification [GENE][2]	Theoretical[2]/ Apparent[3] Mr (kDa)	Access Number[2]	Mascot score	Protein coverage	Mean 0 d[4]	Mean 7 d[4]	Mean 14 d[4]	Mean 21 d[4]	SEM	*P value*
A	Myosin-binding protein C1 slow type, [MYBPC1] **fragment**	129.06/115	XP_023487581.1	701	26	148^{b}	332^{ab}	614^{ab}	822^{a}	81	0.007
B	Tropomyosin 2, [TPM2]	39.43/41.38	XP_003362760.1	945	76%	10944^{a}	7461^{b}	7466^{b}	8261^{ab}	409	0.004
	Fructose-bisphosphate aldolase A, [ALDOA]	32.82/41.38	XP_003364171.1	391	58%						
C	Glyceraldehyde-3-phosphate dehydrogenase, [GAPDH]	35.80/ 36.90	NP_001157328.1	187	36%	6936^{a}	5261^{a}	2912^{b}	1765^{b}	391	<0.001
	F-actin-capping protein subunit alpha-2-like protein, [CAPZA2]	33.30/36.90	AEB61386.1	79	6%						
D	L-lactate dehydrogenase A chain, [LDHA]	39.69/ 34.72	XP_023499905.1	267	34%	1015^{c}	1340^{bc}	1851^{ab}	2096^{a}	109	<0.001
	Malate dehydrogenase mitochondrial, [MDH2]	35.59/34.72	NP_001182455.1	112	18%						
E	Troponin T fast skeletal muscle, [TNNT3] **fragment**	31.52/31.40	XP_023510451.1	236	42%	421^{b}	4131^{a}	4286^{a}	3864^{a}	248	<0.001
	Voltage-dependent anion-selective channel protein 3, [VDAC3]	30.69/31.40	NP_001296239.1	128	19%						
	ATP synthase subunit gamma mitochondrial, [ATP5F1C]	32.84/31.40	XP_001499911.3	90	25%						
F	Carbonic anhydrase 3, [CA3]	30.37/29.75	NP_001157426.1	183	30%	507^{c}	2965^{b}	3294^{ab}	4006^{a}	196	<0.001
	Troponin T fast skeletal muscle, [TNNT3] **fragment**	31.52/29.75	XP_001502572.1	177	10%						
	Phosphoglycerate mutase 2, [PGAM2]	28.60/29.75	XP_001495686.1	89	17%						
G	Myosin light chain 3, [MYL3]	22.30/26.08	XP_001500321.1	281	55%	558^{b}	1504^{a}	1824^{a}	1732^{a}	100	<0.001
	Glyceraldehyde-3-phosphate dehydrogenase, [GAPDH] **fragment**	35.80/26.08	NP_001157328.1	123	24%						
H	Troponin I fast skeletal muscle, [TNNI2]	21.38/22.54	XP_014685731.1	87	18%	4185^{a}	275^{b}	ND	ND	291	
	ATP synthase subunit O, mitocondrial, [ATP5PO]	24.14/22.54	XP_001497214.1	69	16%						
I	Myosin light chain 1/3 skeletal muscle, [MYL1]	21.11/21.94	XP_008542802.1	495	79%	2957^{a}	4823^{a}	1732^{b}	1624^{b}	257	<0.001
J	Myosin regulatory light chain 2 skeletal muscle, [MYLPF]	19.03/18.12	XP_001496245.2	218	40%	596^{b}	810^{b}	1641^{a}	2296^{a}	135	<0.001
	Troponin I fast skeletal muscle, [TNNI2]	21.38/18.12	XP_014685731.1	46	12%						

[SEM] Standard error of the mean.
[1] Colour indicates changes in the relative protein band quantification by ageing (green, increase; red, decrease).
[2] Protein, gene identification, theoretical Mr and accession number were from NCBIprot database interrogation (detailed in section 2.9). All of the identification correspond to *Equus caballus* or *Equus przewalskii*.
[3] Apparent Mr was calculated through band position in the gel using ImageQuant TL analysis.
[4] Means with different superscripts indicate statistically significant differences by Tukey's test ($p<0.05$).
[5] Results were estimated using the General Linear Model from ANOVA.

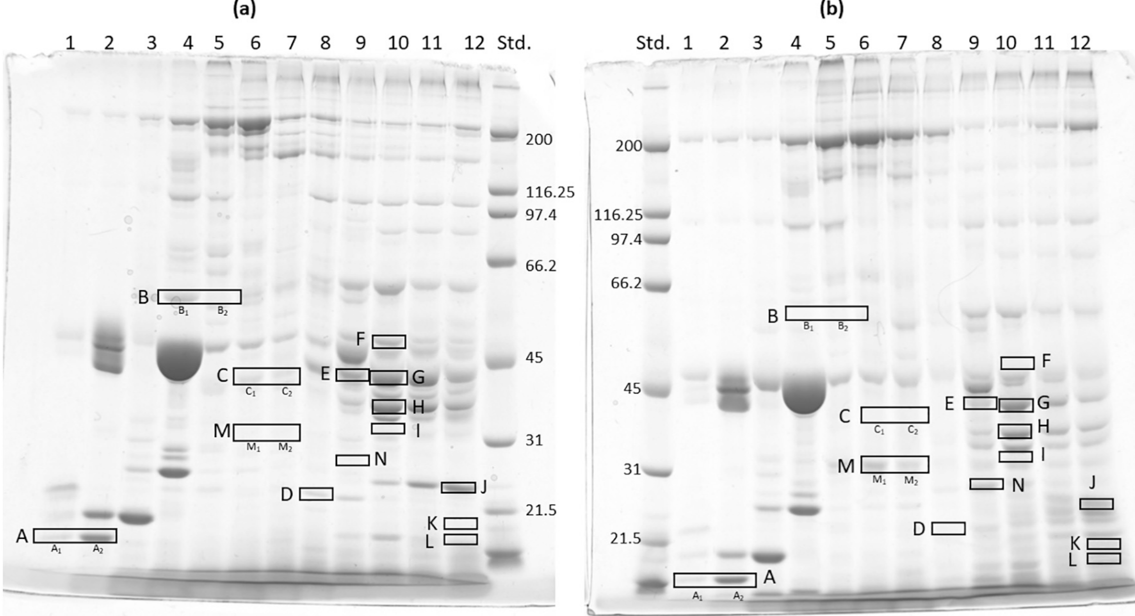

Fig. 2. 5–16% gradient SDS-PAGE gels of the 12 OFFGEL fractions from myofibrillar extracts of unaged **(a)** and 21 d aged **(b)** horse meat samples along the 3–10 pH range assayed. Std: Commercial molecular mass standard.

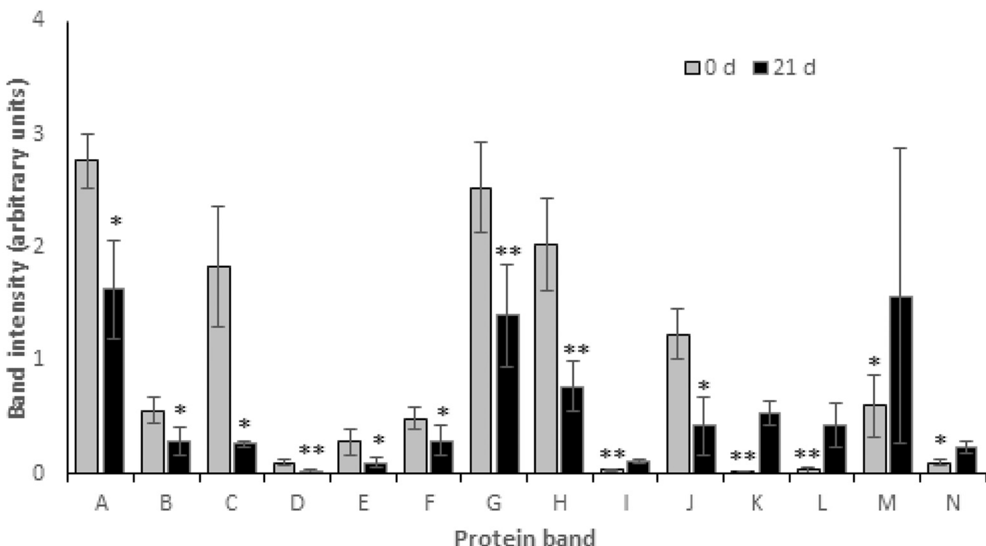

Fig. 3. Quantification by gel image analysis of SDS-PAGE protein bands A-N obtained after OFFGEL fractionation (Fig. 2; Table 2) corresponding to unaged (■) and 21 d aged (■) horse meat samples. Error bars indicate the standard deviation of the means for each group. **p* < 0.05; ***P* ≤ 0.01. Identification of protein bands detailed in Table 2.

et al., 2012). Similarly, abundance of tubulin alpha-4a chain (bands B_1 and B_2), constituent of microtubules and by extension, of centrosome, decreased by ageing (Fig. 3). It plays important role in cellular processes as intracellular tracking, cell division and maintenance of cellular architecture (Becker, Leone, & Engel, 2020). This protein has already been linked to tenderization in beef (Huang et al., 2020) and has a muscle-specific behaviour (Picard et al., 2018). According to present results, it may also be linked to tenderization in horse meat (Fig. 3).

Bands C_1 and C_2 also decreased with ageing. They were identified as troponin T, the tropomyosin-binding subunit of the troponin complex that is constituent of myofibrils. In addition, its degradation product of about 31 kDa was identified in bands M_1 and M_2 (Table 2). As expected, these increased remarkably as the ageing period advanced (Fig. 3). It has been recognized during years that the degradation of troponin T releasing polypeptides migrating at approximately 30 kDa are strongly related to beef (Lonergan et al., 2010) and horse meat (Della Malva et al., 2019) tenderization, as also observed in the present study (Fig. 2). Therefore, it is reasonable to think that troponin T, defined as good biomarker of tenderness in beef (Lana & Zolla, 2016), could also be investigated as an interesting descriptor of horse meat tenderness.

Troponin I has also been proposed, by several authors, as a biomarker of beef tenderness, although to a lesser extent than T subunit (Gagaoua et al., 2021). In this study, troponin I was identified in band J, and two fragments around 19 and 16 kDa were identified in bands K and L, respectively (Table 2). Again, abundance of intact troponin I decreased during ageing (Fig. 3) while its related fragments noticeably increased. Same evolution of intact troponin I during ageing with the inherent increase of the 19 kDa fragment was previously observed by

Table 2
LC-MS/MS protein identification of A-N protein bands from Fig. 2 obtained after OFFGEL fractionation of myofibrillar extracts at 0 and 21 days of ageing.

Band	Protein identification/[GENE[a]]	Theoretical[a]/ apparent[b] Mr (kDa)	Theoretical/ Apparent pI	Access number[a]	Mascot score	Protein coverage	Biological process[c]
A_1	Myosin light chain 1/3 skeletal muscle, [MYL1]	16.68/17	4.62/ (3.59–4.08)	XP_008542803.1	106	47%	Structure
A_2	Myosin light chain 1/3 skeletal muscle, [MYL1]	16.67/17	4.62/ (4.08–4.56)	XP_008542803.1	71	9%	Structure
B_1	Tubulin alpha-4A chain, [TUBB4A], *Bos mutus*	49.55/63	4.78/(5.05–5.53)	XP_001491960.2	52	9%	Structure
B_2	Tubulin alpha-4A chain, [TUBB4A], *B. mutus*	49.55/63	4.78/(5.53–6.02)	XP_001491960.2	102	12%	Structure
C_1	Troponin T fast skeletal, [TNNT3]	37.67/38	6.13/(6.02–6.50)	XP_014685676.1	93	10%	Muscle contraction
C_2	Troponin T fast skeletal, [TNNT3]	37.67/38	6.13 /(6.50–6.98)	XP_014685676.1	59	9%	Muscle contraction
D	Alpha-crystalin B chain, [CRYAB], *B. mutus*	20.02/23	6.76/(6.98–7.46)	XP_001501829.1	89	18%	Heat stress chaperones
E	Creatine kinase M type, [CKM]	43.16/43	6.79/(7.46–7.94)	XP_0015025572.1	174	17%	Energy metabolism
F	Beta enolase, [ENO3]	47.02/47	8.05/(7.94–8.42)	NP_001254531.1	139	18%	Energy metabolism
G	Fructose-bisphosphate aldolase A chain, [ALDOA]	39.43/42	8.30/(7.94–8.42)	XP_003362760.1	120	13%	Energy metabolism
	Glyceraldehyde-3-phosphate dehydrogenase, [GAPDH]	35.80/42	8.22/(7.94–8.42)	NP_001157328.1	66	11%	Energy metabolism
H	Glyceraldehyde-3-phosphate dehydrogenase, [GAPDH]	35.80/37	8.22/(7.94–8.42)	NP_001157328.1	83	12%	Energy metabolism
I	L-lactate dehydrogenase A chain, [LDHA]	36.57/32	8.17/(7.94–8.42)	NP_001138352.1	125	18%	Energy metabolism
J	Troponin I fast skeletal muscle, [TNNI2]	21.38/23	8.86/(8.9–9.38)	XP_014685731.1	141	9%	Muscle contraction
K	Troponin I fast skeletal muscle, [TNNI2] **fragment**	21.38/19	8.86/(8.9–9.38)	XP_014685731.1	147	9%	Muscle contraction
L	Troponin I fast skeletal muscle, [TNNI2] **fragment**	21.38/16	8.86/(8.9–9.38)	XP_014685731.1	128	17%	Muscle contraction
M_1	Troponin T fast skeletal, [TNNT3] **fragment**	37.67/31	6.13/(6.02–6.50)	XP_0145585229.2	66	11%	Muscle contraction
M_2	Troponin T fast skeletal, [TNNT3] **fragment**	37.67/31	6.13 /(6.50–6.98)	XP_0145585229.2	74	11%	Muscle contraction
N	Creatine kinase M type, [CKM] **fragment**	43.16/27	6.79/(7.46–7.94)	XP_0015025572.1	98	9%	Energy metabolism

[a] Protein and gene identification and theoretical Mr. were from NCBInr database interrogation. All of the identification correspond to *Equus caballus* or *Equus przewalskii* unless otherwise indicated.
[b] Apparent Mr. was calculated through band position in the gel using ImageQuant TL.
[c] Proteins were categorized according to their biological pathways using Gene Ontology (GO) slim terms.

Sierra et al. (2012) in beef, suggesting that degradation of troponin I in the first 24 h *post-mortem* could be indicative of the tenderization rate in beef. In our case, further research is necessary to corroborate this assumption in horse meat.

3.3.2. Chaperones

Band D comprised alpha-crystalin B chain, a small Heat Shock protein (HSP) that binds to myofibrils and protects skeletal muscle from protein degradation of protein complexes. HSPs are produced after animal bleeding as a mechanism to preserve cell functions (Ouali et al., 2013), but decreasing abundance of these particular proteins has been described by Ma and Kim (2020) during beef ageing process. Same authors suggested that such decrease could be related to the evolution of tenderness, claiming that small HSPs have no longer the ability to prevent damage of muscle structure (Cramer, Penick, Waddell, Bidwell, & Kim, 2018). In the present study, we found that alpha-crystalin B chain was less abundant at 21 d of ageing compared to non-aged horse meat (Fig. 3).

3.3.3. Proteins related to energy metabolism

Creatine kinase is one of the most cited tenderness biomarker in the literature, especially in LTL muscle (Gagaoua et al., 2021). In our case, this protein was identified in band E. This enzyme is involved in the initial *post-mortem* energy metabolism before glycolysis. It has been reported that it is progressively degraded during meat ageing by calpains (Purintrapiban, Wang, & Forsberg, 2001) and cathepsins (Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004) until its complete inactivation. This seemed to be the case in *post-mortem* horse muscle since abundance of band E decreased over ageing, while an important increase was observed for the 27 kDa creatine kinase fragment (band N in Table 2 and Fig. 3).

Abundance of bands F, G and H decreased from non-aged to 21 d aged horse meat samples, being identified as the glycolytic enzymes beta-enolase, fructose-bisphosphate aldolase A, and glyceraldehyde-3-phosphate dehydrogenase, respectively. The decreasing trend of these enzymes has been previously observed after 14 d of ageing of horse meat (Della Malva et al., 2021). Glycolysis is an important biochemical pathway affecting *post-mortem* meat quality since different metabolic kinetics and degrees of glycolysis can modify lactic acid accumulation rates and, in consequence, pH decline affecting the activity of proteolytic enzymes (Ferguson & Gerrard, 2014). It seems clear that *post-mortem* evolution of glycolytic enzymes may affect meat tenderization. However, since they are not an intrinsic component of the muscle structure, their connection to tenderness is not direct, giving rise to inconsistent results in the literature (Marino et al., 2014).

According to Ouali et al. (2013), immediately after entering into the anoxia state, the concentration of glycolytic enzymes increases as a mechanism to provide energy, but later on, they have been reported to be target of proteolytic degradation. As an example, in beef, *post-mortem* degradation of beta-enolase, fructose-bisphosphate aldolase A, and glyceraldehyde-3-phosphate dehydrogenase have been reported (Laville et al., 2009; Marino et al., 2014). The decrease in abundance of these proteins could also indicate the slowdown of glycolytic activities or further changes in their solubility during the ageing process. In relation to this, it has been recently suggested that the *post-mortem* oxidative damage of glycolytic enzymes greatly affects meat tenderization rate (Malheiros et al., 2019).

Band I also was identified as a glycolytic enzyme, lactate dehydrogenase. However, its abundance increased after three weeks of ageing. This enzyme catalyses the last step of glycolysis, and has been studied with particular interest during the ageing process, due to its role in *post-mortem* meat colour stability by regulating the generation of NADH, that reduces brown metmyoglobin (Kim et al., 2009). The higher abundance of lactate dehydrogenase A chain in 21 d aged horse meat may reflect a higher lactate dehydrogenase activity or, as mentioned for the other glycolytic enzymes, a loss in solubility of this protein as the ageing process advances. Further research is required to understand the relation of glycolytic enzymes with meat tenderization in *post-mortem* muscle.

3.4. Protein-protein interactions of differentially abundant proteins

Considering proteins that significantly changed in abundance between non-aged and 21 d of ageing (Fig. 3), a horse meat interaction network was constructed to unveil the interconnection of the biological pathways involved (Fig. 4). Proteins were mainly clustered into two categories: proteins involved in energy metabolism (in the lower part) and proteins involved in muscle structure and contraction (in the upper part), while creatine kinase remained connected to the centre of the

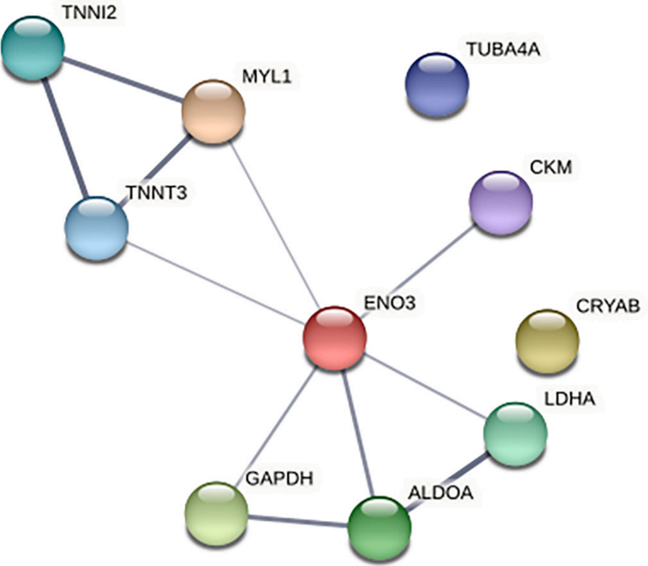

Fig. 4. Protein-protein interaction analysis of differentially abundant proteins found in non-aged and 21 d aged horse meat samples. Network nodes (circles) represent proteins, edges represent known or predicted functional associations and line thickness is an indicator for the strength of the association. Full protein details available in Table 2.

network linked to beta-enolase.

Interrelation of proteins from the glycolytic metabolism was reasonable since fructose bisphosphate aldolase A is a metabolic enzyme of the first stage of glycolysis. On the other hand, beta-enolase, glyceraldehyde-3-phosphate dehydrogenase and L-lactate dehydrogenase are enzymes belonging to the second phase of glycolysis, ensuring the conversion of 2 triose-phosphate to pyruvate and then, to lactate (Ouali et al., 2013). This node of four proteins was connected *via* beta-enolase to creatine kinase because the latter is also a key protein for energy transduction. Regarding the upper node, troponins I and T belonging to the troponin complex (central regulatory protein of muscle contraction) are connected to myosin light chain 1/3, which is part of the motor domain of myosin that protrudes off the surface to interact with actin in muscle contraction events. Interestingly, upper and lower nodes are connected by beta-enolase. This finding was logical since even if the main role of this cytosolic enzyme is the conversion of 2-phosphoglycerate into phosphoenolpyruvate during glycolysis, playing an important role in pH decline and *post-mortem* metabolism, it also regulates skeletal muscle structure regeneration (Merkulova et al., 2000). It also executes a cellular stress response to the deprivation of both oxygen supply and glucose levels *post-mortem*.

Finally, alpha-crystallin and tubulin-4-a chain remained outside of the protein network (Fig. 4), with no interaction between these two proteins and the others. In the case of the HSP, it is associated with the protection of actin filaments in response to stressors (Dimauro, Antonioni, Mercatelli, & Caporossi, 2017) having anti-apoptotic functions. Tubulin-4-a, despite being a structural protein, it is part of another muscle component, the microtubules (Becker et al., 2020).

4. Conclusions

As previously demonstrated in beef, this research revealed how OFFGEL technology can be successfully implemented as a previous step to 1-DE in the study of horse myofibrillar proteins. For the first time, the abundance of several proteins has been reported to change during *post-mortem* horse meat ageing, opening a way for the further study of these proteins as potential biomarkers of horse meat tenderness. Abundances of myosin light chain 1, myosin binding protein C fragment and troponins T and I (and their fragments) significantly changed during ageing, suggesting their role in the development of horse meat tenderness as already proposed in other animal species.

During ageing process, most protein changes found between 0 and 14 d were in accordance with previous instrumental tenderness data, highlighting that, under our conditions, horse meat tenderization primarily occurred during the first two weeks of ageing. Overall, the present proteomic study constitutes a step forward in the understanding of the molecular mechanism of horse meat tenderization.

Author contributions

Conceptualization, L.R.B and M.A.S.; methodology, L.R.B., E.S. and M.A.S.; investigation, L.R.B., N.A. and M.A.S.; data curation, N.A.; writing—original draft preparation, L.R.B.; writing—review and editing, L.R.B., E.S., N.A., and M.A.S.; supervision, N.A. and M.A.S.; project administration, M.A.S.; funding acquisition, M.A.S. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgements

Department of Economic Development and Infrastructures of the Basque Government is acknowledged for the doctoral fellowship of L.R. Beldarrain. Lactiker Research Group (Basque Government IT944-16) funded this work. Funding and contract of E. Sentandreu from project RTI2018-096162-R-C22 (Spanish Agencia Estatal de Investigación) are also fully acknowledged.

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APPENDIX VI

Publication VI

Beldarrain, L.R., Etaio, I., Morán, L., Sentandreu, M.A., Barron,L.J.R., Aldai, N. (2020). Effect of ageing time on consumer preference and sensory description of foal meat. *Food Res. Int.*, 129, 108871.

https://doi.org/10.1016/j.foodres.2019.108871

ISSN: 0963-9969

IF (2020): 6.475

Category: Food Science & Technology (Q1)

Food Research International 129 (2020) 108871

Contents lists available at ScienceDirect

Food Research International

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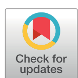
Effect of ageing time on consumer preference and sensory description of foal meat



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A R T I C L E I N F O

Keywords:
Horse meat
Vacuum pack
Consumer study
Visual acceptability
In-mouth acceptability
Sensory drivers

A B S T R A C T

A consumer test (n = 120) was performed in Vitoria-Gasteiz (northern Spain) in order to study the effect of ageing time (0, 7, 14 and 21 days) on the sensory quality of Hispano-Bretón foal meat. Steaks (*Longissimus thoracis et lumborum*) were wet aged and evaluated in-mouth and visually. In both cases, acceptability was scored using a hedonic scale, and sensory drivers related to ageing were characterised by applying check-all-that-apply method in meat. For both, in-mouth and visual acceptability, meat aged for 7 days obtained higher scores than non-aged meat, whereas longer ageing periods did not improve consumer acceptability. Check-all-that-apply method showed to be able to discriminate among samples, both in-mouth and visually. Results revealed that texture related attributes were the most discriminant ones in the in-mouth evaluation, being non-aged meat related to 'dry', 'high residue', 'tough' and 'chewy' terms, whereas aged meat was associated to 'juicy', 'tender' and 'easily dissolving' terms. Visually, consumers perceived that, after 14 days of ageing, meat colour changed to 'brownish'. Under present study conditions, the establishment of a period of 7 days of ageing would be recommended.

1. Introduction

Horse meat is not popular in most countries mainly due to religious and/or cultural concerns (Rossier, 2003; Ursin, 2016). However, it is considerably produced and consumed worldwide, being Asia and America the biggest producers and Asia and Europe the most important consumers, as indicated by Belaunzaran et al. (2015). This study also highlighted that horse meat consumption is slowly increasing in several countries because of the availability of this interesting meat source but also due to its recognized nutritional and environmental value, since horse production is mainly related to grazing systems.

Sensory preference of meat is related to visual appearance and in-mouth perception of texture and flavour (Font-i-Furnols & Guerrero, 2014). More concretely, it has been reported that tenderness, juiciness and flavour are the most important sensory traits affecting meat acceptability (D'Alessandro et al., 2012), being directly correlated with purchase intention and willingness to pay (Banovic, Grunert, Barreira, & Fontes, 2009). Thus, it is important to ensure a homogeneous product quality with acceptable tenderness (Robbins et al., 2003) in order to meet consumer expectations. In this sense, the role of meat ageing in the improvement of meat tenderness has been widely studied (Brewer & Novakofski, 2008; della Malva et al., 2019; Koohmaraie, 1994; Ouali et al., 2013). The level of tenderization will majorly depend on carcass hanging, duration and temperature in the ageing process, together with muscle type and animal species (Cassens, Arnols, Miller, Gehring, & Savell, 2018; Monsón, Sañudo, & Sierra, 2005; Vieira, Cerdeño, Serrano, Lavín, & Mantecón, 2007).

Meat ageing is a common practice that has been applied in the meat industry for decades (Warren & Kastner, 1992). In this line, its implications on consumer acceptability have been extensively studied not only in beef (Brewer & Novakofski, 2008; Li et al., 2014; Monsón et al., 2005) but also in pork (Chanon, D'Souza, & Dunshea, 2016) and sheep meat (Font-i-Furnols et al., 2006). On the contrary, few sensory studies have been focused on horse meat ageing (Gomez & Lorenzo, 2012; Lorenzo & Gomez, 2012; Lorenzo, Pateiro & Franco, 2013; Ruiz et al., 2020; Seong et al., 2016) and there is a clear lack of both standardized ageing practices and sensory research on the field. Particularly, no consumer preference studies concerning horse meat ageing have been published in the scientific literature.

In sensory studies, consumer tests usually focus on hedonic

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https://doi.org/10.1016/j.foodres.2019.108871
Received 16 October 2019; Received in revised form 25 November 2019; Accepted 30 November 2019
Available online 09 December 2019
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measurements, while sensory descriptive analyses are normally performed by trained panels. The high specialization of trained panels allows the collection of detailed, robust and consistent results. However, creating and maintaining the panels can be quite expensive and time-consuming (Cruz et al., 2013). Thereupon, consumer-based methods for sensory descriptive analyses are gaining popularity (Torrico et al., 2018; Varela & Ares, 2012). Among them, Check-all-that-apply (CATA) is a simple approach to gather information about the sensory perception of a product and it is suggested as a valuable alternative to classical descriptive methods that also captures differences among products (Reinbach, Giacalone, Machado, & Bredie, 2014). CATA allows the consumer to choose potential terms from a given list to describe a product, with the advantage of being an easy task that may give rise to more spontaneous descriptions than when intensities are rated (Dooley, Lee, & Meullenet, 2010). The potential of CATA method as a powerful tool for food development has also been pointed (Ares et al., 2017). Actually, the combination of liking and CATA results facilitates the elucidation of the sensory drivers related to the acceptability of a certain product (Ares, Barreiro, Deliza, Giménez, & Gámbaro, 2010). Despite the interesting approach of this sensory technique, few studies have been carried out on meat or meat products (Dos Santos et al., 2015; Jorge et al., 2015; Popoola, Bruce, McMullen, & Wismer, 2019) and, to our knowledge, this is the first time that CATA is applied to investigate the effect of ageing time on consumer meat acceptability.

To this extent, the aim of the present study was to assess, for the first time, the visual and in-mouth consumer acceptability of foal meat aged over 0, 7, 14 and 21 days (d) and to study the sensory drivers affected by meat ageing with CATA approach. This knowledge will contribute to the recommendation of a certain ageing time for the horse meat industry based on consumer preferences.

2. Material and methods

2.1. Animal handling

Ten foals (5 females and 5 males) of Hispano-Bretón breed were used for the present study. Under extensive grazing conditions, foals suckled their mothers from birth (May to June 2017) until 6–8 months of age and then continued under the same grazing conditions until they entered a commercial feedlot at 11–13 months of age. At the feedlot, foals were finished *ad libitum* on a concentrate composed by barley, wheat bran, wheat, soybean flour, dehydrated alfalfa, corn, beet pulp, soybean hulls, molasses, palm oil and salts (13.3% protein, 2.70% fat, 7.60% fibre) for 100–120 d. Foals were cared for in accordance with Directive 2010/63/EU (2010) and were slaughtered in a commercial abattoir at 15–17 months of age (August–October 2018). They were stunned with a captive bolt and slaughtered and dressed according to the specifications outlined in the European legislation (Council Regulation 1099/2009). The average carcass weight was of 246.2 ± 14.0 kg (249.9 ± 15.0 kg for females and 242.5 ± 12.5 kg for males). All carcasses were classified as U (conformation) and 2 (fat cover) according to the Community scale for the classification of carcasses of adult bovine animals (Council Regulation 1208/81) as there is no specific classification system for meat horses at EU level.

2.2. Sampling

Two foals (female and male) were slaughtered per week during 5 consecutive weeks. After 48 h (4 °C), both right and left rib joints were removed from each carcass and transported to the laboratory under refrigeration conditions. The loin, *Longissimus thoracis et lumborum* (LTL) muscle, was subsequently excised and sliced into 1.5 cm thick steaks. From each loin, 26 steaks were cut and destined for different analyses. Among them, 8 steaks beginning from the 10th rib were employed for the present study. Those steaks were vacuum packed and randomly assigned to an ageing time: 0, 7, 14 or 21 d (2 steaks per ageing day). Ageing was performed in a refrigerated room (4 ± 1 °C) without illumination.

When each ageing day was reached, steaks were unpacked and covered with an oxygen-permeable polyvinylchloride film (oxygen permeability of 580 mL/m^2/h at standard pressure and temperature). After 1 h of exposure to air (4 °C), 3 photographs of each steak (uncooked) were taken under controlled artificial illumination conditions (911 ± 10 lx) using a professional digital camera (Nixon-700, Nikon Corporation, Tokyo, Japan). Same white plate was used as background in order to avoid differences in contrast, intensity or direction of the illumination. From the whole set of photographs, 4 representative photographs were selected for each ageing time by a group of experts, taking into account the variability among animals, sexes and loin sides. Same batch of selected photographs were used for visual acceptability and visual CATA of all sessions. Moreover, the microbial safety of foal steaks aged for 21 d was checked and it was ensured that samples met the established safety requirements (TR CU 021/2011).

Then, all samples from each ageing time were vacuum packed and frozen at −80 °C until the consumer study.

2.3. Consumer study

2.3.1. Consumer recruitment and session organization

For recruitment purposes, consumer databases from the Sensory Laboratory of the University of the Basque Country (LASEHU) were used. At the same time, intensive efforts were made to reach potential consumers by e-mailing, posters, broadcast, social media, personal contacts, etc. Interested people fulfilled out a basic recruitment questionnaire (gender, age and foal meat consumption frequency). From among them, 120 volunteers from Vitoria-Gasteiz and surrounding areas (northern Spain) were recruited, balancing as much as possible for age and sex.

Ten sessions of 12 consumers were carried out during three consecutive days in LASEHU facilities, which are equipped with individual booths and computers. Sessions lasted approximately 1 h and were organized in the following order: in-mouth acceptability (IMA), visual acceptability (VA), in-mouth CATA, visual CATA and a final questionnaire. The alternation between visual and in-mouth tests avoided consumer's fatigue and provided enough time to cook the second set of samples for in-mouth CATA. Data were collected using Fizz Acquisition Software Version 2.40 (Biosystemes, Couternon, France).

2.3.2. In-mouth evaluation

For each session, batches of 16 steaks (8 steaks per loin; one loin from a female and another loin from a male foal) were used. The 8 steaks contained samples from all ageing times (0, 7, 14 or 21 d); 4 steaks were used for IMA and another 4 steaks were used for in-mouth CATA. All samples evaluated by each consumer came from the same animal. Samples were thawed overnight (4 °C). Two hours before cooking, each set of samples (2 animals × 4 ageing times) was taken out of the vacuum pack, covered with an oxygen-permeable PVC film (to avoid surface dryness) and kept at room temperature. Samples were cooked in two rounds; first, the IMA set and second the in-mouth CATA set. All samples per set were cooked at the same time (2 steaks per grill) in 4 double grills (one grill per ageing time) (Dalkyo MB-30, Sogo, Spain). Grills were set at 200 °C and steaks were cooked until they reached an internal temperature of 71 ± 1 °C. Individual internal temperature was monitored using 4-channel thermocouples (Lutron electronic, Pennsylvania, USA). Cooked steaks were immediately trimmed and cut into 2 × 1.5 × 2 cm cuboids, which were wrapped in aluminium foil and labelled with randomly established three-digit codes. Samples were kept in heaters (heat diffusion by glass microspheres) (Indoterm, Indo, Sant Cugat, Barcelona) at 60–65 °C until they were served (less than 5 min).

For IMA and in-mouth CATA, samples (one per ageing time) were

provided randomly and monadically. Water and unsalted crackers were available as palate cleansers between samples. Tasting was performed under red light (16 ± 1 lx) in order to avoid colour bias. Acceptability (IMA) was evaluated by answering the question 'How much do you like this steak?' in a 10 cm continuous hedonic scale with an extra cm in both sides, ranging from 'I extremely dislike' to 'I extremely like'. In-mouth descriptive terms were discussed and selected by a group of experts, with background in meat, from LASEHU. Previously an in-depth bibliographical search of terms was performed (Maughan, Tanasawat, Cornforth, Ward, & Martini, 2012; Rodbotten, Kubberod, Lea, & Ueland, 2004). The following 19 terms were selected: 'cowshed', 'fatty', 'intense', 'livery', 'low intensity', 'rancid', 'roasted' and 'unpleasant' for odour/aroma category; 'chewy', 'dry', 'easily dissolving', 'high residue', 'juicy', 'tough' and 'tender' for texture category; and 'bitter', 'metallic', 'salty' and 'sweet' for taste and trigeminal sensations category. Consumers were asked to tick off all the terms that considered appropriate to describe each sample (terms from the three categories were presented together and in different order along the sessions). After assessing the 4 samples for in-mouth CATA, consumers were asked to tick off all the attributes that relate to an 'ideal foal meat' sample, using the same list of terms.

2.3.3. Visual evaluation

Visual acceptability and CATA were assessed by the evaluation of selected photographs for each ageing period. Consumers were asked to consider the average impression of the batch of 4 selected photographs. In the booths, a correct position and a fixed distance to the screen were established in order to avoid as much as possible differences among consumers and no illumination was provided.

Visual CATA terms were defined beforehand and selection was performed as described for in-mouth CATA. Initially, the following 9 terms were selected: 'brownish', 'dark', 'fresh', 'maroon', 'not uniform colour', 'pinkish', 'red', 'spoilt', 'uniform colour'. The questions and scale for VA were the same as described for IMA, and consumers were asked to tick off all the terms that considered appropriate to describe each sample. After assessing the samples of the 4 ageing times for visual CATA, consumers were asked to tick off all the terms that relate to an 'ideal foal meat' sample.

2.3.4. Final questionnaire

After sensory evaluations, consumers were asked to complete a questionnaire concerning demographic and socio-economic information, and their familiarity with foal and aged meat. The following questions were included in the questionnaire: gender (female, male), age (18–35, 36–55, > 55), occupation (student, unemployed, employed, retired), how often do you consume foal meat? (once a week or once a month, once a year or I have not tried), how do you purchase foal meat? (steak, cut in cubes, minced, no answer), how do you prepare foal meat? (deep fried, grilled, oven roasted, barbequed, stewed, no answer), do you do the shopping yourself? (yes, no), have you ever tried aged meat? (yes - at restaurants, yes - at home and restaurants, no - I don't know what aged meat is), do you like aged meat? (yes, no, I have not tried/I don't know what aged meat is). Questionnaires were available in both original languages (Spanish/Basque) of the Basque region (northern Spain).

2.4. Statistical analysis

Analyses were conducted using IBM-SPSS Statistics Software Version 25.0 (SPSS Inc., IBM Corporation, Armonk, USA) and XLSTAT Statistics Software Version 2011.2 (Addinsoft, Bordeaux, France).

2.4.1. In-mouth and visual acceptability

The General Linear Model (GLM) of the Analysis of Variance (ANOVA) was used to determine the presence or absence of significant differences ($p \leq 0.05$) in the IMA and VA scores among different ageing times. Normality and homoscedasticity of the variables within each group (ageing times) were checked.

The following linear models were used:

For IMA scores: $Y_{ijkl} = \mu + AT_i + AS_j + (AT * AS)_{ij} + C_k(A_l(AS_j)) + A_l(AS_j) + \varepsilon_{ijkl}$

For VA scores: $Y_{ijk} = \mu + AT_i + C_j(S_k) + \varepsilon_{ijk}$

Models included ageing time (AT), animal sex (AS), animal (A) and session (S) as fixed factors and consumer (C) as a random factor.

Tukey test ($p \leq 0.05$) was applied for multiple comparisons of means.

2.4.2. Agglomerative Hierarchical Clustering and internal preference mapping

Agglomerative Hierarchical Clustering (AHC) was performed on the IMA and VA scores in order to identify groups of consumers with different patterns (Varela, 2014). Euclidean distance and Ward's agglomeration methods were employed. The number of selected clusters was determined by the observation of the dendrogram (MacFie, 2007). Then, the non-parametric Chi-squared test ($p \leq 0.05$) was applied in order to explore relationships among the information gathered from consumers (questionnaires) and the clusters selected.

Finally, Internal Preference Mapping (IPM) was constructed by performing Principal Component Analysis (PCA) with Varimax rotation on the covariance matrix of IMA and VA consumer individual scores. Kaiser criterion (eigenvalue > 1) was applied to extract the principal components. Bi-plot graphs were used to depict the associations among ageing times, consumers, and studied variables.

2.4.3. In-mouth and visual CATA

A contingency table was generated by counting the number of assessors (consumers) that used each term to describe meat from each ageing time. Following the criteria suggested by Meyners, Castura, and Carr (2013), only terms with effective sample size equal or over 24 were considered in the analysis. In order to evaluate the correct effective sample size (number of assessors for each term), assessors who tick one term from all four samples and those who did not tick any of them were excluded. Then, the non-parametric Cochran's Q test ($p \leq 0.05$) was applied to determine the presence or absence of significant differences in the IMA and VA CATA scores among different ageing times (Manoukian, 1986) and McNemar's test ($p \leq 0.05$) was applied for multiple comparisons (McNemar, 1947). The scores of the 'ideal foal meat' were not included in the aforementioned analysis. Moreover, based on the contingency table built by terms that showed significant differences among the 4 aged meat groups, a Correspondence Analysis was conducted using Chi-squared distances in order to obtain a perceptual mapping of meat samples and terms.

3. Results and discussion

3.1. Consumer acceptability

Foal meat acceptability scores for each ageing time are shown in

Table 1
Effect of ageing time (0, 7, 14 and 21 days) on foal meat in-mouth and visual acceptability scores.

Acceptability	0 d	7 d	14 d	21 d	SEM	*p*-value
In-mouth	5.49^{b}	6.20^{a}	5.90^{ab}	5.86^{ab}	0.11	0.029
Visual	5.74^{b}	6.44^{a}	6.22^{ab}	3.97^{c}	0.11	< 0.001

d: day; SEM: standard error of the mean.
Within a row, means with different superscripts indicate statistically significant differences by Tukey's test ($p \leq 0.05$).

Table 1. Both, IMA and VA, were significantly affected by ageing ($p \leq 0.05$). Besides, as it is common in consumer studies, consumer had also a significant effect ($p \leq 0.05$) over IMA and VA when nested with other factors: C(A(AS)) and C(S), respectively (data not shown).

Meat aged for 7 d obtained significantly higher IMA score than non-aged meat (0 d), while meat aged for longer periods, 14 and 21 d, showed intermediate acceptability scores. In agreement with IMA results, meat aged for 7 d also obtained the highest VA scores. Foal meat aged for 7 d was visually more acceptable than the non-aged (0 d) and meat aged for 21 d, which was the least acceptable for consumers (3.97; $p \leq 0.05$). Finally, foal meat aged for 14 d showed intermediate scores, being significantly better scored than meat aged for 21 d but not significantly different from meat aged for 7 d and non-aged meat (Table 1). From the scientific literature, several studies about acceptability of aged foal meat have not been considered, as according to the Society of Sensory Professionals (2018), trained and semi-trained panels should not be used for liking or acceptance studies. For this reason, these studies have not been included in the discussion section.

In the present study, one week of ageing improved both IMA and VA scores, and longer ageing periods did not improve aforementioned scores. This may indicate that, from a consumer point of view, no extra ageing time is needed to improve the acceptability of foal meat, and that ageing for 21 d would negatively affect VA scores, mainly due changes in colour (Beldarrain et al., 2019; Ruiz et al., 2018).

3.2. Consumer segmentation

In order to understand consumer behaviour and identify different preference patterns AHC was performed using IMA and VA scores separately. Three clusters were identified using IMA scores while four clusters were identified using VA scores (Table 2).

Regarding IMA-related clusters, cluster 1 was composed by 31 consumers which, in general, scored better the aged compared to non-aged meat (4.55). These consumers rated the meat aged for 7 d as the one with the best IMA (7.55). Cluster 2 was composed by 54 consumers which, opposite to cluster 1, rated all foal meat samples with high scores (6.66–7.54). These consumers preferred non-aged meat compared to meat aged for 21 d, while intermediate and not significantly different scores were found for other ageing times. Both, cluster 1 and 2, had the ability to discriminate among foal meat samples. On the other hand, cluster 3, composed by 35 consumers, did not appreciate significant differences among foal meat samples and, overall, they scored all of them quite negatively (3.14–4.26).

These cluster information was matched with the information collected in the final questionnaire (Table 3). Chi-squared test revealed that, among all the aspects considered in the questionnaire, only consumption frequency (‘How often do you consume foal meat?’ question) was significantly different ($p \leq 0.05$) among clusters (data not shown). Consumers from clusters 2 (79.6%) and 3 (88.6%) stated that they seldom consumed foal meat (once a year or have never tried), while consumers of cluster 1 (58%) consumed foal meat once a week or once a month. This lack of familiarity with foal meat consumption may have been the reason for the low discrimination and low rating of foal meat by consumers in cluster 3. However, the reason seemed to be different for consumers included in cluster 2. This cluster was a more multitudinous group with scattered liking scores.

Table 2
Effect of ageing time (0, 7, 14 and 21 days) on foal meat in-month and visual acceptability scores depending on clusters (Agglomerative Hierarchical Clustering).

Acceptability	Cluster	n	0 d	7 d	14 d	21 d	SEM
In-mouth	1	31	4.55^{c}	7.55^{a}	5.53^{bc}	6.62^{ab}	0.18
	2	54	7.54^{a}	6.94^{ab}	7.10^{ab}	6.66^{b}	0.12
	3	35	3.14^{a}	3.83^{a}	4.26^{a}	3.91^{a}	0.16
Visual	1	33	3.95^{a}	4.70^{a}	4.16^{a}	2.51^{b}	0.16
	2	44	5.90^{cb}	6.70^{ab}	6.92^{a}	5.62^{c}	0.13
	3	30	6.35^{a}	7.07^{a}	6.36^{a}	1.50^{b}	0.24
	4	13	7.52^{a}	9.80^{a}	9.68^{a}	8.00^{a}	0.15

d: day; SEM: standard error of the mean.
Within a row, means with different superscripts indicate statistically significant differences by Tukey’s test ($p \leq 0.05$).

These results can be visually interpreted in the multidimensional IPM representation, including consumers (Fig. 1A) and non-aged/aged foal meat samples (Fig. 1B), which explained 75.2% of the total variability. Additionally, the average acceptability scores of each cluster obtained by AHC were plotted as vectors. As indicated by Varela (2014), the direction of each vector represents the direction of increasing acceptability scores for each consumer cluster (Fig. 1A), and is linked to the position of the foal meat samples in the plane (Fig. 1B).

Regarding VA-related clusters (Table 2), cluster 1 (n = 33) and 3 (n = 30) followed a similar pattern and discriminated the foal meat aged for 21 d from the rest of the samples ($p \leq 0.05$). However, in cluster 3 the scores were extreme compared to cluster 1. Consumers included in cluster 2 (n = 40) preferred the visual appearance of foal meat aged for 7 and 14 d compared to non-aged foal meat or meat aged for 21 d. Consumers included in cluster 4 (n = 13) rated quite or very positively the visual appearance of all foal meat samples (7.52–9.80) and did not discriminate among them. When these cluster information was matched with the information collected in the final questionnaire (Table 3), it was observed that none of the information gathered was significantly related to any of the clusters.

Fig. 2 represents the aforementioned data using an IPM multidimensional approach where consumers (Fig. 2A) and non-aged/aged foal meat samples (Fig. 2B) have been depicted. It explained 88.3% of the total variability and average acceptability scores of each cluster were plotted as vectors.

In general, there is lower homogeneity in IMA than VA scores, as for VA consumers were located primarily on the first and fourth quadrants, whereas for IMA they were distributed all over the sensory plane (Figs. 1 and 2).

3.3. Sample description by CATA

From the initial 19 in-mouth CATA terms 13 showed an effective sample size equal or over 24 (at least 24 consumers discriminated among foal meat samples using this term; Meyners et al., 2013) and were the ones statistically analysed (Table 4). ‘Cowshed, ‘fatty’, ‘rancid’ and ‘unpleasant’ odour/aromas, and ‘metallic’ and ‘salty’ taste and trigeminal sensations did not reach the necessary effective sample size.

For odour/aroma related terms, ‘intense’ and ‘roasted’ presented higher citation frequencies (CF) at 21 d aged steaks compared with non-aged or steaks aged for 7 d, while 14 d presented intermediate CF values, being not significantly different from 0 or 21 d aged meat. The opposite trend was observed for ‘low intensity’, since it was significantly more cited in non-aged and meat aged for 7 d compared with 21 d aged meat, while intermediate citation frequencies were observed for 14 d aged meat. These results are comprehensive, as besides its effect on texture, ageing is also known to have an effect on volatile compounds from the Maillard reaction, increasing the concentration of substances that play an important role in the aroma of cooked meat (Watanabe et al., 2015). Some other consumer studies, however, found no change in flavour over time in aged beef (Brewer & Novakofski, 2008; Laster et al., 2008). ‘Livery’ term had a considerable CF value (34.1), although no statistically significant differences were observed among ageing times. Thus, ‘livery’ could be considered merely descriptive as a typical aroma presented in foal meat as stated in previous studies (Sarriés & Beriain, 2005).

For the terms included in the texture category, ‘juicy’, ‘tender’, and ‘easily dissolving’ ($p \leq 0.01$) exhibited similar trends with the lowest CF values in non-aged samples, while non-significant differences were

Table 3
Number of responses and frequencies from the questionnaire distributed according to the clusters obtained from in-mouth and visual acceptability scores (Agglomerative Hierarchical Clustering).

Question	Possible answers		In-mouth acceptability			Visual acceptability			
		TOTAL	Cluster 1	Cluster 2	Cluster 3	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Gender	Female	69(57.5)	18(58.1)	29(53.7)	22(62.9)	19(57.6)	27(61.4)	18(60.0)	5(38.5)
	Male	51(42.5)	13(41.9)	25(46.3)	13(37.1)	14(42.4)	17(38.6)	12(40.0)	8(61.5)
Age	18–35	39(32.5)	9(29.0)	18(33.3)	12(34.3)	6(18.2)	17(38.6)	14(46.7)	2(15.4)
	36–55	53(44.2)	17(54.8)	22(40.8)	14(40.0)	15(45.5)	18(40.9)	13(43.4)	7(53.8)
	Over 55	28(23.3)	5(16.1)	14(25.9)	9(25.7)	12(36.4)	9(20.5)	3(10.0)	4(30.8)
Occupation	Student	23(19.2)	5(16.1)	11(20.4)	7(20.0)	3(9.1)	11(25.0)	7(23.3)	2(15.4)
	Unemployed	74(61.7)	21(67.7)	31(57.4)	22(62.9)	22(66.7)	25(56.8)	19(63.3)	9(69.2)
	Employed	3(2.5)	1(3.2)	2(3.7)	0(0.0)	0(0.0)	1(2.3)	1(3.3)	0(0.0)
	Retired	20(16.7)	4(12.9)	10(20.4)	6(17.1)	8(24.2)	7(15.9)	3(10.0)	2(15.4)
How often do you consume foal meat?	Once a week or once a month	28(23.3)	13(41.9)	11(20.4)	4(11.4)	5(15.2)	9(20.5)	9(30)	3(23.1)
	Once a year or I have not tried it	92(76.7)	18(58.1)	43(79.6)	31(88.6)	28(84.8)	35(79.5)	21(70)	10(76.9)
How do you purchase foal meat?	Steak	26(21.7)	12(38.7)	9(16.7)	5(14.3)	9(27.3)	9(20.5)	4(13.3)	2(15.4)
	Cut in cubes	15(12.5)	9(29.0)	3(5.6)	3(8.6)	2(6.1)	0(0.0)	1(3.3)	1(7.7)
	Minced	6(5.0)	5(16.1)	0(0.0)	1(2.9)	0(0.0)	2(4.5)	4(13.3)	2(15.4)
	No answer	73(60.8)	5(16.1)	42(77.8)	26(74.3)	22(66.7)	33(75.0)	21(70.0)	8(61.5)
How do you prepare foal meat?	Deep fried	5(4.2)	2(6.5)	2(3.7)	1(2.9)	2(6.1)	3(6.8)	0(0.0)	0(0.0)
	Grilled	26(21.7)	13(41.9)	9(16.7)	4(11.4)	7(21.2)	7(15.9)	8(26.7.0)	4(30.8)
	Oven roasted	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Barbequed	1(0.8)	0(0.0)	0(0.0)	1(2.95)	1(3.0)	0(0.0)	0(0.0)	0(0.0)
	Stewed	5(4.2)	1(3.2)	2(3.7)	2(5.7)	1(3.0)	2(4.5)	1(3.3)	1(7.7)
	No answer	83(69.2)	15(48.4)	41(75.9)	27(77.1)	22(66.6)	32(72.7)	21(70.0)	8(61.5)
Do you do the shopping yourself?	Yes	81(67.5)	25(80.7)	32(59.3)	24(68.6)	23(69.7)	31(70.5)	18(60.0)	9(69.2)
	No	39(32.5)	6(19.3)	22(40.7)	11(31.4)	10(30.3)	13(29.5)	12(40.0)	4(30.8)
Have you ever tried aged meat?	Yes, at restaurants	13(10.8)	2(6.5)	8(14.8)	3(8.6)	4(12.1)	5(12.8)	3(10.0)	2(15.4)
	Yes, at home and restaurants	16(13.3)	5(16.1)	8(14.8)	3(8.6)	4(12.1)	9(23.1)	2(6.6)	1(7.7)
	No	16(13.3)	3(9.7)	5(9.3)	8(22.9)	6(18.2)	6(15.4)	4(13.3)	1(7.7)
	I don't know what aged meat is	75(62.5)	21(67.7)	33(61.1)	21(60.0)	19(57.6)	24(48.7)	21(70.0)	9(69.2)
Do you like aged meat?	Yes	20(16.7)	5(16.1)	11(20.4)	4(11.4)	6(18.2)	9(20.5)	4(13.8)	2(15.4)
	No	11(9.2)	3(9.7)	5(9.7)	3(8.6)	3(9.1)	5(11.4)	2(6.9)	1(7.7)
	I have not tried/I don't know what aged meat is	89(74.2)	23(74.2)	38(70.4)	28(80.0)	24(72.7)	30(68.2)	23(79.3)	10(76.9)

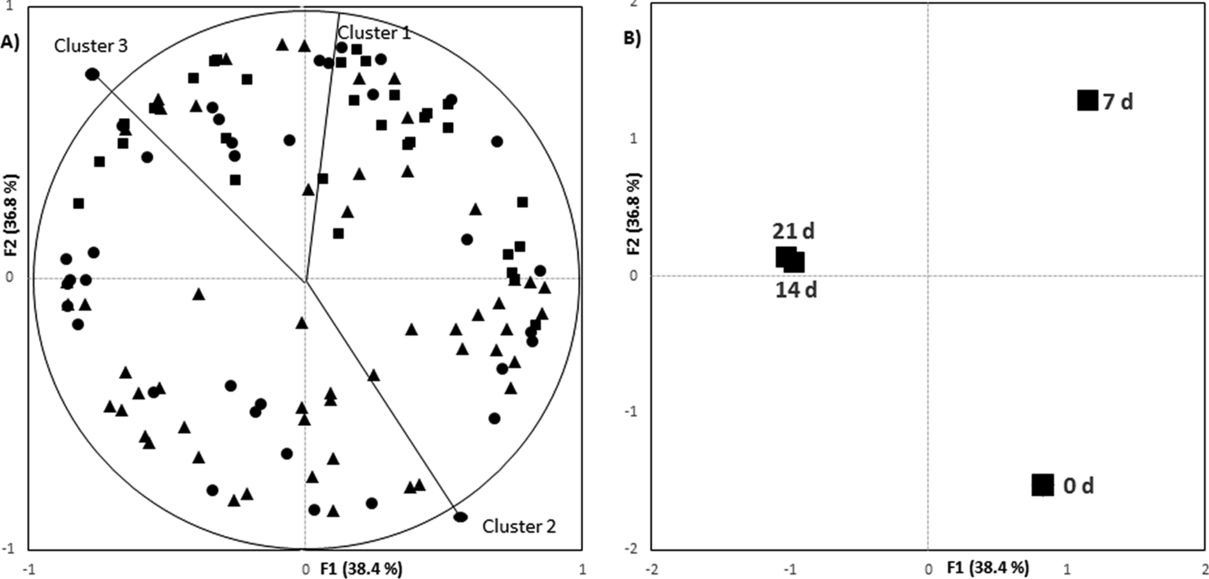

Fig. 1. Internal preference map of consumer IMA scores of foal meat samples aged over 0, 7, 14 and 21 days including the representation of (**A**) individual consumers depending on the cluster they belong to and clusters projected as supplementary variables, and (**B**) non-aged and aged foal meat samples.

observed among aged samples. Opposite trend was observed for 'dry', 'tough' and 'chewy' terms ($p \leq 0.05$), with the highest CF values in non-aged foal meat and non-significant differences among aged samples. Citation frequencies of 'high residue' term ($p = 0.027$) were significantly higher for non-aged and 14 d aged meat samples, and lower for 7 d aged meat. Intermediate CF value was observed for meat aged for 21 d.

These results demonstrate the improvement in meat tenderness

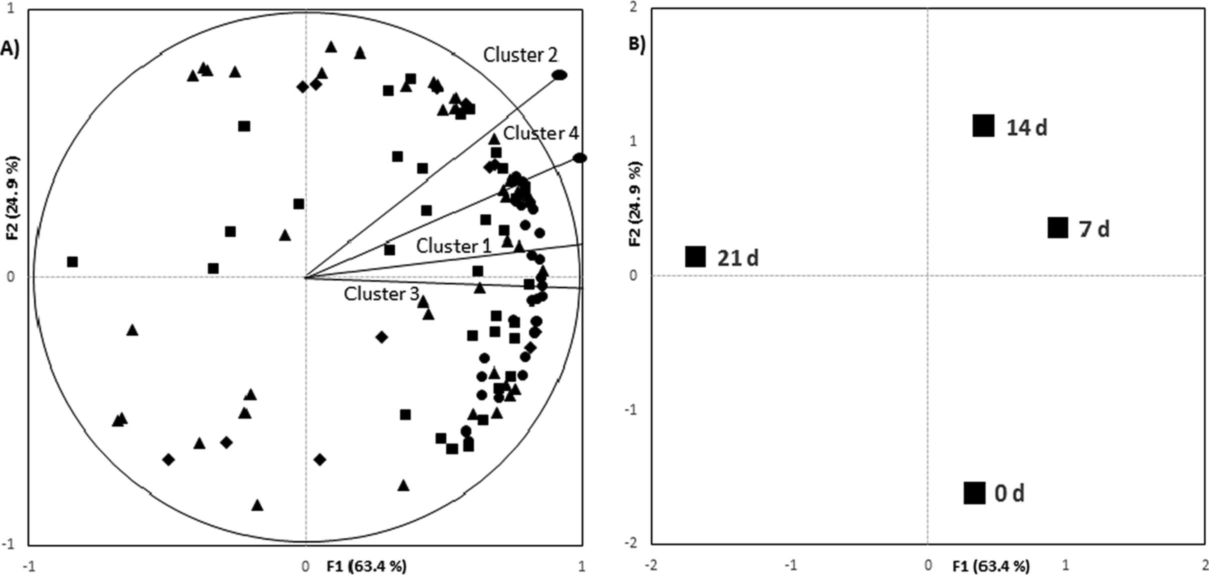

Fig. 2. Internal preference map of consumers VA scores of foal meat samples aged over 0, 7, 14 and 21 days including the representation of **(A)** individual consumers depending on the cluster they belong to and clusters projected as supplementary variables, and **(B)** non-aged and aged foal meat samples.

during the ageing process, which is explained by *post mortem* proteolysis processes (Koohmaraie, 1994; Ouali et al., 2013). In this sense, della Malva et al. (2019) found that instrumental tenderness (by measuring Warner Bratzler shear force) of meat from the *Longissimus lumborum* of 12-month Italian Draft Horses increased after 6 days of ageing, and no statistically significant differences were observed for longer ageing periods. Moreover, this is coherent with the results obtained for IMA, as the acceptability of meat aged for 7 d was higher compared to non-aged meat, and no differences were found when meat was aged for longer periods of time. This also indicates that texture related attributes had a high relevance when evaluating IMA of cooked foal meat. Among the terms included in taste and trigeminal sensations category, 'bitter' and 'sweet' (both taste sensations) showed no statistically significant differences among ageing times.

For the description of ideal foal meat, for the odour/aroma category, 'intense' and 'roasted' were the most cited terms (72.5% and 57.5% of the consumers, respectively). However, 'low intensity' and 'livery' were not chosen by the consumers as important attributes in their definition of ideal foal meat. Texture terms obtained higher CF values compared to odour/aroma and taste and trigeminal sensations categories being, therefore, critical for the consumer. Within this category, the terms 'juicy', 'tender', and 'easily dissolving' were the most cited terms (98.3, 94.2 and 87.5%, respectively), and conversely, 'dry', 'tough', 'chewy' and 'high residue' were almost not included in the description of the ideal foal meat. Finally, in terms of taste and trigeminal sensations category, 37.5% of the consumers defined 'sweet' as an attribute of ideal foal meat; very close to the 44.6% of citations of the non-discriminant 'sweet' term as observed by Ruiz et al. (2020).

With the 10 IMA CATA terms that showed statistically significant differences ($p \leq 0.05$) among ageing times further multidimensional correspondence analysis was performed. A two-dimensional bi-plot of terms and samples, preserving the 97% of the original inertia of data is depicted in Fig. 3. As it can be observed, selected attributes enabled the spatial separation of samples depending on ageing time. Texture attributes defined the first dimension and clearly separated non-aged samples, characterized as 'chewy', 'tough', 'dry' and 'high residue', from the rest of the aged meat samples, characterized as 'tender', 'easily dissolving' and 'juicy'. Moreover, on the left side of the sensory map, samples

Table 4
Effect of ageing time (0, 7, 14 and 21 days) on foal meat in-mouth CATA term citation frequencies and the overall citation frequencies of the 'ideal foal meat'.

Category	CATA TERMS	ESS	CF 0 d	CF 7 d	CF 14 d	CF 21 d	ACF	CF (%)	*p*-value	'ideal foal meat'	
										CF	CF (%)
Odour/Aroma	Intense	67	24^{b}	28^{b}	35^{ab}	44^{a}	131	48.9	0.004	87	72.5
	Livery	58	14	24	18	23	79	34.1	0.250	6	5.0
	Low intensity	73	43^{a}	36^{a}	26^{ab}	22^{b}	127	43.5	0.004	11	9.2
	Roasted	71	23^{bc}	18^{c}	33^{ab}	39^{a}	113	39.8	0.003	69	57.5
Texture	Easily dissolving	86	20^{b}	44^{a}	48^{a}	45^{a}	157	45.6	≤0.001	105	87.5
	Dry	99	63^{a}	46^{b}	44^{b}	45^{b}	198	50.0	0.027	0	0.0
	High residue	80	42^{a}	26^{b}	41^{a}	30^{ab}	139	43.4	0.031	2	1.7
	Juicy	87	25^{b}	49^{a}	45^{a}	41^{a}	160	46.0	0.003	118	98.3
	Chewy	95	71^{a}	38^{b}	35^{b}	29^{b}	173	45.5	≤0.001	1	0.8
	Tender	91	14^{b}	45^{a}	53^{a}	52^{a}	164	45.1	≤0.001	113	94.2
	Tough	75	59^{a}	21^{b}	12^{b}	19^{b}	111	37.0	≤0.001	0	0.0
Taste sensations	Bitter	24	6	6	7	12	31	32.3	0.289	0	0.0
	Sweet	46	23	23	21	15	82	44.6	0.335	45	37.5

ESS: effective sample size; CF: citation frequency; d: day; ACF: absolute citation frequency (sum of citation frequencies in foal meat aged of 0, 7, 14 and 21 d). CF (%) = (ACF/ESS * 4 ageing times) * 100.
Within a row, means with different superscripts indicate statistically significant differences by Cochran's Q test ($p \leq 0.05$).
Ideal sample was analysed separately and effective sample size was 120.

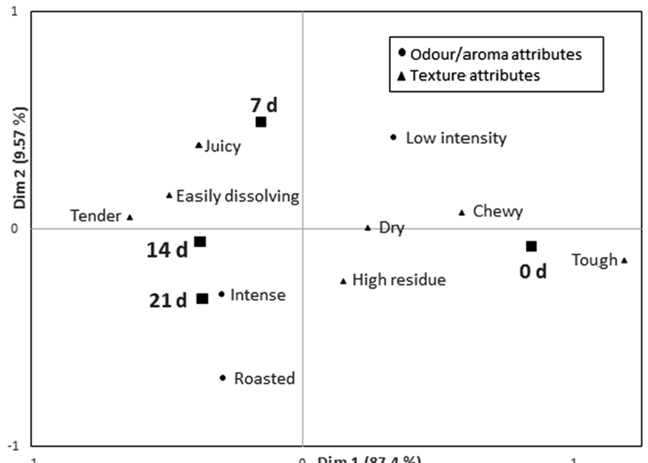

Fig. 3. Representation of the in-mouth CATA terms and samples in the first and second dimensions of the Correspondence Analysis based on Chi-squared distance.

aged for 14 and 21 d were located closer from each other than meat samples aged for 7 d. Regarding odour/aroma related terms, non-aged samples located close to 'low intensity' term while meat samples aged for 21 d, and to a lower extent meat samples aged 14 d, located close to 'intense' and 'roasted' terms, being indicative of aroma development over ageing time.

Citation frequencies of visual CATA terms are gathered in Table 5. In this case, all 9 terms initially selected resulted in an effective sample size equal or over 24 (Meyners et al., 2013), and statistically significant differences were observed in all of them among studied ageing times ($p \leq 0.001$).

As it could be expected, meat samples aged for 21 d obtained the highest CF values for undesirable terms such as 'brownish', 'not uniform colour', and 'spoilt', confirming that this meat was not visually attractive for consumers. On the other hand, CF values of terms like 'fresh' and 'red' were significantly higher in meat aged for 7 and 14 d compared to non-aged or meat aged for 21 d. Similarly, in beef studies, the consumer is able to discriminate the meat that is not red, as they associate it with a lack of freshness (Mancini & Hunt, 2005).

The term 'dark' presented significantly higher CF values in non-aged and meat aged for 21 d compared to meat aged for 7 and 14 d, and 'maroon' was also more cited in non-aged foal samples compared to others. It seems that the consumer understood 'dark' as intense maroon in non-aged samples and as intense brown in samples aged over 21 d. For the rest of the terms, significantly higher 'pinkish' CF values were observed in meat aged for 14 d compared to non-aged or meat aged for 21 d, while intermediate values were observed for meat aged for 7 d. Similarly, higher 'uniform colour' CF values were observed for meat aged for 14 d compared to meat aged for 21 d, while intermediate values were observed in non-aged and meat aged for 7 d.

As reviewed by Mancini and Hunt (2005), myoglobin is a key component of meat colour. When *post mortem* time increases, enzyme activity and NADH pool in charge of brown metamyoglobin reduction are decreased and depleted, respectively, leading to meat to discoloration going from 'red' to 'brownish' and passing through 'pinkish' (Faustman & Cassens, 1990; McKenna et al., 2005). The time needed for the process to take place depends on both intrinsic and extrinsic factors, but the results suggest that under the conditions of the present study, the change to 'brownish' was mainly perceived by consumers after 14 d of ageing.

Regarding the consumer description of ideal foal meat (Table 5), 'fresh', 'uniform colour' and 'red' were the most cited terms (96.7%, 89.2% and 83.3%, respectively), while 'not uniform colour', 'brownish' and 'spoilt' were the least desired terms (below 3.5%). Others such as 'maroon', 'pinkish' and 'dark' obtained intermediate CF values (20.8%, 20.8% and 14.2%, respectively).

Correspondence Analysis was performed on the contingency table of visual CATA data, and the first two dimensions explained the 97.2% of the original inertia enabling the spatial separation of samples depending on ageing time (Fig. 4). As it can be noted, visual CATA terms performed a good discrimination of the four ageing times. The first dimension showed the differentiation of meat aged for 21 d and the rest of the meats. Meat aged for 21 d was clearly associated with 'spoilt' and 'brownish' terms. The rest, and especially meat aged for 7 d and 14 d, were characterized by 'fresh', 'red' and 'uniform colour' terms. The second dimension seemed to be related to the evolution of colour where non-aged meat was related to 'maroon', meat aged for 7 d to 'red', meat aged for 14 d to 'pinkish' and meat aged for 21 d to 'brownish' terms.

4. Conclusions

Sensory methodologies based on consumer perception like CATA have shown to be useful to characterize and discriminate foal meat aged for different time periods. For the first time, a hedonic evaluation combined with a CATA characterization of aged foal meat is provided, which highlights the relevance of texture attributes on the acceptability of foal meat. Moreover, it evidences that changes in colour happening during the ageing process are perceived by the consumer. According to these results, the best ageing time could be established at 7 days, since it would be enough to obtain a good in-mouth and visual acceptability. Indeed, longer ageing periods would require an economic investment that would not be translated into higher acceptability by the consumer. Further research would be necessary in order to thoroughly understand

Table 5
Effect of ageing time (0, 7, 14 and 21 days) on foal meat visual CATA term citation frequencies and the overall citation frequencies of the 'ideal foal meat'.

CATA TERMS	EES	CF 0 d	CF 7 d	CF 14 d	CF 21 d	ACF	CF (%)	*p*-value	'ideal foal meat'	
									CF	CF (%)
Brownish	77	16^{b}	15^{b}	12^{b}	64^{a}	107	34.7	≤0.001	3	2.5
Dark	87	61^{a}	22^{b}	4^{c}	69^{a}	156	44.8	≤0.001	17	14.2
Fresh	107	68^{b}	88^{a}	81^{a}	10^{c}	247	57.7	≤0.001	116	96.7
Maroon	81	63^{a}	33^{b}	19^{c}	25^{bc}	140	43.2	≤0.001	24	20.8
Not uniform colour	94	46^{b}	44^{b}	25^{c}	62^{a}	177	47.1	≤0.001	4	3.3
Pinkish	50	10^{c}	20^{b}	37^{a}	6^{c}	73	36.5	≤0.001	25	20.8
Red	100	43^{b}	69^{a}	64^{a}	17^{c}	193	48.3	≤0.001	100	83.3
Spoilt	105	31^{b}	11^{c}	20^{bc}	97^{a}	159	37.9	≤0.001	0	0.0
Uniform colour	95	47^{b}	43^{b}	68^{a}	20^{c}	178	46.8	≤0.001	107	89.2

EES: effective sample size; CF: citation frequency; d: day; ACF: absolute citation frequency (sum of citation frequencies in foal meat aged of 0, 7, 14 and 21 d).
CF (%) = (ACF/ESS * 4 ageing times) * 100.
Within a row, means with different superscripts indicate statistically significant differences by Cochran's Q test ($p \leq 0.05$).
Ideal sample was analysed separately and effective sample size was 120.

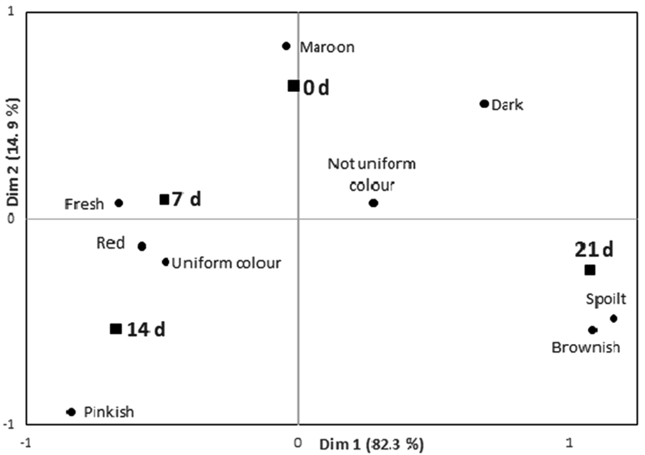

Fig. 4. Representation of the visual CATA terms and samples in the first and second dimensions of the Correspondence Analysis based on Chi-squared distance.

the ageing process under other conditions/factors such as breed, feeding systems, muscle type and others.

CRediT authorship contribution statement

Lorea R. Beldarrain: Investigation, Data curation, Writing - original draft, Visualization. **Iñaki Etaio:** Methodology, Formal analysis, Resources, Data curation, Visualization. **Lara Morán:** Conceptualization, Methodology, Investigation, Writing - original draft. **Miguel Ángel Sentandreu:** Writing - review & editing, Supervision. **Luis Javier R. Barron:** Formal analysis, Data curation, Funding acquisition. **Noelia Aldai:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Department of Economic Development and Infrastructures of the Basque Government is acknowledged for the doctoral fellowship of L.R. Beldarrain. Lactiker Research Group (Basque Government IT944-16) funded this work as well as the contract of L. Morán. The authors wish to thank the staff from the Information and Communication Technologies services of UPV/EHU for the technical support with photographs.

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