ZALDI-HARAGIAREN LIPIDOMIKA: ASPEKTU METODOLOGIKOAK ETA BERE KONPOSIZIOARI ERAGITEN DIZKIOTEN FAKTOREAK

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LIPIDOMICS OF HORSE-MEAT: METHODOLOGICAL ASPECTS AND FACTORS AFFECTING ITS COMPOSITION

eman ta zabal zazu

Universidad del País Vasco

Euskal Herriko Unibertsitatea

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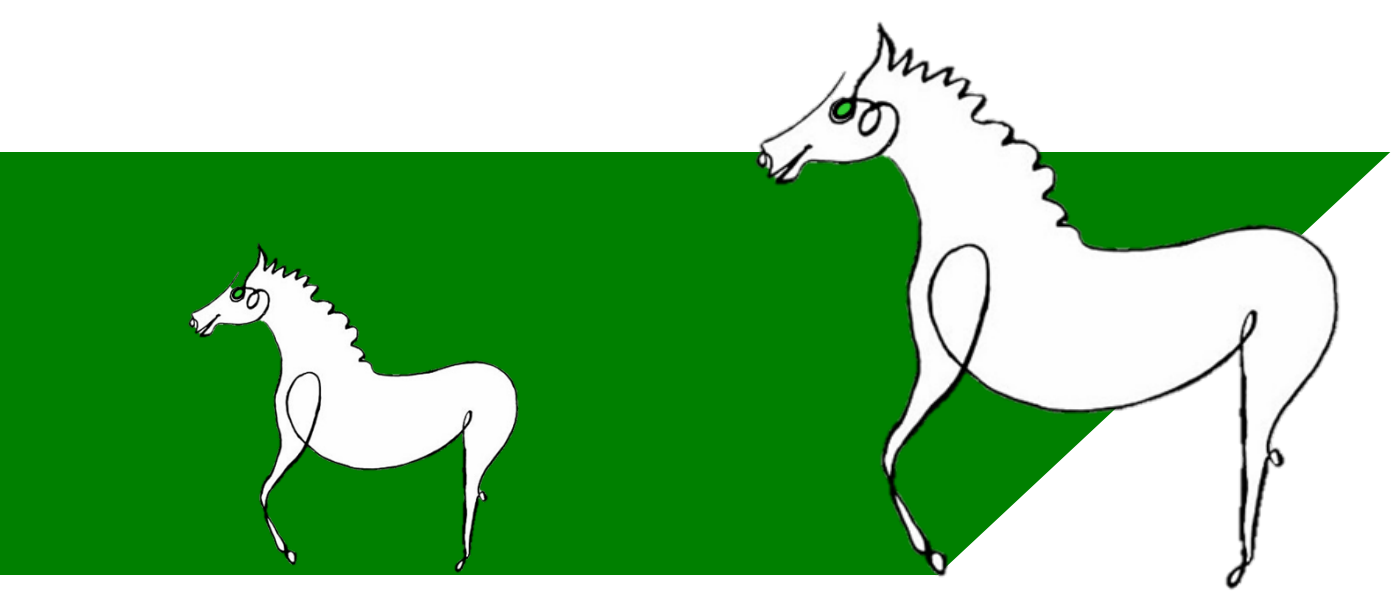

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Universidad del País Vasco
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FARMAZIA FAKULTATEA
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FACULTAD DE FARMACIA
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TESIS DOCTORAL

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Elikagaien Kalitatea eta Segurtasuna
Doktore Gradua lortzeko memoria

Memoria para optar al Grado de Doctor en Calidad y Seguridad Alimentaria

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FATTY ACID NOMENCLATURE

	Systematic name	Common name
10:0	Decanoic acid	Capric acid
12:0	Dodecanoic acid	Lauric acid
13:0	Tridecanoic acid	
14:0	Tetradecanoic acid	Myristic acid
16:0	Hexadecanoic acid	Palmitic acid
18:0	Octadecanoic acid	Stearic acid
23:0	Tricosanoic acid	
i-16:0	14-methyl-pentadecanoic acid	Isopalmitic acid
i-17:0	15-methyl-hexadecanoic acid	
ai-17:0	14-methyl-hexadecanoic acid	
i-18:0	16-methyl-hepadecanoic acid	Isostearic acid
9*c*-16:1	9*c*-hexadecenoic acid	Palmitoleic acid
6*c*-18:1	6*c*-octadecenoic acid	Petroselinic acid
9*c*-18:1	9*c*-octadecenoic acid	Oleic acid
11*c*-18:1	11*c*-octadecenoic acid	
9*t*-16:1	9*t*-hexadecenoic acid	
9*t*-18:1	9*t*-octadecenoic acid	Elaidic acid
11*t*-18:1	11*t*-octadecenoic acid	Vaccenic acid
9*c*,11*t*-18:2	9*c*,11*t*-octadecadienoic acid	Rumenic acid
16:2n-6	7*c*,10*c*-hexadecatrienoic acid	
18:2n-6	9*c*,12*c*-octadecadienoic acid	Linoleic acid
20:2n-6	11c,14*c*-eicosadienoic acid	
20:3n-6	8*c*,11*c*,14*c*-eicosatrienoic acid	Dihomo-Ɣ-linolenic acid
20:4n-6	5*c*,8*c*,11*c*,14*c*-eicosatetraenoic acid	Arachidonic acid
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	
16:3n-3	7*c*,10*c*,13*c*-hexadecatrienoic acid	
18:3n-3	9*c*,12*c*,15*c*-octadecatrienoic acid	α-linoleic acid
20:3n-3	11c,14*c*,17*c*-eicosatrienoic acid	
20:4n-3	8*c*,11*c*,14*c*,17*c*-eicosatetraenoic acid	
20:5n-3	5*c*,8*c*,11*c*,14*c*,17*c*-eicosapentaenoic acid	
22:5n-3	7*c*,10*c*,13*c*,16*c*,19*c*-docosapentaenoic acid	
22:6n-3	4*c*,7*c*,10c,13*c*,16*c*,19*c*-docosahexaenoic acid	

ABBREVIATION

Ag^+	Silver ion
ai	*anteiso*
AL	Algae
ANOVA	Analysis of variance
ArA	Arachidonic acid
AS	Asturias
BA	Basque
BCFA	Branched-chain fatty acid
BU	Burguete
BW	Body weight
c	*cis*
CA	Cantabria
CI	Chemical ionization
CL	Castile & Leon
CLA	Conjugated linoleic acid
CO	Coconut oil
CO_2eq	Carbon dioxide equivalents
CV	Coefficient of variation
DHA	Docosahexaenoic acid
DHGLA	Dihomo-γ linolenic acid
DM	Dry matter
DMA	Dimethylacetal
DP	Deposition preference
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FID	Flame ionization detector
FO	Fish oil
FXO	Flax oil
FXS	Flaxseed
GA	Galicia
GC	Gas chromatography
GHG	Greenhouse gas
GM	Galician Mountain
HA	Haflinger
HB	Hispano-Breton
HPLC	High performance liquid chromatography
i	*iso*

IHDH	Italian Heavy Draft
IM	Intramuscular
IS	Internal standard
K	Konik
LA	Linoleic acid
LC	Long chain
LD	Limits of detection
LNA	Linolenic acid
LQ	Limits of quantification
LTL	*Longissimus thoracis et lumborum*
LW	Live weight
ME	Methyl ester
min	Minute
mr	Maintenance requirements
MS	Mass Spectrometry
MSHB	Mixed stock horse breed
MU	Murgese
MUFA	Monounsaturated fatty acid
NA	Navarre
NC	Non-conjugated
ND	Not detected
NL	Neutral lipid
PC	Principal component
PCA	Principal component analysis
PL	Polar lipid
PLRP2	Pancreatic lipase related to protein 2
PUFA	Polyunsaturated fatty acid
R	Region
RA	Rumenic acid
rf	Response factor
S	Season
SA	Sanfratello
SC	Subcutaneous
SCD	Stearoyl Co-A desaturase
SD	Standard deviation
SEM	Standard error of the mean
SFA	Saturated fatty acid
SPE	Solid-phase extraction
t	*trans*
T	Tissue (adipose)
TFA	*trans* -fatty acid
TG	Triacylglycerol
TLC	Thin layer chromatography
UPV/EHU	University of the Basque Country
VA	Vaccenic acid

LABURPENA

Doktorego Tesi hau animalia jatorriko elikagaien kalitatea eta segurtasuna ikertzea helburu duen Euskal Herriko Unibertsitateko (UPV/EHU) Lactiker jakintza arlo anitzeko ikertaldean burutu da, CSIC "Consejo Superior de Investigaciones Científicas" - Leoneko Unibertsitateko IGM "Instituto de Ganadería de Montaña" zentroarekin elkarlanean.

Doktorego Tesi honen helburu nagusia, lipidoen osagaiak ardatz izanik, zaldi-haragiaren kalitate nutrizionala ebaluatu, eta animaliaren ekoizpen praktikak, zaldi-haragiaren nutrizio kalitatearengan izandako eragina ikertzeko erabilitako lipidomika alorreko analisi-tekniken baliagarritasuna baieztatzea izan da. Era berean, kalitatezko, eskuragarri, osasuntsu eta kontsumitzailearen ikuspuntutik onartua (animalien ongizatea eta ingurugiroaren kontserbazioa) den produktua lortzeko zaldi-haragiaren ekoizpen jasangarria sustatzeko lagungarria izatea. Ikerketa hau espainiar iparraldean giza kontsumora bideratutako zaldi-haragi ekoizpenaren alorrean burutu da, eta ezaugarri sentsorial, kontsumitzaileen onargarritasuna eta hauentzat garrantzitsuak izan daitezkeen, genomika, elikadura, egiaztatzea, trazabilitatea eta bestelako kontzeptu etiko eta sozio-ekonomikoak jorratzen dituen ikerketaren parte izan da.

Doktorego Tesi honen lehenengo zatian, zaldi-haragiaren kalitatearen inguruko aurrekariak aztertu dira; momentuko ezagutza zientifikoetatik abiatuz, informazio falta nabaritu, eta jarraipen ikerketa zehaztu da. Berrikuspen prozesuan, zaldi-haragiaren ekoizpen-, merkataritza- eta biztanleriarekiko eskuragarritasun-balioak laburbildu dira, kontsumitzaileen onargarritasuna eta zaldiak ez-hausnarkariak izatearen ondorioak aztertuz. Zaldiaren digestio fisiologia, mendiko larreak erabiltzeko aukera eta abantailekin lotu da. Alde batetik, larreko n-3 gantz-azidoak haragian barneratzeko daukaten gaitasun eraginkorra behatuz, eta bestetik, ekoiztutako haragi unitateko berotegi-efektuko gasen emisio baxua aztertuz. Gainera, zaldi-haragian, hausnarkariekin alderatuz, *trans* konfigurazioko gantz-azido kopuru baxua da. Zaldiak haragitarako ere ekoiztu daitezkeela ondorioztatu da, eta ez soilik aisialdirako, herrialde askotan egin ohi den bezala. Gainera, zaldi-haragia behi-haragiarekin alderatuta aukera interesgarria izan

daitekeela behatu da. Era berean, zaldi-haragiaren gantz-azidoen konposizio zehatza ezarri, honen konposizioa hobetzeko faktoreak aztertu, eta gantzaren digestio eta metabolismo prozesuek gantz-azidoen profilarekin duten erlazioa ulertzeko ikerketa berriak ezinbestekoak direla zehaztu da.

Erronka hauei aurre egiteko, hiru helburu nagusi ezarri ziren. Lehena, lipidoen analisi sakon eta zehatza burutzeko beharrezko den metodologia egokia erabili, optimizatu eta garatzea izan zen. Helburu hau lortzeko, hainbat teknika hobetu eta garatu ziren, eta ikertutako molekulen identitatearen berrespena gas-kromatografiaren teknika aurreratuak erabiliaz eratu zen. Zaldi-haragiaren laginak analizatzeko erabilitako tresna eta teknika aurreratu hauek, Estatu batuetako FDA-ren "Food and Drug Administration" zentroan egindako egonaldi internazionalean izan zituen eskuragarri Doktorego ikasleak. Egonaldian, Pierluigi Delmonte Doktorearen gainbegiratzepean, zaldi-haragiaren dimetilazetalak gantz-azido metil esterretatik isolatzeko metodoa garatu zen. Haragiak lipido plasmalogenikoak dituela jakina da, oro har, ester loturetariko bat eter batekin ordezkatzen da glizerol molekularen *sn*-1 posizioan. Haragiaren konposatu hauen edukia aztertzen duten lan zientifikoen kopurua oso mugatua da, eta guk dakigula, inoiz ez dira zaldi-haragian identifikatu. Ugaztunen ehunetan, plasmalogenoak, batez ere lipidoen frakzio polarrean ageri dira, eta kopuru baxuak behatu dira frakzio neutroan. Konposatu hauek, animalien zelulen mintzean aurkitzen den fosfatidiletanolamina fosfolipioaren osagai nagusiak dira. Egonaldian garatutako metodoak lipidoen konposatuak bereizi eta kuantifikatzeko erabilgarria dela erakutsi zuen (metilazioan azil ester eta alkenil eter-etatik eratorritako produktuak). Bestalde, bertan landutako teknika kromatografiko aurreratuak beste helburuak lortzeko oso lagungarriak izan ziren.

Bigarren helburua, Espainiar iparraldeko Kantauri Mendilerroaren bi aldeetan kokaturiko herrialdeetan komertzializatutako zaldi-haragia ezaugarritzea, eta haragi-mota honen kalitate nutrizionalaren inguruko informazio zehatza eskuratzea izan da. Horretarako, zona geografiko honetan kokaturiko herrialdeetan (Euskadi, Nafarroa, Kantabria, Asturias, Galizia, eta Gaztela eta Leon) laginketa burutu zen. Gainera, urtaro desberdinetan komertzializatutako

haragiaren kalitatean egon daitezkeen aldaketak aztertzeko asmoz, laginketa bi urtarotan egin zen (udaberrian eta neguan).

Lortutako emaitzen arabera, Kantauri Mendilerroko herrialdeen arteko laginen gantz kopurua desberdina izan da. Nafarroa eta Gaztela Leonekoak izan ziren koipetsuenak, Galizia eta Asturiaskoak gantz urrienekoak, eta Euskadi eta Kantabriakoak aldiz, gantz kopuru ertainekoak. Ikusitako herrialdeen arteko ezberdintasun esanguratsuek, agerian jarri zituzten maneiu praktika ezberdinak; arraza mota, animalien elikadura eta adina faktore garrantzitsuenetarikoak izan zitezkeelarik. Gainera, n-3 motako gantz-azido poliasegabeen edukiari zegokionez, aldakortasun nabaria ikusi izan zen, muskuluko kopuruak % 1,17 eta % 18,9 bitartean kokatu ziren eta % 1,52 eta % 27,9 bitartean ehun adiposokoak (larruazalpeko gantza). Oro har, neguan jasotako laginen n-3 motako gantz-azido poliasegabeen kopurua nabariagoa izan zen, eta emaitzak, lagin hauen jatorria mendietako larreetan ekoiztutako animaliak zirenaren adierazgarri izan daitezke. Analizatutako laginen % 5k, n-3 iturri bezala etiketatzeko aukera izango lukete, Europako Batzordearen 116/2010 Erregelamenduak zehazten duen azido linolenikoaren gutxieneko 300 mg/100 g haragiko kopurura iritsi baitziren. Bestalde, larruazalpeko gantz zati bat gehituko bagenio haragiari, lagin guztien n-3 gantz-azidoen kopurua hainbat Europar elkarteek ezarritako eguneroko gomendiora iritsiko litzateke (eguneko eta pertsonako 1 g n-3 gantz-azido poliasegabe).

Hirugarren eta azken helburua, animaliaren adinak eta honekin loturiko elikadurak, zaldi-haragiaren kalitate nutrizionalean izan dezakeen eragina ebaluatzea izan zen. Helburu hori lortzeko, bi zaldi-haragi ekoizlerekin elkarlanean, ikerketa erdi kontrolatuari ekin genion. Ekoizleek, bai lagin eta baita ekoizpen parametroak erraztu zizkiguten. Gurutzatutako bi moxal talde ikertu ziren; lehenengo taldea, larrean behorrarekin hazitako 4 hilabeteko esne-moxalek osatzen zuten, eta bigarren taldea, aldiz, 7 hilabetez larrean behorrarekin hazi eta ondoren 12 hilabete izan arte pentsuz elikatutako moxalez osatua. Ikerketa honetan, zaldi muskuluaren lipidoak ezaugarritzeaz gain, lipidoen frakzio neutroa eta polarra bereizi ziren eta bakoitzaren gantz-azidoen konposizioa ikertu zen. Gantz-azido hauek nagusiki frakzio neutro edo polarrean esterifikatzen ziren

ikertu, eta animalien adinak eta honekin lotutako elikadurak izan dezakeen eragina aztertu zen.

Lortutako emaitzetatik esne-moxalen haragia gantz urriko produktua dela ikusi zen. Gainera, lagin hauen dimetilazetal, azido linoleniko eta kate luzeko n-3 eta n-6 gantz-azido poliasegabeen kopuru altuak, profil osasuntsuagoaren adierazgarri izan ziren. Bestalde, pentsuz elikatutako moxalen muskulua koipetsuagoa zela, eta honek eragin zuzena izan zuela lipido neutro eta polarren gantz-azidoen konposizioan ondorioztatu zen. Era berean, lagin hauen dimetilazetal eta kate luzeko gantz-azido poliasegabeen kopuruak baxuagoak izan ziren. Koipetsuagoak ziren laginetan, frakzio neutroa areagotua zegoen, eta polarra, aldiz, konstante mantendu zen. Ondorioz, frakzio polarraren osagai nagusiak ziren dimetilazetal eta kate luzeko gantz-azido poliasegabeen kopuruak diluituta ageri ziren. Bestalde, azido linolenikoa (18:3n-3) nagusiki frakzio neutroan esterifikatu zela behatzea interesgarria izan zen. Zaldi muskuluaren eta gantzaren azido linoleniko kopuru esanguratsuak, zaldi-haragiari n-3 jatorriko gantz-azido poliasegabeen iturri bezala merkaturatzeko aukera paregabea eman diezaioke.

Ugaztunen ehunetan, plasmalogenoetako alkenil eter konposatuak ase eta monoasegabe eran aurkitu izan dira. Halere, Doktorego lan honetan erabilitako kromatografia teknika aurreratuek 2 eta 3 lotura bikoitz dituzten (18:2 eta 18:3) alkenil eter konposatuak identifikatzea ahalbidetu zuten. Lan honetan ikertutako zaldi-haragi langinen eter lipidoen edukia % 1,40 eta % 8,99 bitartean kokatu zen. Beraz, plasmalogeno hauek lipido totalari egindako ekarpena esanguratsua denez, zaldi-haragiaren kalitate nutrizionala ebaluatzean, konposatu hauek aztertzea ezinbestekoa litzake.

Oro har, Doktorego Tesi honetan lortutako emaitzek zaldi-haragiaren balio nutrizional zehatza adierazteko base-datuen eraketari ekarpen garrantzitsua egin diezaioke. Emaitza hauek kontsumitzaileek produktu honekiko duten harrera hobetu, kalitatezko eta haragi aukera osasuntsu bezala baietsi, eta haragi honen kontsumo eta merkatua sustatzea eragin dezakete. Horrez gain, ikerketa honetan erabilitako lipidomika arloko teknikek, animalien ekoizpen prozesuen ondorioz,

zaldien lipido eta haragiaren kalitate nutrizionalean egon daitezkeen aldaketak zehazki aztertzeko erabilgarriak izan dira.

RESUMEN

La presente Tesis Doctoral se ha realizado dentro del Grupo de Investigación multidisciplinar Lactiker de la Universidad del País Vasco (UPV/EHU) cuya actividad está centrada en la Calidad y Seguridad de los Alimentos de Origen Animal, y así mismo, en colaboración con el Instituto de Ganadería de Montaña del Consejo Superior de Investigaciones Científicas - Universidad de León.

El objetivo general de esta Tesis Doctoral ha sido evaluar la calidad nutricional de la carne de caballo, en particular, la calidad de la fracción grasa y demostrar la utilidad de la lipidómica para valorar de manera precisa las diferencias en la calidad nutricional de la carne producida bajo diferentes sistemas de manejo, contribuyendo con ello a promover la producción de carne de caballo en condiciones sostenibles de manejo con el fin de obtener un alimento de alta calidad que al mismo tiempo sea una alternativa asequible y saludable para el consumidor y aceptada desde el punto de vista de las condiciones de producción (bienestar animal y conservación medioambiental). Esta investigación se ha centrado en la producción de carne de caballo destinada al consumo humano en el norte de España y forma parte de un proyecto de investigación más amplio en el que se abordan otros aspectos como las características sensoriales de la carne y la aceptabilidad de los consumidores, así como otros factores relevantes como la genómica, la alimentación, la autentificación, la trazabilidad y los aspectos éticos y socioeconómicos.

La primera parte de la Tesis Doctoral consistió en una revisión bibliográfica sobre la calidad de la carne de caballo con el objetivo de conocer el estado del arte, las lagunas de conocimiento y las posibles oportunidades para la investigación. Durante la revisión se recogieron datos de producción, comercialización y disponibilidad por habitante de carne de caballo a nivel mundial, así como las implicaciones de la utilización del ganado caballar como especie de gran formato y su aceptabilidad por los consumidores. Estos animales podrían contar con ventajas a la hora de utilizar los pastos de montaña en comparación con otras especies de rumiantes debido a su fisiología digestiva la cual se asoció con una mayor eficiencia en la transferencia de ácidos grasos poliinsaturados de tipo n-3

de los pastos a la carne y con una menor emisión de gases efecto invernadero por unidad de carne producida. Además, la carne de caballo presenta un bajo contenido de ácidos grasos tipo *trans*. De la revisión bibliográfica se concluyó la posibilidad de promover la cría de caballos para la producción de carne y no sólo para actividades de ocio, como es el caso que existe en muchos países, al mismo tiempo que podría ser una alternativa a la carne de vacuno, pero que era necesario realizar un mayor esfuerzo de investigación para determinar con profundidad la composición lipídica de la carne de caballo, estudiar los factores que afectan a su composición y entender la relación del perfil de ácidos grasos con el proceso digestivo y el metabolismo de la grasa en el ganado equino.

Para abordar algunos de los retos descritos, se propusieron tres objetivos principales. El primer objetivo fue optimizar y desarrollar metodologías analíticas para un análisis preciso, detallado y completo de los lípidos de la carne de caballo. Las metodologías analíticas utilizadas se complementaron, a su vez, con técnicas cromatográficas de alta resolución con fines confirmatorios. Para poder llevar a cabo estas técnicas complementarias el doctorando realizó una estancia internacional en el U.S. Food and Drug Administration (FDA) bajo la supervisión del Dr. Pierluigi Delmonte. Durante la estancia, se desarrolló un método para separar los dimetilacetales de los ésteres metílicos de los ácidos grasos de la carne de caballo. La carne contiene lípidos plasmalogénicos donde uno de los enlaces éster está sustituído por un éter vinílico generalmente unido en la posición *sn*-1 del glicerol. El contenido de estos compuestos apenas se ha descrito en la carne, en general, y nunca habían sido antes identificados en la carne de caballo. Los plasmalógenos están presentes en pequeñas cantidades en la fracción neutra de la grasa de los mamíferos, y se encuentran sobre todo en la fracción polar, principalmente en fosfolípidos (*p.e.,* fosfatidiletanolamina) presentes en las membranas celulares. El método de separación desarrollado durante la estancia demostró ser muy útil para identificar y cuantificar exhaustivamente los lípidos del caballo, tanto los productos de metilación tipo éster de acilo como los de tipo alquenil éter. Por otro lado, las técnicas cromatográficas de alta resolución empleadas ayudaron de forma significativa en el avance y el logro de los otros objetivos de esta Tesis Doctoral.

El segundo objetivo fue evaluar la calidad nutricional de la carne de caballo comercializada a ambos lados de la Cordillera Cantábrica en el norte de España. El diseño experimental consistió en un muestreo en dos épocas diferentes del año (primavera e invierno) de la carne de caballo comercializada en carnicerías especializadas y grandes superficies. Con objeto de abarcar toda la variabilidad posible, el muestreo se llevó a cabo en seis comunidades autónomas del norte peninsular; Euskadi, Navarra, Cantabria, Asturias, Galicia, y Castilla y León.

Los resultados del estudio pusieron de manifiesto que las muestras más grasas fueron las de Navarra y Castilla y León, y las muestras más magras fueron las de Asturias y Galicia, mientras que las de Euskadi y Cantabria presentaron valores de grasa intermedios. La variabilidad observada entre las regiones evidenció diferencias claras en el manejo de los animales donde la raza, la alimentación y la edad de sacrificio parecían ser los factores más relevantes. Además, se observó una alta variabilidad en el contenido de ácidos grasos poliinsaturados de tipo n-3 que osciló entre el 1,17 % y el 18,9 % en el músculo, y entre el 1,52 % y el 27,9 % en el tejido adiposo (grasa subcutánea). En general, se observó un mayor contenido de ácidos grasos poliinsaturados de tipo n-3 en las muestras recogidas en invierno, posiblemente debido a que los animales fueron criados en pastos de montaña hasta finales del otoño. Es interesante resaltar que el 5% de las muestras de carne de caballo analizadas alcanzaron el contenido mínimo de 300 mg de ácido linolénico por 100 g de carne fresca que exige el Reglamento 116/2010 de la Comisión Europea para poder etiquetar el producto como fuente de ácidos grasos de tipo n-3. Por otro lado, si en el cómputo total de n-3 se incluyera parte de la grasa subcutánea, las muestras de carne podrían alcanzar y superar la recomendación de ingesta diaria de n-3 establecida por algunas Sociedades Europeas (1 g de ácidos grasos poliinsaturados de tipo n-3 por día y persona).

El tercer y último objetivo consistió en evaluar el efecto de la alimentación asociado a la edad del animal sobre la calidad nutricional de la carne de caballo. Para ello se llevó a cabo un estudio semi-controlado en colaboración con dos productores de ganado equino que proporcionaron las características de producción de los animales y las muestras de carne de los dos grupos de potros. El primer grupo consistió en animales cruzados criados con sus madres en

condiciones de pastoreo y lactancia natural hasta el sacrificio (4 meses de edad). El segundo grupo consistió en animales cruzados que fueron criados con sus madres en condiciones de pastoreo y lactancia natural hasta los 7 meses de edad, en que fueron destetados y acabados con un concentrado comercial hasta el sacrificio (12 meses de edad). En este estudio se llevó a cabo una caracterización más completa de la fracción lipídica de la carne, separando la fracción neutra y polar con objeto de determinar su composición específica en ácidos grasos, la preferencia de deposición de éstos en una fracción u otra y el efecto de la alimentación asociado a la edad del animal sobre dichas fracciones y su composición.

A partir de los resultados obtenidos se observó que la carne de los potros lechales presentó un bajo contenido en grasa y un perfil más saludable con un mayor contenido en dimetilacetales, ácido linolénico y todos los ácidos grasos poliinsaturados de cadena larga de tipo n-3 y n-6. La carne de los potros acabados con pienso concentrado, sin embargo, presentó un contenido de grasa intramuscular significativamente mayor, lo cual afectó directamente al contenido de las fracciones neutra y polar y a su composición en ácidos grasos. Estas muestras presentaron un menor contenido de lípidos plasmalogénicos (identificados como dimetilacetales) y ácidos grasos poliinsaturados de cadena larga que se depositaron principalmente en la fracción polar. La fracción neutra aumentó considerablemente su contenido cuando los potros eran acabados con pienso concentrado, mientras que el contenido de lípidos polares se mantuvo constante. Esto ocasionó un efecto de dilución de los dimetilacetales y ácidos grasos poliinsaturados de cadena larga depositados en la fracción polar. Llamó la atención la deposición preferencial del ácido linolénico (18:3n-3) en la fracción lipídica neutra lo que indicó que su acumulación en músculo y grasa pueden hacer de la carne de caballo una buena opción para su comercialización como fuente de ácidos grasos de tipo n-3.

En los mamíferos los compuestos alquenil éter de los plasmalógenos se han descrito generalmente como saturados y monoinsaturados. Sin embargo, las técnicas cromatográficas avanzadas utilizadas en esta Tesis Doctoral han permitido la identificación de compuestos alquenil éter con 2 y 3 dobles enlaces (18:2 y 18:3). Las muestras de carne de caballo analizadas en este trabajo han

mostrado un contenido en lípidos éter entre un 1,40 y un 8,99 % de los lípidos totales. Este contenido no debería ser ignorado a la hora de evaluar la calidad nutricional de la carne ya que contribuye de forma relevante a la cantidad total de grasa de la carne.

En general, los resultados obtenidos en esta Tesis doctoral contribuyen de forma significativa a la creación de bases de datos precisas en cuanto al valor nutricional de la carne de caballo. Estos resultados pueden también promover la aceptación por parte del consumidor de este producto como una carne alternativa y saludable, e impulsar la comercialización y/o la venta de este tipo de carne. Por otra parte, las técnicas de lipidómica empleadas han demostrado ser útiles para una evaluación exhaustiva de la calidad nutricional de la carne de caballo y de cambios asociados a diferencias en el sistema de manejo.

ABSTRACT

This Ph. D. Thesis has been conducted within the Lactiker Research Group at the University of the Basque Country (UPV/EHU), which is dedicated to perform multidisciplinary research in the field of Quality and Safety of Food from Animal Origin, and in close collaboration with the Mountain Livestock Institute of the Spanish National Research Council – University of León consortium.

The main objective of this Ph. D. Thesis was to evaluate the nutritional quality of horse-meat, specifically the quality of the lipid fraction, and to prove the usefulness of a lipidomic approach to accurately monitor the changes in the nutritional quality of horse-meat as a result of different management practices. This would help to promote horse production under sustainable farming systems in order to obtain a high quality food that simultaneously provides an alternative, affordable and healthy animal product, while accepted by the consumer in terms of production practices (animal welfare and protection of the environment). The investigation was performed in the area of horse-meat production destined for human consumption in the northern Spain which is part of a wider research project in which additional quality aspects such as sensory characteristics and consumer acceptability are being addressed, together with many other relevant aspects of horse-meat production for human consumption such as genomics, feeding strategies, authentication, traceability, and ethical and socio-economic aspects.

Several stages were incorporated into the present Ph. D. study. The first was a literature review on horse-meat in order to understand and update the current knowledge of horse meat and to find opportunities to conduct further research because the information was limited. In the review process, several issues were identified that needed further attention, i.e., horse-meat acceptance as edible meat, horse-meat production, trade and supply data were evaluated, and the implications of horse as a large framed non-ruminant species. These animals could advantageously compete with ruminants for the utilization of pastures and rangelands due to their digestive physiology that allow them to efficiently transfer the n-3 polyunsaturated fatty acids from pasture into meat while producing low

greenhouse gas emissions per unit of meat produced compared to ruminants. Moreover, horse-meat provides a very low *trans* fatty acid content. From the literature review, it was concluded that horses could be bred for meat production and not only for leisure activities as it is the case of many countries. It was also concluded that horse-meat could be an alternative to beef but further studies were needed to establish a more accurate fatty acid composition of horse-meat and factors to improve the lipid composition to understand the relationship of the fatty acid profile with the characteristic digestive process and fat metabolism in horse.

To address several of the proposed challenges, the following three main objectives were undertaken. The first one was to optimize, develop and utilize the most appropriate analytical methodology in order to achieve an accurate, detailed and complete analysis of lipid constituents in horse-meat. In the process to achieve this goal, several techniques were improved and developed, and novel and comprehensive gas chromatography techniques were applied for complementary and confirmatory purposes. In order to have access to these equipments and master the techniques, the Ph. D. candidate visited the U.S. Food and Drug Administration (FDA) under the supervision of Dr. Pierluigi Delmonte to acquire experience to perform advanced analytical techniques for the analysis of horse-meat. During this international stay, a method was developed for dimethylacetal isolation. Meat is known to contain plasmalogenic lipids where one of the ester linkages is replaced by a vinyl ether (alk-1-enyl moiety), generally linked to the *sn*-1 position of the glycerol molecule. These compounds are seldom reported in meat, and to the best of our knowledge have never been reported in horse-meat. In mammalian tissues, the plasmalogens are only present in small amounts in the neutral lipid fraction, while they are major components of the polar lipid fraction. They are particularly found in phosphatidylethanolamine fraction, which is an important structural component of the cell membranes in animals. The method proved to be a successful tool to comprehensively identify and quantify horse lipids (i.e., methylation products of acyl ester and alkenyl ether moieties). The mastering of these techniques helped greatly in the performance and achievement of the other objectives.

The second objective was to assess the nutritional quality of horse-meat commercialized at both sides of the Cantabrian Mountains in northern Spain. In order to do so, a survey was designed where horse steaks were collected at the retail level in butcher shops and grocery stores at two different times of the year, spring and winter seasons. Moreover, along the Cantabrian Mountains, samples were collected in six different Spanish regions; Basque, Navarre, Cantabria, Asturias, Galicia, and Castile & Leon, in order to sample most of the compositional variability.

The results from the survey study demonstrated that along the Cantabrian Mountains, the fattest muscle samples were collected in the Navarre and the Castile & Leon regions, the leanest samples were collected in Asturias and Galicia, while the intermediate ones were collected in Basque Country and Cantabria regions. The significantly variability among regions revealed clear differences in management practices where the type of breed, feeding and age at slaughter might be the most relevant factors. Furthermore, high overall variability was observed in the n-3 polyunsaturated fatty acid content which ranged from 1.17 % to 18.9 % in muscle, and from 1.52 % to 27.9 % in adipose tissue (subcutaneous fat). In general, in samples collected in early winter the n-3 polyunsaturated fatty acid level was higher presumably because animals were reared under mountain grazing conditions until late fall. Interestingly, 5 % of the surveyed horse muscles reached the minimum of 300 mg of linolenic acid per 100 g of fresh meat that made it possible to make a claim for this meat product and market it as a source of n-3 fatty acids according to the European Commission Regulation 116/2010. Also, if trimming fat derived from subcutaneous fat is included to the loin, most of the horse-meat could meet or exceed the recommended daily intake established by other European Societies (*i.e.*, 1 g of total n-3 polyunsaturated fatty acids per day).

The third and last objective was to evaluate the effect of age, and associated differences in feeding practices on the nutritional quality of horse-meat. In order to achieve this objective, a semi-controlled experiment was undertaken in collaboration with a horse-meat producer who provided us with samples and production parameters. Two groups of crossbred horses were studied; the first group was selected from foals managed under grazing conditions and naturally

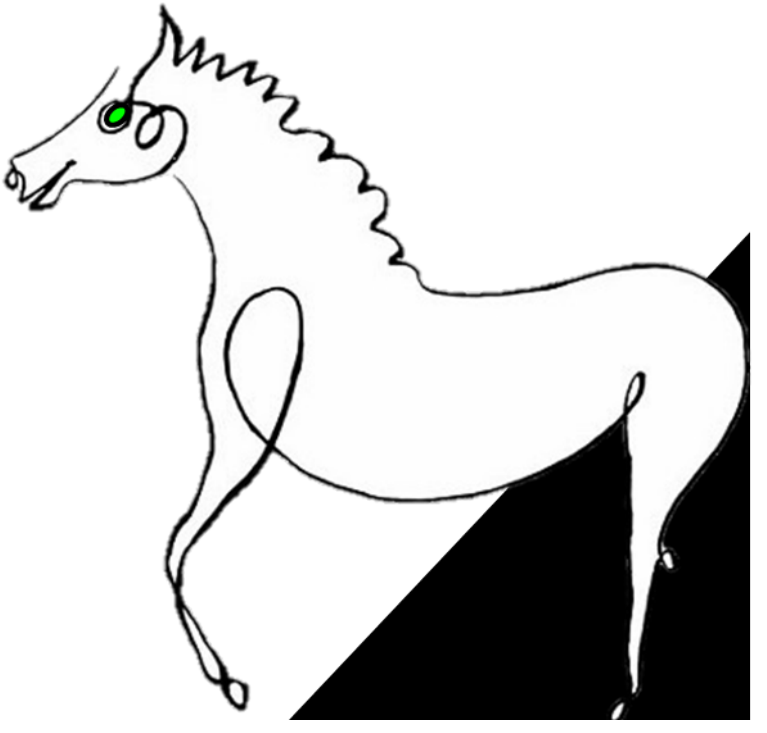
suckled by their mothers from birth to slaughter at 4 months of age. The second group was selected from foals managed under grazing conditions and naturally suckled by their mothers from birth to 7 months when they were concentrate-finished until 12 months of age. In this experiment, a more thorough characterization of the horse-meat was undertaken in which the total lipid fraction of muscle was fractionated into neutral and polar lipids. The isolated fractions were separately analyzed for their total fatty acid composition and the selective preference of individual fatty acid into the neutral and polar lipid fractions. The effects of age and associated feeding practices were also examined.

From the results it was observed that meat obtained from suckling foals was the leanest and provided a healthier profile with higher dimethylacetal, linolenic acid, and all (n-3 and n-6) long chain polyunsaturated fatty acid content. Meat obtained from concentrate-finished foals, however, presented a significantly higher intramuscular fat content which directly affected the fatty acid composition of the neutral and polar lipid fractions. At the same time, there was a lower content of plasmalogenic lipids (identified as dimethylacetals) and long chain polyunsaturated fatty acids due to their favorable deposition in the polar lipid fraction. The fatty acid composition of the polar lipids, a fairly constant lipid fraction, was largely affected by the dilution effect exerted by the neutral lipids. It was interesting to note that linolenic acid (18:3n-3) was preferentially deposited in the neutral lipid fraction and, therefore, its significant accumulation in muscle and subcutaneous fat makes horse-meat a good option to be marketed as a source of n-3 fatty acids.

In mammalian species, the alkenyl ether chain from plasmalogens is generally reported to consist of saturated and monounsaturated moieties. However, advanced gas chromatographic analysis performed during the Ph. D. study it allowed for the identification of alkenyl ether chain with 2 and 3 double bonds (18:2 and 18:3). All the horse-meat samples analyzed in the present investigation showed that the ether lipid content represented about 1.40 to 8.99 % of the total lipids. The plasmalogen content should not be ignored in a nutritional quality evaluation of horse-meat, since the alkenyl ether chain will meaningfully contribute to the overall fat content of the product.

Overall, it was concluded that the results obtained in the present Ph. D. work are significant contribution to create an accurate database to assess the nutritional value of horse-meat. It could also promote consumer acceptance of horse-meat as an edible and healthy alternative food, and can contribute to marketing purposes of horse-meat. In addition, lipidomic tools developed and improved in this study proved to be a useful approach to accurately evaluate the changes occurring in horse lipids as affected by management practices that can, at the same time, affect the nutritional quality of horse-meat.

Chapter I. GENERAL FRAMEWORK AND BRIEF INTRODUCTION



Chapter I. GENERAL FRAMEWORK AND BRIEF INTRODUCTION

Current livestock production needs to be committedly complemented with all available meat-producing species in order to fulfill the high quality protein needs driven by the increased population (McLeod, 2011). The global population is expected to surpass 9 billion by 2050 (United Nations, 2013), from 7.3 billion today, and it is expected that at least 3 billion people will join the middle class in the coming years (Kharas, 2010).

In order to meet the protein demand, in the last 50 years, the main driving force defining production and breeding policies of main livestock species in western countries has been primarily focused on productivity and, therefore, intensification practices, which had a huge impact on many native or local breeds, some of them becoming endangered or even extinct. Nowadays, at least in Europe, the overall tendency is to reverse this situation towards a more resilient and sustainable production practices providing, simultaneously, ecosystem services. This strategy has been clearly detailed in the European Research Area on Sustainable Animal production as well as in the H2020 – Work Program 2016-2017: Food security, sustainable agriculture and forestry, marine and maritime and inland water research and the bioeconomy.

Linked to the equine sector as a meat-producing specie, very few actions have been taken at European and national level even though the environmental impact in terms of greenhouse gas (GHG) emissions of this species is insignificant compared to ruminants. Overall, our understanding is that there is a clear lack of information about equine management strategies focused on quality horse-meat production for human consumption and its associated socio-economic, ethical and welfare issues, and yet, horse-meat consumption is slightly increasing in western European countries.

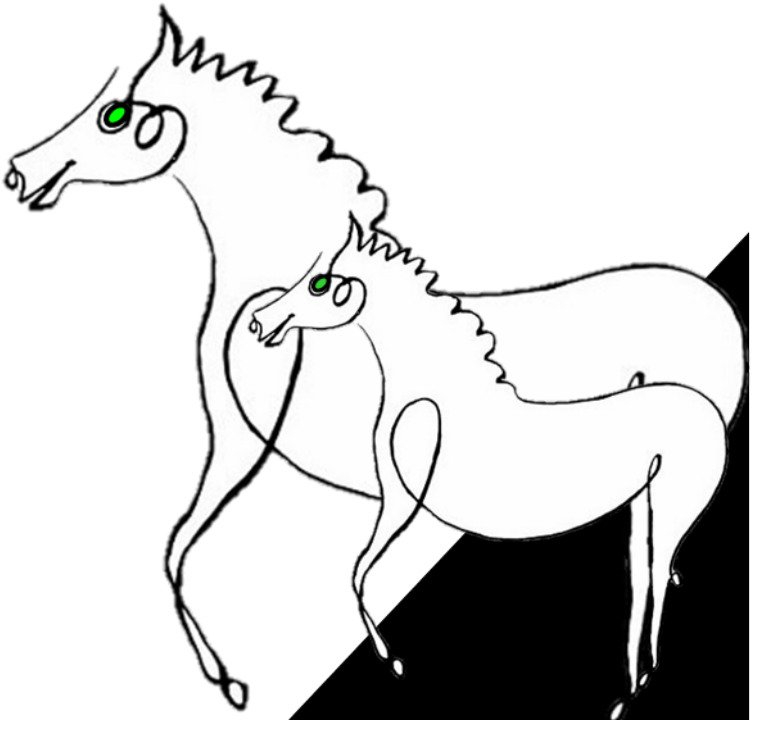
Horses are monogastric herbivores and hindgut fermenters, and their digestive tract is adapted to ingest large quantities of forage in a continuous fashion. Microbial metabolism occurs in a post absorptive part of the gut (cecum and colon) and, therefore, biohydrogenation activity is low compared to ruminants. The digestive physiology of equines is characterized by an efficient transfer of n-3 polyunsaturated fatty acids (PUFAs) from pasture into the meat (Gupta *et al.*, 1951), with a very low formation and deposition of *trans* fatty acids (FA) (Clauss *et al.*, 2009) and conjugated linoleic acids (CLA) (Cicognini *et al.*, 2014; Jahreis *et al.*, 1999).

First studies on the effect of dietary PUFA intake on the FA composition of horse tissues were performed in the fifties, when Shorland *et al.* (1952) evidenced high levels of linolenic acid (LNA) compared to linoleic acid (LA) in grass-fed horse adipose tissue. Moreover, Guil-Guerrero (2013a) suggested that horse backfat could have been a relevant source of energy and n-3 PUFA for the Upper Paleolithic man, time where plants and marine foods availability was minimal due to recurrent glaciations. Horse-meat has been, recently, defined as a "healthy meat" due to its high content in essential and long chain (LC) PUFAs (Lorenzo, 2013). However, it is important to mention that horse-meat consumption has been interrupted throughout history by religious, social and/or cultural reasons while presently, this animal generates positive emotions, such as affection and closeness, and therefore, horse is considered as a pet more than as a domestic meat-producing animal, minimizing and even preventing its consumption in several countries (Fernández de Labastida, 2011; Hintz, 1995). Therefore, despite the horse-meat consumption back in olden times and its reported beneficial nutritional quality, the consumer acceptance of horse-meat as an edible meat is still very minor compared to other red meats.

Currently, below twenty scientific studies about horse-meat quality have been published (Badiani *et al.*, 1997; De Palo *et al.*, 2014; Franco *et al.*, 2013; Franco *et al.*, 2014; Guil-Guerrero *et al.*, 2013a; He *et al.*, 2005; Juárez *et al.*, 2009; Lanza *et al.*, 2009; Lorenzo *et al.*, 2010; Lorenzo *et al.*, 2013; Pinto *et al.*, 2004; Sarriés *et al.*, 2006; Tateo *et al.*, 2008; Tonial *et al.*, 2009) which is a significantly minor number

compared to research activities undertaken in other meat producing species (*i.e.*, beef, sheep, pork, poultry). This evidences a lack of thorough characterization of horse-meat quality, especially as it relates to its lipid fraction. Even though satisfactory methodological techniques have been used in most of the aforementioned studies, several minor but relevant FAs (*i.e.*, branched-chain FAs (BCFA), PUFA biohydrogenation intermediates, and dimethylacetals (DMA, generated from ether lipids) have been neglected. In this sense, a better understanding of the horse lipid fraction and its constituents is necessary as it relates to the oxidative stability of the meat during retail, processing and storage (Mahecha *et al.,* 2010), negatively affecting the meat colour as well as its sensory and nutritional characteristics (Addis, 1986; Wood *et al.*, 2004). For this lipid profiling purpose, the utilization of appropriate but also complementary methodological approaches is of major importance.

Chapter II. LITERATURE REVIEW



Chapter II. LITERATURE REVIEW

1. Current horse-meat production, trade and supply at national level

According to the Statistical Yearbook of Spanish Agriculture, Fishery, Food and Environment Ministry (MAPAMA, 2017), which is updated until 2015, the majority of the registered living horses are located in Andalusia region (216,994 heads; 34.3 % of the total), followed by Castile & Leon (9.81 %), Galicia (7.28 %), Extremadura (7.00 %), Asturias (5.7 %), Basque (5.24 %), Catalonia (5.06 %) and Cantabria (4.98 %) as depicted in Figure II.1.

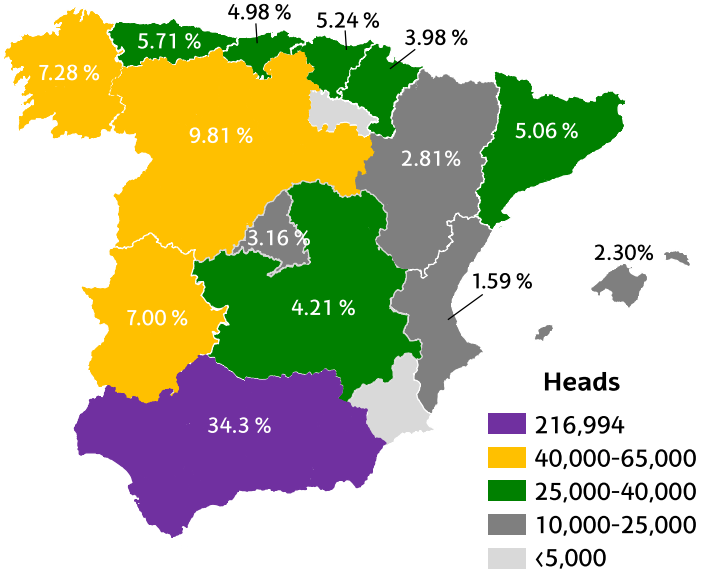


Figure II.1. National registered living horses by regions in 2015 (632,366 heads; MAPAMA, 2017).

However, it is of interest to note that many horse farms are not even cataloged (21.1 %), while most of them are classified as noncommercial (35.7 %) or private farms (15.9 %), and only 9.74 % are registered as horse-meat producing farms, and farms classified as mixed productions where animals for meat production but also riding animals are combined (6.98 %; MAPAMA, 2017). Nevertheless, the number of registered Spanish horse-meat producing farms increased from 10,463 in 2010 to 18,275 in 2015, and most of them are located in the northern regions at both sides

of the Cantabrian Mountains: Asturias (7,598), Basque (3,427), Cantabria (2,600), Galicia (1,394), Navarre (1,303) and Castile & Leon (786). The significant increase could have been related to the recent registration of many farms as meat producing farms. Additionally, the number of current horse farmhouses classified as mixed producers is considerable in Castile & Leon (7,207), Galicia (3,678), and Asturias (1,125) regions.

Regarding the national horse-meat production, it was maintained quite constant until 2010 at an average value of 6,000 tonnes of horse-meat per year. Then, over the next 5 years, the production increased considerably reaching 12,940 tonnes in 2015, where Navarre was the major horse-meat producer (26.6 %), followed by Aragon (23.1 %), Valencia (12.1 %), Cantabria (11.9 %), Catalonia (11.5 %), Castile & Leon (7.92 %), and Asturias (3.84 %; Figure II.2). In general, the enlargement of horse-meat production happened in the last years is associated greatly with increased exports. Exports reached 9,454 tonnes in 2015 with Italy (6,128 tonnes) and France (1,440 tonnes) as main destination countries, while imports to Spain were negligible (328 tonnes) (ITC, 2017). Also, previous two figures (Figure II.1. and II.2.) clearly indicate that the production region does not necessarily coincide with the regions where the horses are being slaughtered.

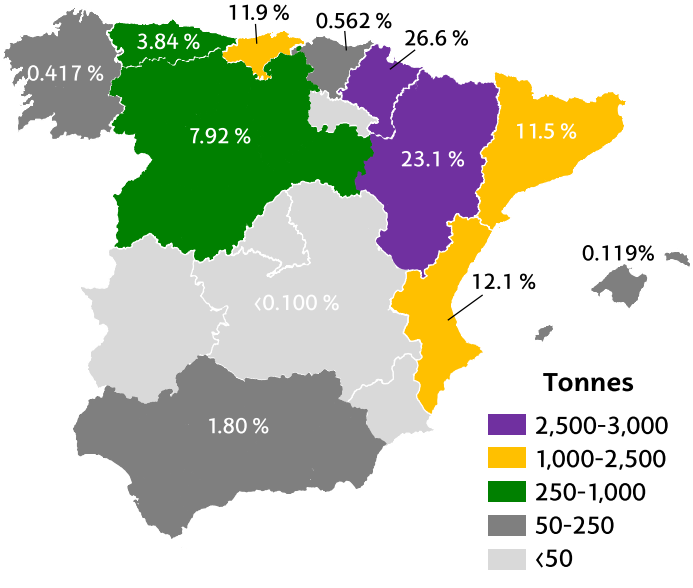


Figure II.2. National horse-meat production by regions in 2015 (12,940 tonnes; MAPAMA, 2017).

In Figure II.3 the national horse-meat supply per capita (2015) has been depicted. Horse-meat supply was calculated using net production values (production plus imports minus exports) relative to the population data. Overall, it was estimated that the average national horse-meat supply was below 0.100 kg per capita. This value was considerably low compared to other highly consumed meat from species like poultry (13.8 kg per capita), pork (10.9 kg per capita) or beef (5.69 kg per capita). These data clearly demonstrate the still low acceptance of horse-meat as a food source in Spain.

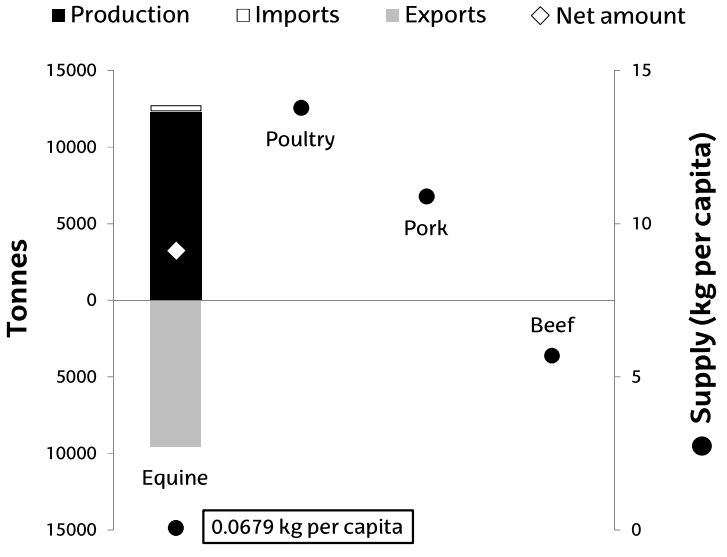


Figure II.3. National horse-meat trade and supply in 2015. The primary axis (left) represents the production, imports, exports and net amount while the secondary axis (right) represents the meat supply.

Estimated net amount (tonnes) = production + exports - imports. Estimated supply (kg per capita) = net amount/population (MAPAMA, 2017).

2. Horse breeds and equine management at both sides of the Cantabrian Mountains

The most relevant local horse breeds in northern Spanish regions are the Basque Mountain Horse in Basque, Burguete and Jaca Navarra in Navarre, Hispano-Breton and Monchina in Cantabria, Asturcón in Asturias, Galician Mountain Horse in

Galicia, and Hispano-Breton in Castile & Leon (Tragsega, 2003). However, it is also very common to see crossbred animals. During the past century, Breton breed, developed in Brittany (France), was introduced in Spain. Over time, and as a result of crossbreeding with other local mares, it was recognized as Hispano-Breton which is actually one of the main national heavy horse breed (Franco *et al.,* 2013).

Historically, horse-meat was obtained from old animals that were used for farm-work (draft animals). However, current horse-meat is supplied primarily from foals that are descendants of ancient heavy draft horses with good carcass and meat yields (Badiani *et al.,* 1997; De Palo *et al.*, 2013; Tateo *et al.,* 2008). In this sense, due to the replacement of autochthonous breeds with more productive ones, several breeds are endangered and threatened with disappearance (García *et al.*, 2013). For example, Jaca Navarra and Monchina autochthonous breeds have been considered in danger and, therefore, included in the list of Domestic Animal Diversity Information System hosted by Food and Agriculture Organization (FAO, 2014).

In terms of horse-meat production systems, equine farms could be classified in two well differentiated but broad groups:

1) Extensive and semi-extensive production systems in mountain regions: Farms located at both sides of the Cantabrian Mountains are mainly characterized by this type of production system, which is associated with the conservation of natural resources (mountain grasslands) and agroecological practices (Figure II.4). Foals are normally born in spring which are suckled by their mothers and reared in mountain conditions. In late autumn, weaned foals are moved to valley pastures and can be directly slaughtered, supplemented with concentrate rations before slaughter, or exported to other regions for fattening and slaughter.

2) Intensive production system: Farms can be divided into small family farms and industrial farms. The first ones normally have around 20-25 horses of relevant northern Spanish breeds. The industrial farms, on the other hand, are exclusively specialized in horse-meat production with 100-800 heads of mixed breeds. At national level in 2015, 258 out of 187,538 horse producing farms were focused on the intensive production (0.138 % of the total farms). The vast majority of these

industrial farms are located in Catalonia (81) and Valencia (42) regions (MAPAMA, 2017).

Figure II.4. Horses grazing in mountain grasslands of the Aralar Natural Park, Basque region.

In terms of horse-meat commercialization, the most commonly commercialized carcasses come from suckling foals of 4 to 8 months of age, fifteenth foals of 10 to 15 months of age, and adult horses of over 15 months of age (Fabregas, 2002). Moreover, it is worth noting that the average horse carcass weights are significantly greater in main production regions such as Aragon (339 kg of carcass weight), Navarre (277 kg), Catalonia (245 kg), Valencia (234 kg), and Cantabria (224 kg), compared to lighter carcasses commercialized in Galicia (153 kg), Basque (153 kg), and Asturias (145 kg; MAPAMA, 2016), while carcasses commercialized in Castile & Leon (192 kg) are of intermediate weights. These differences are more than likely associated to differences in production system.

3. Horse-meat for human consumption – Current research and future opportunities

Publication I compiles worldwide horse-meat production, trade and supply data. Additionally, other advantages of horse production have been evaluated, such as the lower footprint of greenhouse gas emissions and the efficient uptake and deposition of dietary PUFA in muscle and adipose tissues compared to ruminants. Especial emphasis has been made on the nutritional quality of the lipid fraction and its unique FA composition.

PUBLICATION I

Horse-meat for human consumption – Current research and future opportunities

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Review

Horse-meat for human consumption — Current research and future opportunities

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ABSTRACT

The consumption of horse-meat is currently not popular in most countries, but because of its availability and recognized nutritional value consumption is slowly increasing in several western European countries based on claims that it could be an alternative red meat. In this review, horse-meat production, trade and supply values have been summarized. In addition, the advantage of horse production is noted because of its lower methane emissions and increased uptake, particularly of n-3 polyunsaturated fatty acids (PUFAs), which is based on its digestive physiology. Of particular interest in this review is the unique fatty acid composition of horse-meat with its high level of the nutritionally desirable PUFAs in both the adipose and muscle fat. Because of its large frame size and digestive physiology, the horse can be considered an alternative to bovine meat, with large advantages regarding the maintenance of less favored mountain grazing areas and its facility to transfer PUFA from feed to meat.

Contents

1. Horse — its acceptance as edible meat

Horses have been associated with humans for many years. It is thought that all domestic horses are descended from two wild horses (*Equus ferus*): 1) Tarpan, which was native to eastern Europe and Russian steppes and 2) Przewalski from Mongolia (Peplow, 1998; Valderrábano, 1970). There is evidence that before domestication, horses were already used as a food source by humans. Discovered cave paintings and horse bones dated the Paleolithic era (10,000 B.C.), evidence that horses were hunted for food in western Europe (Edwards, 1998; Hintz, 1995; Peplow, 1998). Furthermore, horse tissues might have been a valuable source of essential fatty acids (FAs) for the Upper Paleolithic people, time where plants and marine foods availability were minimal due to recurrent glaciations (Guil-Guerrero, Rincón-Cervera, Venegas-Venegas, Ramos-Bueno, & Suárez-Medina, 2013a). Animal domestication dates at the end of Neolithic era (6000–5000 B.C.), where nomadic tribes of central Asia were mainly involved. Afterwards, horse carcasses and milk, were used as a food source, as

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well as a draft and working animals. Horse riding, however, dates from a much later era, approximately 1500–1000 B.C. (Brown, Pilliner, & Davies, 2003; Peplow, 1998).

Horse-meat consumption has been interrupted throughout history due to religious, social and/or cultural reasons (Fernández de Labastida, 2011). As a consequence, it was not contemplated as a popular meat and was generally associated with poor social classes, famine and periods of short food supply. Presently, this animal can generate positive emotions, such as affection, closeness or tenderness, and for this reason horses are considered as a pet which has further stopped its consumption in several countries.

Historically, horse breeds noted for meat production came from old animals used for farm-working, while nowadays, selected horses for this purpose are bred (Martuzzi, Catalano, & Sussi, 2001; Tateo, De Palo, Ceci, & Centoducati, 2008). Furthermore, nowadays meat production is generally supplied by young animals that are offspring of mainly ancient heavy draft breeds (Tragsega, 2003) which are characterized by high dressing percentages as well as good meat yields (Badiani, Nanni, Gatta, Tolomelli, & Manfredini, 1997; Tateo et al., 2008). In the past century, however, in several European regions, indigenous breeds were replaced by more productive breeds resulting in a high risk of disappearance of the more traditional ones (García et al., 2013). Consequently, some local horse breeds have been classified as endangered and included in the list of Domestic Animal Diversity Information System hosted by FAO (FAO, 2015a).

2. Horse-meat production, trade and supply

According to the information provided by the Food and Agriculture Organization of the United Nations (FAO), worldwide horse-meat production kept quite constant between 1965 and 1990 at an average of 500,000 tonnes per year (FAO, 2015b) (Fig. 1). Since that time, due to the increase of Asian and European production, horse-meat overall production increased by 40%, averaging 700,000 tonnes per year. The significant increase in Asia could have been related to the inclusion of Kazakhstan and Kyrgyzstan in the FAO's database after 1992, as well as an important increase of horse-meat production in China. On the other hand, the main increase in Europe occurred in the nineties, mostly associated with the bovine spongiform encephalopathy crisis (Fernández de Labastida, 2011). By comparison to other meat-producing species like pork, poultry, bovine or ovine, current horse-meat production represents only 0.25% of the total worldwide meat production (Fig. 2; FAO, 2015b).

Production by continents shows that 46% was produced in Asia, 30% in America, 18% in Europe, 4% in Oceania and 2% in Africa (2013 data; FAO, 2015b). Looking at individual countries, China was the major producer (26% of the world production), followed by Kazakhstan (12%),

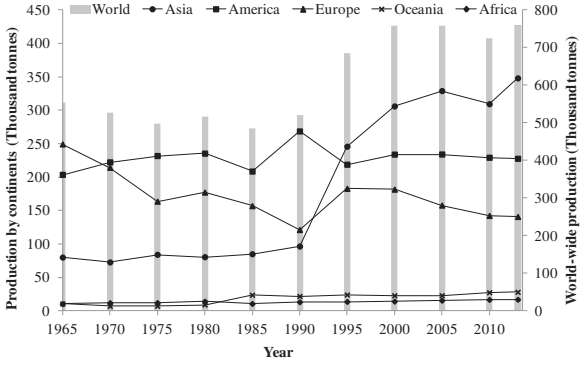

Fig. 1. Worldwide horse-meat production by continents from 1965 to 2013. The lines represent the production per continent with axis on the left in thousand tonnes, while the bars represent the world total production with axis on the right in thousand tonnes.

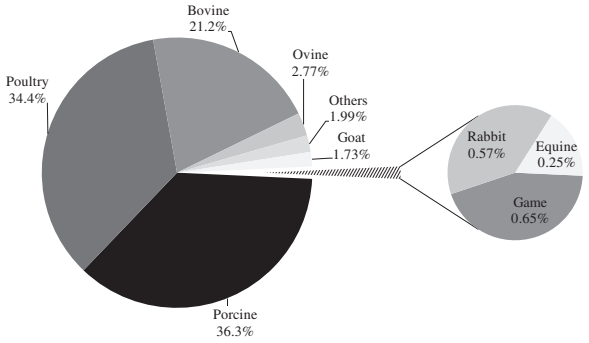

Fig. 2. Worldwide meat production (%) by species in 2013 (FAO, 2015b). Poultry group is represented by chicken, turkey, duck, birds, geese and guinea fowl. The other group represents ass, buffalo, camel, mules, other camelids, and other rodents but not sea snails.

Mexico (11%), Russia (7%) and Argentina (4%); representing 58% of the total word horse-meat production (FAO, 2015b).

Horse-meat trade among continents, shown in Fig. 3, is also considerable (2011 data; FAO, 2015b). The Asian continent is the major producer, with very low imports and exports of 13,651 and 9007 tonnes, respectively. Imports into America are negligible, while exports reached 59,977 tonnes with Europe being the main destination. Horse-meat trade in Europe is important with eastern countries exporting to western European countries. Considering worldwide horse-meat trade, the 10 major importing (a) and exporting (b) countries are summarized in Table 1. Italy, Russia, Belgium and France were the main importing countries, while, Argentina, Belgium, Canada and Mexico were the main exporting countries with each over 10% of the total worldwide imports and exports, respectively.

The fraction of the horse-meat destined for human consumption may in some cases be uncertain, especially in countries where the acceptability of horse-meat as a food source is non-existent (Gill, 2005). Horse-meat supply per capita (2011) was calculated using net production values (production plus imports minus exports) relative to human population data. Overall, it was estimated that the average worldwide horse-meat supply is about 0.10 kg per capita. Countries where the horse-meat supply per capita values are clearly above the world average are Mongolia (5.81 kg), Kazakhstan (4.92 kg), Kyrgyzstan (3.50 kg), Iceland (2.19 kg), Switzerland (0.73 kg), Italy (0.70 kg), Croatia (0.69 kg), Belgium (0.58 kg), Russia and Finland (0.51 kg, each), France (0.27 kg), Malta (0.26 kg), Ukraine and Greece (0.25 kg, each) (Fig. 3). In spite of being the largest producer, the supply value in China is only 0.14 kg per capita. These data demonstrate the regional differences of horse-meat production and consumption, and the globally low utilization of this resource even though there is a huge potential to make a more rational and efficient use of this valuable resource. A partial replacement of beef consumption by horse-meat could be less stressful on the environment since the enteric emission of methane is lower in horses, and they provide a good source of essential FAs due to the high transfer efficiency of PUFAs from pastures to meat. These two aspects will be reviewed in the following sections.

3. Horse — the large framed non-ruminant domestic grazer and browser

Grass and rangelands are traditionally used to maintain ruminant herds that support most ruminant meat production. Ruminants are the most specialized and efficient digesters of grassland and range vegetable biomass (Clauss, Hume, & Hummel, 2010; Janis, 1976). The high efficiency of fiber digestion of ruminants is mainly due to the long retention time in the large gastric fermentative chambers where fibrous particles are subjected to extensive microbial cellulolysis (Van Soest, 1994). However, the high microbial digestive efficiency of ruminants comes at

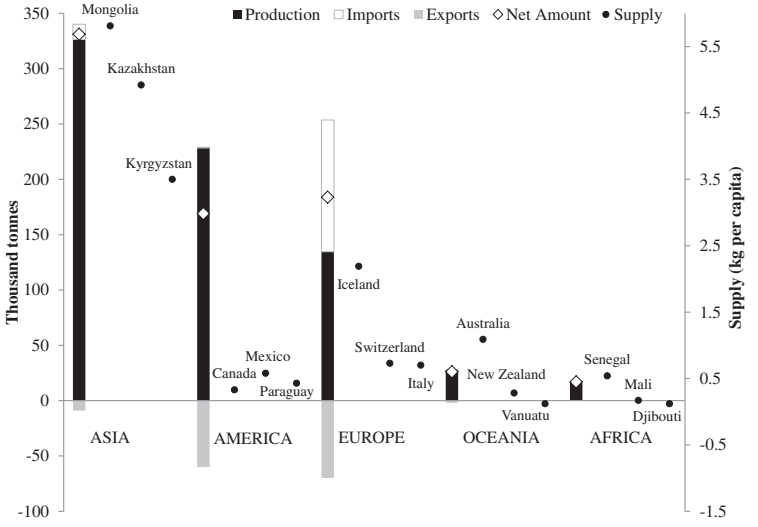

Fig. 3. World horse-meat trade (production, imports, exports, net amount) and the three major supplier countries of each continent in 2011 (FAO, 2015b). The primary axis (left) represents the production, imports, exports and net amount (as bars in thousand tonnes), while the secondary axis (right) represents the horse-meat supply (for individual countries in kg per capita). Estimated amount in tonnes = production + exports − imports. Estimate of supply = net amount/population.

the cost of physical intake constrains when the nutritional quality of feed material is low (Allen, 1996), production of methane that is associated with cellulolysis (Krause, Nagaraja, Wright, & Callaway, 2013), and loss of dietary PUFAs before absorption due to biohydrogenation in the rumen that results in a high content of saturated and *trans*-FA in ruminant edible fats (Aldai, de Renobales, Barron, & Kramer, 2013; Harfoot & Hazlewood, 1997; Lourenco, Ramon-Morales, & Wallace, 2010).

The horse digestive tract and digestive physiology follows an opposite digestive strategy compared to ruminants, with location of the large digestive fermentative chambers in the hindgut (Janis, 1976; Van Soest, 1994). This is usually associated with a lower efficiency of fiber utilization, but a much higher feed intake capacity (Menard, Duncan, Fleurance, Georges, & Lila, 2002), a lower methane emission (Franz et al., 2010), and a capacity to absorb dietary PUFA before the anaerobic microbial hydrogenation.

Table 1
The ten major importing (a) and exporting (b) countries worldwide in 2011 (tonnes; FAO, 2015b).

	Country	Production	Imports	Exports	Net amount	Imports (%)	Exports (%)
(a)	Italy	16,527	28,962	3000	42,489	21.7	2.13
	Russia	47,942	25,562	0	73,504	19.1	0.00
	Belgium	2115	25,206	20,986	6335	18.9	14.9
	France	5000	18,122	5686	17,436	13.6	4.04
	The Netherlands	380	5945	6324	1	4.45	4.50
	Switzerland	775	5003	0	5778	3.74	0.00
	Japan	4868	4512	0	9380	3.38	0.00
	Kazakhstan	75,600	3680	0	79,280	2.75	0.00
	Finland	460	2306	22	2744	1.73	0.02
	Luxembourg	13	1965	1924	54	1.47	1.37
(b)	Argentina	25,970	–	21,225	4745	–	15.1
	Belgium	2115	25,206	20,986	6335	18.9	14.9
	Canada	27,000	0	15,787	11,213	0.00	11.2
	Mexico	83,350	0	14,026	69,324	0.00	10.0
	Poland	12,600	80	11,832	848	0.06	8.41
	Mongolia	24,964	–	8977	15,987	–	6.38
	Uruguay	8170	0	6673	1497	0.00	4.74
	The Netherlands	380	5945	6324	1	4.45	4.50
	Romania	9540	3	6151	3392	0.00	4.37
	France	5000	18,122	5686	17,436	13.6	4.04

Estimated amount in tonnes = production + exports − imports.
–, data not reported.

3.1. Digestive physiology and lipid metabolism in horses

The peculiar PUFA content and profile of horse tissues are associated with their unique digestive system. The digestive physiology of horse clearly indicates that they are herbivores adapted to continuous eating (high chewing efficiency), mainly free-ranging in grassland environments. Equines are hindgut fermenters with a relatively small stomach. The absence of a gall bladder and the continuous secretion of pancreatic juice are also a reflection of their adaptation to continuous eating. The digesta passage rate through their stomach and small intestine is quite fast in comparison to other ruminants (Udén, Rounsaville, Wiggans, & Van Soest, 1982), reaching the hindgut fermentative compartments 3 h after feed consumption (Van Weyenberg, Sales, & Janssens, 2006). Fibrous feed portions are submitted to microbial fermentation in the cecum and colon (Santos, Rodrigues, Bessa, Ferreira, & Martin-Rosset, 2011) with extensive absorption of volatile FAs produced that can contribute to 60–70% of their energy supply (Vermorel & Martin-Rosset, 1997). Equine hindgut has also the ability for selective retention of coarse particles in the cecum, and fluids and small particles in the colon as previously reviewed by Santos et al. (2011). As indicated before, in comparison to ruminants, horses have faster passage rates (Udén et al., 1982; Van Soest, 1994) and, therefore, greater feed intakes (Menard et al., 2002) but only mild digestive efficiencies (Munn, Streich, Hummel, & Clauss, 2008). These abilities allow them to adapt and sustain more efficiently to a broader range of foods, including low quality feeds (Koene, 2006), by increasing their intake (Demment & Van Soest, 1985). This may be critical when quality feed is limited, or when competing with cattle and sheep for the same preferred plant resources (Osoro et al., 2012).

The capacity of horses to digest lipids has been demonstrated to be quite efficient even when lipid supplementation reached about 20% of the dry matter intake (NRC, 2007). Contrary to ruminants, due to the post-gastric localization of digestive fermentative chambers in horses, dietary FAs are absorbed before being submitted to extensive microbial metabolism. This allows an efficient absorption and deposition of PUFAs from pasture species (rich in 18:3n-3, linolenic acid, LNA) into tissues before the PUFAs are subjected to biohydrogenation in the hindgut, as explained earlier. Due to the continuous secretion of biliary salts by the liver together with the pancreatic juice, which is particularly rich in lipases, the horse seems to be capable of efficiently digesting high amounts of dietary lipids in the small intestine.

Table 2
The effect of ante-mortem factors on the fatty acid composition of intramuscular (IM, clear) and subcutaneous (SC, shaded) adipose tissues.

Breed	Age (mo)	Sex	LW (kg)	Weaning at (months)	Grazing (months)	Finishing (months)	IM or SC (%)	16:0	18:0	SFA	9c-16:1	9c-18:1	11c-18:1	11t-16:1	9t-18:1	11t-18:1	9c, 11t-18:2	MUFA	18:2 n-6	20:2 n-6	20:3 n-6	20:4 n-6	n-6	18:3 n-3	20:3 n-3	20:5 n-3	22:5 n-3	22:6 n-3	n-3	PUFA	n-6/n-3	Reference
–	72 – 120	–	–	–	–	–	6.63	26.6	3.72	34.8	10.3	34.1	–	–	–	–	–	46.5	12.0	0.33	0.30	1.09	13.7	4.92	–	–	–	–	4.92	18.6	*2.79*	(1)
MU	10	–	379	6	–	4 ((70 concentrate: 30 hay)[a] + 1.5% CO)	5.12	–	–	–	5.22	31.0	1.60	–	–	–	–	38.9	13.6	0.30	0.10	0.01	14.5	2.00	0.10	0.10	–	0.10	2.30	17.1	6.32	(2)
MU	10	–	386	6	–	4 ((70 concentrate: 30 hay)[a] + 1.5% FO&FXO)	4.95	–	–	–	5.27	32.6	1.65	–	–	–	–	40.9	14.1	0.33	0.20	0.20	15.6	2.17	0.10	0.15	–	0.10	2.52	18.4	6.17	
B	36	–	1124	–	–	12 (commercial concentrate[a])	12.7	26.4	2.52	34.2	11.1	37.1	–	–	–	–	0.15	50.2	13.2	0.26	0.09	0.33	13.9	1.53	–	–	–	–	1.53	15.6	*9.10*	(3)
							82.2	24.5	2.71	31.4	7.56	35.5	–	–	–	–	0.16	44.7	20.5	0.45	0.07	0.18	21.2	2.50	–	–	–	–	2.50	23.9	*8.48*	
BU	16	M	412	7 – 8	8 – 9	7–8 (7–8 kg concentrate/head/day + hay[a])	3.01	29.4	8.73	43.4	3.96	20.3	–	0.11	0.78	1.66	–	28.4	18.8	3.01	0.70	2.53	26.2	0.75	0.10	0.17	–	0.75	1.78	27.9	15.56	
							–	27.9	3.35	37.1	5.82	32.1	–	0.18	0.97	1.38	–	41.6	14.0	4.93	0.22	0.14	19.7	1.37	0.09	0.07	–	0.11	1.57	21.2	13.16	
BU	16	F	411	7 – 8	8 – 9	7–8 (7–8 kg concentrate/head/day + hay[a])	3.32	29.6	8.24	43.5	4.58	20.3	–	0.16	0.80	1.68	–	29.2	17.3	3.69	0.86	2.39	25.1	1.07	0.15	0.15	–	0.60	1.99	27.1	15.43	
							–	27.8	3.40	36.2	6.47	33.9	–	0.09	0.63	1.50	–	43.9	13.7	3.38	0.16	0.20	17.8	1.98	0.08	0.04	–	0.06	2.15	19.9	9.51	(4)
BU	24	M	394	–	16 – 17	7–8 (7–8 kg concentrate/head/day + hay[a])	5.21	28.6	6.80	41.5	4.99	23.0	–	0.13	0.79	1.54	–	31.9	16.6	3.71	0.63	1.66	23.3	2.43	0.16	0.12	–	0.40	3.12	26.4	9.04	
							–	29.5	2.95	39.5	5.75	27.4	–	0.19	0.80	1.21	–	37.0	14.0	5.24	0.34	0.12	20.2	2.90	0.13	0.07	–	0.20	3.15	23.3	7.17	
BU	24	F	397	–	16 – 17	7–8 (7–8 kg concentrate/head/day + hay[a])	4.76	29.8	5.52	41.1	6.70	25.8	–	0.16	0.80	1.67	–	36.6	13.5	3.65	0.35	1.34	19.4	2.18	0.11	0.11	–	0.39	2.81	22.2	8.00	
							–	28.2	2.91	37.6	6.45	29.9	–	0.14	0.60	1.33	–	40.1	14.3	4.19	0.17	0.11	19.2	2.76	0.13	0.06	–	0.09	2.99	22.2	7.08	
IHDH	11	M	–	6	–	5 (concentrate[a])	4.52	29.8	7.80	44.2	1.13	26.8	–	–	–	–	–	31.1	19.8	–	–	–	–	4.43	–	–	–	–	–	24.8	*4.59*	
							–	27.9	4.17	37.2	6.59	35.9	–	–	–	–	–	44.3	16.4	–	–	–	–	1.71	–	–	–	–	–	18.5	*9.81*	(5)
IHDH	11	F	–	6	–	5 (concentrate[a])	4.01	31.1	6.52	44.7	1.16	29.6	–	–	–	–	–	33.9	16.6	–	–	–	–	4.18	–	–	–	–	–	21.4	*4.11*	
							–	27.5	3.09	35.2	8.37	36.8	–	–	–	–	–	47.1	15.5	–	–	–	–	1.84	–	–	–	–	–	17.8	*8.66*	
SA	18	–	411	7 – 8	15	3 (5 kg concentrate/head/day + hay[a])	2.29	26.8	6.43	36.9	5.45	24.8	2.01	–	0.18	0.02	0.45	33.3	20.9	0.19	–	3.84	25.1	1.88	0.17	0.52	1.26	0.37	4.18	29.9	6.70	(6)
HA	18	–	350	7 – 8	15	3 (5 kg concentrate/head/day + hay[a])	2.44	26.2	6.26	36.3	5.13	25.1	1.73	–	0.05	0.00	0.47	32.9	21.0	0.16	–	2.74	23.9	3.90	0.22	0.43	1.34	0.40	6.29	30.8	4.09	
BU	24	M	675	7 – 8	16 – 17	7–8 (7–8 kg concentrate/head/day + hay[a])	2.08	25.1	7.47	38.0	4.99	27.4	–	–	–	–	0.46	34.4	20.3	–	–	–	–	5.50	–	–	–	–	–	27.6	*3.70*	
							–	28.3	4.13	38.5	7.97	31.5	–	–	–	–	0.50	41.1	13.9	–	–	–	–	5.63	–	–	–	–	–	20.5	*2.48*	(7)
HB	24	M	715	7 – 8	16 – 17	7–8 (7–8 kg concentrate/head/day + hay[a])	2.22	26.1	7.15	38.7	6.23	28.0	–	–	–	–	0.39	37.6	16.3	–	–	–	–	4.27	–	–	–	–	–	23.8	*3.83*	
							–	25.7	3.82	35.8	6.71	30.1	–	–	–	–	0.47	39.5	16.0	–	–	–	–	7.47	–	–	–	–	–	24.7	*2.15*	
–	108 – 132	M	460 – 530	–	–	–	2.90	25.9	7.47	36.8	2.88	22.2	1.58	–	–	–	–	27.4	22.9	–	0.34	2.97	34.9	0.43	–	0.43	–	–	0.86	35.8	40.6	(8)
GM	9	M,F	–	–	6	3 (3 kg concentrate/head/day + hay[a])	0.31	29.2	4.36	38.6	6.01	29.3	–	–	–	–	–	36.1	15.2	0.24	0.09	0.59	15.9	8.90	0.08	–	–	0.09	9.07	25.2	1.86	(9)
GM	9	M,F	–	–	9	0 (extensive system)	0.15	25.7	4.97	36.2	4.00	17.9	–	–	–	–	–	22.7	14.1	0.18	0.19	1.85	16.2	23.9	0.31	–	–	0.21	24.4	40.7	0.74	
GM, GM x HB	15	–	273	6 – 8	11	4 (1.5 kg concentrate/head/day + hay[a])	0.15	24.7	6.08	36.3	3.63	27.1	–	–	–	–	–	32.0	17.5	0.29	0.36	0.16	19.5	11.8	0.49	0.55	–	0.34	13.2	33.0	1.47	(10)
GM, GM x HB	15	–	287	6 – 8	11	4 (3 kg concentrate/head/day + hay[a])	0.58	25.8	5.01	35.3	5.58	34.0	–	–	–	–	–	40.8	13.6	0.28	0.26	0.12	15.1	6.52	0.34	0.27	–	0.10	7.23	22.6	2.08	
GM	15	M	195	6 – 7	15	0 (extensive system)	0.10	23.0	6.60	40.2	2.60	14.0	–	–	–	–	–	18.1	20.6	–	0.80	4.90	26.4	11.8	0.60	1.30	–	0.80	14.7	41.5	1.80	(11)
GM	15	F	185	6 – 7	15	0 (extensive system)	0.10	24.3	6.50	39.1	3.00	15.9	–	–	–	–	–	20.3	18.9	–	0.60	3.40	23.0	14.5	0.60	1.00	–	0.70	17.1	40.5	1.30	
GM	15	–	–	6 – 7	–	0 (extensive system)	0.22	21.1	5.72	35.5	2.53	1.26	–	–	–	–	–	16.4	17.1	0.28	0.66	3.75	21.5	11.4	0.60	1.08	–	0.68	13.7	35.6	1.64	(12)
GM	24	M,F	–	–	–	3 (5 kg of concentrate/head/day + hay[a])	0.60	17.6	10.0	–	0.70	6.30	2.00	–	–	–	–	–	32.4	–	–	6.70	39.1	0.90	–	0.90	ND	0.50	2.30	41.4	*17.0*	
							63.7	27.9	4.6	–	5.50	25.1	0.70	–	–	–	–	–	15.9	–	–	ND	15.9	7.60	–	ND	0.20	ND	7.80	23.7	*2.04*	(13)
GM	24	M,F	–	–	–	0 (extensive system)	0.70	15.8	10.7	–	0.80	7.70	1.90	–	–	–	–	–	29.9	–	–	5.80	35.7	4.50	–	1.70	3.30	0.80	10.3	46.0	*3.47*	
							60.5	23.4	7.4	–	2.80	27.5	0.60	–	–	–	–	–	7.6	–	–	ND	7.60	24.3	–	ND	0.20	ND	24.5	32.1	*0.31*	
GM	15	–	–	6 – 8	11	4 (1.5 kg concentrate/head/day + hay[a])	0.58	24.4	6.02	35.9	3.58	26.6	–	–	–	–	–	31.5	17.4	0.36	0.48	0.21	19.3	11.6	1.58	0.33	–	–	13.0	32.6	1.48	(14)
GM	15	–	–	6 – 8	11	4 (3 kg concentrate/head/day + hay[a])	0.15	26.0	5.13	35.8	5.68	34.1	–	–	–	–	–	41.0	14.2	0.27	0.35	0.03	15.7	6.51	1.21	0.12	–	–	7.27	23.2	2.15	
IHDH	11	M	448	4	–	– (65 concentrate: 35 hay) at 150% m.r.	2.96	33.3	6.81	47.8	1.25	25.8	–	–	–	–	–	27.1	17.7	–	–	0.77	*19.4*	4.48	–	–	–	–	*4.48*	28.4	*4.33*	(15)
IHDH	11	M	456	4	–	– (65 concentrate: 35 hay) at 180% m.r.	3.11	31.8	7.02	45.4	1.11	26.0	–	–	–	–	–	28.5	18.8	–	–	0.56	*20.0*	4.50	–	–	–	–	*4.50*	25.8	*4.45*	
MSHB	60 – 168	F	525 – 673	–	1[c]	3 ((1.8 g barley/kg BW) + hay[b])	–[d]	–	–	–	–	–	–	–	–	–	–	–	35.1	–	–	7.05	–	1.77	–	1.08	2.66	4.88	–	–	*3.38*	
MSHB	60 – 168	F	525 – 673	–	1[c]	3 (1.1 g barley/kg BW/day + hay[b] + AL&FO)	–[d]	–	–	–	–	–	–	–	–	–	–	–	28.6	–	–	6.54	–	1.18	–	2.72	1.54	8.96	–	–	*1.99*	(16)
MSHB	60 – 168	F	525 – 673	–	1[c]	3 (0.51 g barley/kg BW/day + hay[b] + FXS)	–[d]	–	–	–	–	–	–	–	–	–	–	–	34.8	–	–	7.73	–	1.97	–	1.09	2.89	4.62	–	–	*3.29*	
K		F	364	–	–	During weaning (1.5kg oats + 5–6kg hay/day)	2.27[e]	26.0	2.34	49.6	5.16	21.0	1.49	–	–	–	0.00	29.5	8.29	–	–	0.37	8.66	12.3	–	–	–	–	12.3	20.9	0.96	(17)
K		F	364	–	–	During weaning (1.5kg oats + 5–6kg hay/day)	1.51[f]	21.7	0.97	46.1	5.72	16.5	0.86	–	–	–	0.03	25.5	7.02	–	–	0.41	7.46	20.9	–	–	–	–	20.9	28.4	0.37	
Survey performed in the northern Spain (n = 82)							1.98	25.7	6.15	36.8	5.55	24.3	1.90	0.04	0.08	0.03	0.03	33.9	14.1	0.25	0.41	1.53	16.5	6.3	0.31	0.40	0.93	0.25	8.5	25.3	2.95	(18)
						(n = 74)	78.3	25.8	4.05	37.8	6.16	26.9	1.48	0.01	0.06	0.03	0.06	36.8	11.6	0.25	0.05	0.09	12.1	11.5	0.34	0.03	0.17	0.04	12.3	24.4	2.54	

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; GM, Galician Mountain; HB, Hispano-Breton; SA, Sanfratello; HA, Haflinger, BU, Burguete; IHDH, Italian Heavy Draft; MU, Murgese; MSHB, mixed stock horse breed; K, Konik.
[a]ad libitum.
[b]1.6% of BW on a DM basis (BW, body weight; DM, dry matter).
[c]Basal diet of free-choice alfalfa grass hay; m.r., maintenance requirements; FO, fish oil; FXO, flax oil; CO, coconut oil; FXS, flaxseed; AL, algae.
[d]Biopsy of *middle gluteal* muscle.
[e]Colostrum (average of two lactation days).
[f]Milk (average of six lactation months).
–, data not reported; ND, not detected.
Data in italics are based on limited information.
(1) Badiani et al., 1997; (2) Pinto et al., 2004; (3) He et al., 2005; (4) Sarriés et al., 2006; (5) Tateo et al., 2008; (6) Lanza et al., 2009; (7) Juárez et al., 2009; (8) Tonial et al., 2009; (9) Lorenzo et al., 2010; (10) Franco, Crecente, Vázquez, Gómez, & Lorenzo, 2013; (11) Lorenzo et al., 2013; (12) Lorenzo & Pateiro, 2013; (13) Guil-Guerrero et al., 2013a; (14) Franco & Lorenzo, 2014; (15) De Palo et al., 2014; (16) Hess et al., 2012; (17) Pikul et al., 2008; (18) Belaunzaran et al., unpublished.

Most of the LNA found in pasture species is esterified in galactolipids located in choloroplasts and thylakoid membranes of higher plants that are composed primarily of phospholipids (PL) and galactolipids. The higher ability of the horse to hydrolyse these galactolipids has been associated either with pregastric microbial activity or with a specific pancreatic lipase related to protein 2 (PLRP2; Amara et al., 2013; Andersson, Carrière, Lowe, Nilsson, & Verger, 1996). It has been reported that PLRP2 is absent in pigs, turkeys and ruminants but surprisingly present in horses (De Caro et al., 2008). This enzyme could potentially explain the high content of LNA deposition in horse tissues in comparison to porcine and poultry tissues (He, Ishikawa, & Hidari, 2005; Ribeiro et al., 2013). Based on the studies by Lorenzo, Fuciños, Purriños, and Franco (2010) and Guil-Guerrero et al. (2013a) a large accumulation of LNA in horse tissues was evident when fed under extensive conditions (grass feeding) compared to animals finished with concentrates (3–5 kg/day for the last 3 months before slaughter (Table 2)). These data indicate that the horse might be one of the best species to transfer n-3 PUFAs from pastures to humans as suggested by Guil-Guerrero et al. (2013b).

3.2. Greenhouse gas (GHG) emissions from horses

It is known that livestock activity contributes significantly to the global GHG emissions and climate change (Garnett, 2009; McMichael, Powles, Butler, & Uauy, 2007). However, worldwide meat demand is constantly increasing associated with population growth and income level. Currently the average meat supply is around 43.3 kg per capita (2012 data; FAO, 2015b). As global consumption of products from animal origin is increasing, less 'climate-harmful' and more sustainable meat production systems should be encouraged to reduce the GHG emissions to the atmosphere. In this regard, horse-meat production could be environmentally friendlier since equines produce significantly less GHGs than ruminants.

It has been estimated that 18% of global GHG emissions measured in carbon dioxide equivalents (CO_2eq) is attributed to animal production. Livestock activity emits 9% of anthropogenic CO_2, 37% of CH_4 and 65% of N_2O (Steinfeld et al., 2006).

Based on the domestic animal population, Asia is responsible for the 38.9% of CH_4 emissions associated with the fermentation of the digestive tract, followed by America (31.8%), Africa (15.3%), Europe (10.5%) and Oceania (3.5%). Among countries, India, Brazil, China, USA and Pakistan are the major emitters (FAO, 2015b). Regarding N_2O emissions related to manure management, Asia is responsible for 57.8% of the emissions followed by America (17.9%), Europe (17.5%), Africa (5.3%) and Oceania (1.6%); with China, USA, India, Russia and Brazil as the main emitting countries (FAO, 2015b). It is worth noting that the amount of GHG emissions differ between livestock species and production systems (Kramer, Moll, Nonhebel, & Wilting, 1999; Lesschen, van den Berg, Westhoek, Witzke, & Oenema, 2011; Steinfeld et al., 2006). Ruminants are the greatest contributors of CH_4 emissions (Lesschen et al., 2011; Zervas & Tsiplakou, 2012). It is estimated that cattle (dairy and non-dairy) are responsible for about 73.0% of CH_4 emissions followed by buffaloes (11.0%), sheep (6.6%), goats (5%), horses (1.1%) and pork (1.0%) (FAO, 2015b). Regarding N_2O emissions associated with manure management, it is estimated that 51.0% come from cattle (dairy and non-dairy), followed by pork (23.3%), chickens (11.2%), buffaloes (7.3%), sheep (2.9%), turkeys (1.6%), ducks (1.3%), goats (1.1%) and horses (0.2%) (FAO, 2015b).

The potential advantage of horses compared to big body size ruminants is that they produce less CH_4 (Crutzen, Aselmann, & Seiler, 1986; Franz et al., 2010; Moss, Jouany, & Newbold, 2000). In general, energetic losses in horse due to CH_4 production average 3.5% of digestible energy of feeds compared to 10–13% in adult ruminants (Vermorel, 1997) which could be explained by a lower retention time of digesta and lower microbial population density in the digestive fermentation chambers in horses compared to ruminants (Franz et al., 2010). Moreover, hindgut fermentation is characterized by a much higher reductive acidogenesis than in the rumen fermentation, which results in lower methanogenesis (Demeyer, Graeve, Durand, & Stevani, 1989; Franz et al., 2010; Kienzle & Zeyner, 2010). The environmental part is not the only benefit of horse production versus other traditional productions (i.e., bovine, ovine, caprine). In countries like Spain, equine production has been an interesting option in less favored mountain areas where human population is very low. All year round mountain grazing extensive systems make the management simple with no need of indoor housing.

3.3. Quality of horse-meat lipids

The protein and mineral value of horse-meat will not be reviewed here but in general it compares well with other meats (Lorenzo et al., 2014). On the other hand, the lipid profile has distinct features that have not been critically reviewed, specifically as it relates to PUFAs and *trans*-FAs.

The FA composition of the horse, a hindgut fermenter herbivore, was shown to have the ability to efficiently transfer PUFA from the diet into meat (Gupta & Hilditch, 1951). In general, its FA profile is described as 'healthy' due to its high content in essential and other PUFAs (Lorenzo, 2013; Mordovskaya, Krivoshapkin, Pogozheva, & Baiko, 2005). Some researchers even claimed horse-meat as a 'dietetic meat' since it could serve as an alternative to red meat from beef (Badiani et al., 1997; Lorenzo et al., 2010).

Most of the parameters related to the quality of horse-meat have been recently reviewed by Lorenzo et al. (2014). These authors have compiled the data obtained from scientific studies where the effects of ante- or post-mortem factors on horse carcass and meat quality were investigated. These studies were generally conducted in countries where horse-meat consumption is considerable, i.e., Brazil, Canada, Japan, Korea, Russia, Italy, Switzerland, France, Poland and Spain. In most of these investigations the primary focus was on local breeds and on factors such as age (or live weight) at slaughter, sex, and production system (mainly related to feeding) (Lorenzo et al., 2014).

In relation to intramuscular (IM) and subcutaneous (SC) FA composition of horse-meat, our interest has been to critically discuss the FA composition of horse-meat particularly as it relates to FAs that provide a health image (Table 2). For most of the studies, post-mortem IM fat composition of the *longissimus thoracis et lumborum* muscle was reported (Badiani et al., 1997; De Palo, Tateo, Maggiolino, & Centoducati, 2014; Franco, Crecente, Vázquez, Gómez & Lorenzo, 2013; Franco & Lorenzo, 2014; Guil-Guerrero et al., 2013a; He et al., 2005; Juárez et al., 2009; Lanza, Landi, Scerra, Galofaro, & Pennisi, 2009; Lorenzo et al., 2010; Lorenzo & Pateiro, 2013; Pinto, Schiavone, & Marsico, 2004; Sarriés, Murray, Troy, & Beriain, 2006; Tateo et al., 2008; Tonial et al., 2009). However, Hess et al. (2012) also determined the ante-mortem FA profile from biopsies taken from the *gluteus medius* muscle. From the aforementioned studies, only few reported the SC fat profile (Table 2 shaded; Guil-Guerrero et al., 2013a; He et al., 2005; Juárez et al., 2009; Sarriés et al., 2006; Tateo et al., 2008). The FA profiles of colostrum (average of two lactation days) and milk (average of six lactation months) of primitive horses (Pikul, Wójtowski, Danków, Kuczyńska, & Łojek, 2008) were also included for purpose of comparison. We have included some of our own data obtained from SC and IM fat (Belaunzaran et al., unpublished), which is part of an ongoing survey of horse loin steaks that were collected in the northern regions of Spain.

The total FA content of SC adipose tissue was reported in only few studies (Table 2 shaded) and this value ranged between 60.5% in Galician mountain horse (Guil-Guerrero et al., 2013a) to 82% in the Breton breed (He et al., 2005), and in most cases the exact sampling site was not indicated. In our survey, where samples from different breeds were collected, we observed a range from 457 to 904 mg/g of SC adipose tissue from the *longissimus thoracis et lumborum* muscle (average value of 715 mg/g).

In reference to muscle fat content (Table 2), it is noteworthy to point out the huge variability in the IM fat content that ranged from 0.1% (Lorenzo, Sarriés, & Franco, 2013) to 12.7% (He et al., 2005) depending primarily on breed and feeding. Values of 0.1% appear questionable since the content of PLs in muscle is relatively independent of the total fat content and is known to vary between 0.2% and 1% of muscle weight (De Smet, Raes, & Demeyer, 2004). The content of muscle triacylglycerol on the other hand is strongly related to the total fat content and varies from 0.2% to more than 5%. Some of the reported variations in fat content may be related to differences in extraction methods. The use of appropriate extraction methods, which also extract PLs, is highly recommended to obtain a more accurate and correct value of the total fat content of the muscle (Aldai et al., 2012a). For this reason we decided to disregard the extremely low values of fat content reported in extensively reared foals (from 0.1 to 0.3%). Based on the remaining data, the IM fat content in extensively reared horses appear to range from 0.5 to 0.7% depending on the age at slaughter, while for concentrate-fed horses it ranged from 4% (11 months at slaughter) to 12.7% (36 months at slaughter). In our survey we obtained values ranging from 0.47 to 8.43% of muscle IM fat (Belaunzaran et al., unpublished). The variability is also reflected in the main FA groups and major individual FAs that depend mainly on the diets fed (Lorenzo et al., 2014). The saturated fatty acid content varied from 34.2% to 47.8%, while the monounsaturated fatty acid (MUFA) content ranged from 16.4% to 50.2%; this variability was generally accounted for by differences related to animal age and feeding strategies. Oleic acid (9*c*-18:1) was the major *cis*-MUFA representing between 70 and 90% of the total MUFA, while *trans*-MUFAs were reported only in a few studies (Lanza et al., 2009; Sarriés et al., 2006). Of the conjugated linoleic acid (CLA), only the 9*c*,11*t*- and 10*t*,12*c*-18:2 isomers were reported in some studies (He et al., 2005; Juárez et al., 2009; Lanza et al., 2009). Unfortunately the GC columns and conditions used do not appear to be sufficient to separate potentially coeluting CLA and *trans*-FA isomers (Cruz-Hernandez et al., 2004; Delmonte et al., 2011; Kramer, Hernandez, Cruz-Hernandez, Kraft, & Dugan, 2008). CLA content in horse-meat is generally very low in comparison to ruminant species because the latter are produced from PUFAs by the action of rumen microbiota. However, the accumulation of low amounts of *trans*-FAs (all FAs with at least one double bond in *trans*-configuration) in horse tissues is not unexpected since the mechanism of *trans*-FA formation occurs in the post-absorptive region of the gut rather than in the rumen as occurs in ruminants (Clauss, Grum, & Hatt, 2009; Hartam, Shorland, & Moir, 1956). Some bacterial activity has been identified in the large intestine of horse (Dougal et al., 2013), and 'rumen-like' metabolites have been, therefore, reported in bacterial fractions collected from cecal and colon contents (Santos, Jeronimo, Ferreira, Rodrigues, & Bessa, 2013) and feces (Hartam et al., 1956). In the data published by Santos et al. (2013), vaccenic acid (VA; 11*t*-18:1) and 6–8*t*-18:1 were reported as major *trans*-18:1 isomers, while branched-chain FAs (BCFAs) were also found in the bacterial (liquid and solid associated-) fractions. On the other hand, others have reported 9*t*- and 11*t*-18:1 as major 18:1 isomers in horse fat (Lanza et al., 2009; Sarriés et al., 2006), but there was no mention of the individual or total contents of BCFAs in horse. Our results from SC and muscle FA analyses support the muscle data published by Lanza et al. (2009), indicating that the content of 9*t*- was higher than 11*t*-18:1. However, our total content of these isomers was 10 to 50 times smaller than the values reported by Sarriés et al. (2006) in SC and muscle tissues.

Finally, the variability in PUFA percentage in horse-meat was quite significant ranging from 15.6% in 3-year-old horses fed commercial concentrates (He et al., 2005), to 46% in 2-year-old grass fed animals (Guil-Guerrero et al., 2013a). In absolute amounts, however, concentrate-fed animals provided a meat with a higher PUFA content (1.98 g/100 g of fresh meat), due to the greater IM fat content compared to grass fed animals (0.32 g/100 g of fresh meat). High linoleic (LA; 18:2n-6) and LNA (18:3n-3) acid contents were observed in horse fat (Brooker & Shorland, 1950; Gupta & Hilditch, 1951) mainly related to the low biohydrogenation activity in the gut and efficient uptake of PUFAs (Brooker & Shorland, 1950; Clauss et al., 2009). In hindgut fermenter herbivores, most dietary PUFAs are absorbed in the small intestine before being subjected to microbial processes in the hindgut. This was clearly evident in Table 2 in which the relative amount of total n-6 PUFAs ranged from 13.7% (Badiani et al., 1997) to 39.1% (Guil-Guerrero et al., 2013a), while the values of n-3 PUFAs ranged from 0.86% (Tonial et al., 2009) to 24.4% (Guil-Guerrero et al., 2013a; Lorenzo et al., 2010) depending primarily on the breed type and feed consumed. In some cases, higher contents (%) of n-3 relative to n-6 PUFAs in horse tissues (Guil-Guerrero et al., 2013a; Lorenzo et al., 2010) and milk (Pikul et al., 2008) resulted in n-6/n-3 ratios of <1.

A comparison of several domestic species showed that the LA percentages of horse-meat ranged between 12 and 32.4%, which was somewhat similar to that found in pork (12.8–23.4%; Enser, Richardson, Wood, Gill, & Sheard, 2000; Raes, De Smet, & Demeyer, 2004; Raj et al., 2010; Wood et al., 2008) and chicken (13.7–24.7%; Azcona et al., 2008; Woods & Fearon, 2009) muscle. A comparison of LNA and other long-chain n-3 PUFA percentages clearly showed that horse-meat had higher values than beef cattle. The muscle LNA content in horse ranged between 0.43 and 23.9% while in beef it ranged between 0.26 and 3.96% (Aldai, Dugan, Rolland, & Kramer, 2009; Aldai, Lavín, Kramer, Jaroso, & Mantecón, 2012b; French et al., 2000; Mach et al., 2006; Raes et al., 2004). Similar differences were observed for long-chain n-3 PUFA contents (20:5n-3, 22:5n-3, 22:6n-3). The range in horse meat was between 0.10–1.70%, 1.26–3.30% and 0.09–0.80% for 20:5n-3, 22:5n-3, and 22:6n-3, respectively, while in beef it ranged between 0.10–1.90%, 0.27–1.73% and 0.01–0.28, respectively (Aldai et al., 2009; Aldai et al., 2012b; French et al., 2000; Mach et al., 2006; Raes et al., 2004).

In the literature (Table 2), several n-6 PUFAs other than LA have been reported, but it is not clear which of these n-6 PUFA is the second most abundant, i.e., 20:2n-6, 20:3n-6 or 20:4n-6, since considerable variations were observed between these reports. Unfortunately, most of the studies report only a limited FA profile of horse tissues. The same was found for the n-3 PUFAs. Apart from LNA, it is not clear which other n-3 PUFA is the second most abundant isomer, i.e., 20:3n-3, eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) or docosahexaenoic acid (DHA; 22:6n-3). Again, there are large differences between studies. Some report 22:5n-3 as the most abundant n-3 metabolite, while others report 22:6n-3. For example, in meat from extensively reared Galician Mountain horse, 20:3n-3 was reported as the second major n-3 PUFA when animals were slaughtered at 9 months (Lorenzo et al., 2010), 20:5n-3 when animals were slaughtered at 15 months (Lorenzo & Pateiro, 2013; Lorenzo et al., 2013), and 22:5n-3 when animals were slaughtered at 24 months (Guil-Guerrero et al., 2013a). In general, DPA was the least reported long-chain n-3 PUFA metabolite (only 3 studies; Guil-Guerrero et al., 2013a; Hess et al., 2012; Lanza et al., 2009) even though it is generally recognized to be quantitatively higher than DHA in most mammals. As indicated by Guil-Guerrero et al. (2013a,b), if both the horse-meat and backfat are consumed, the total contents of EPA, DPA and DHA could meet the daily long-chain n-3 PUFA requirements of humans as specified in some recommendations. For years it was believed that there was a real benefit of horse as a source of n-3 PUFAs, especially for population's dependant mostly on terrestrial mammals for subsistence and with little access to marine mammals (Stiner, Munro, Surovell, Tchernov, & Bar-Yosef, 1999). The debate is still ongoing as to which n-3 PUFA should be included in any recommendation, whether it should be restricted to the content of EPA and DHA, or whether LNA (Barceló-Coblijn & Murphy, 2009) or DPA (Byelashov, Sinclair, & Kaur, 2015) should be included. This will depend on the human body's ability to convert adequate amounts of LNA to EPA and DHA, and which long-chain n-3 PUFAs provide biological functions. The recommended daily intake (or more accurately availability) of EPA plus DHA ranges from 250 mg/day by the European Food Safety Authority to 650 mg (minimum 220 mg of each)/day by the National Institute of Health

(see http://www.goedomega3.com/healthcare). Even though only few studies reported the FA composition of horse backfat, this tissue is known to contain appreciably higher LNA levels than muscle tissue, while the content of long-chain n-3 PUFAs is higher in muscle (Table 2).

4. Conclusions

Horse, as a domestic animal, could be bred not only for leisure activities but also for meat production as is already the case in many countries. Equines are large framed and hindgut fermenter herbivores that can compete advantageously with ruminants for the utilization of pastures and rangelands. The digestive physiology of equines allow then to efficiently transfer the n-3 PUFAs from pasture into meat with very low *trans*-FA deposition and low methane emissions per unit of meat produced in comparison to ruminants.

More studies will be necessary to establish a more accurate FA composition of horse-meat and factors to improve its composition. Further research will also be required to clarify the FA profile in horse and relate them to the characteristic digestive processes and fat metabolism in the horse that is still not fully understood.

Equine meat production under sustainable extensive systems should be encouraged 1) to maintain endangered local horse breeds, 2) to obtain a healthy product in terms of its FA profile (n-3 PUFA content), 3) to reduce GHG emissions to the atmosphere, and 4) to conserve natural resources (mountain areas), as grazing could provide greater diversity of habitats benefiting fauna and flora that bring environmental and social benefits to rural areas.

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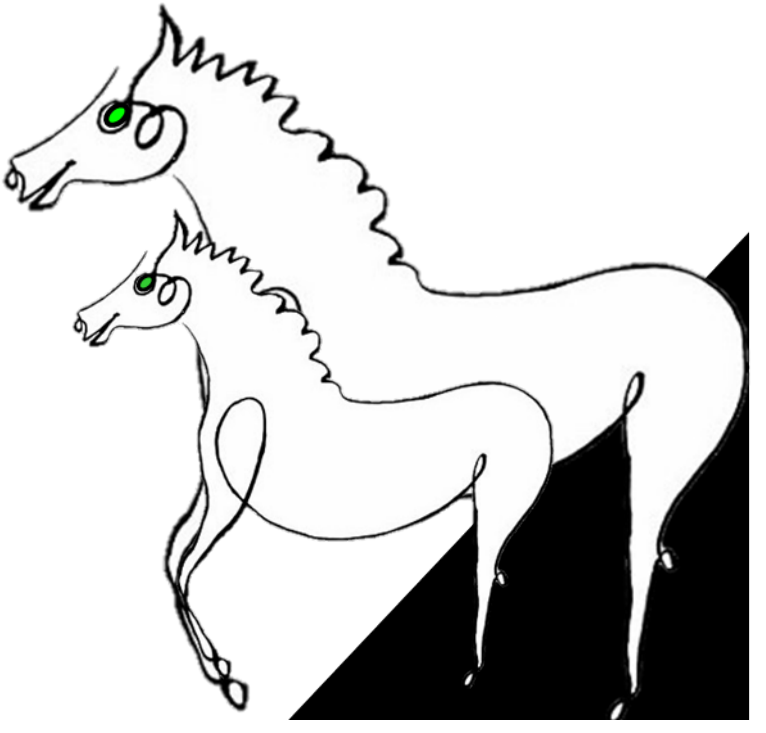
Chapter III. OBJECTIVES



Chapter III. OBJECTIVES

This Ph. D. Thesis has been conducted within Lactiker Research Group at the University of the Basque Country (UPV/EHU), which is dedicated to perform multidisciplinary research on the field of Quality and Safety of Food from Animal Origin, and in close collaboration with the Mountain Livestock Institute of the Spanish National Research Council – University of León consortium.

The overall aim of the present Ph. D. Thesis was to evaluate the nutritional quality of horse-meat, specifically the quality of the lipid fraction, and to prove the usefulness of a lipidomic approach to accurately monitor the changes in the nutritional quality of horse-meat as a result of different management practices. This would help to promote horse production under sustainable farming systems in order to obtain a high quality food that simultaneously provides an alternative, affordable and healthy animal product, while accepted by the consumer in terms of production practices (animal welfare and protection of the environment).

The investigation was performed in the area of horse-meat production destined for human consumption in the northern Spain which is part of a wider research project in which additional quality aspects such as sensory characteristics and consumer acceptability are being addressed together with many other relevant aspects of horse-meat production for human consumption such as genomics, feeding strategies, authentication, traceability, and ethical and socio-economic aspects.

The present work has been performed following three main objectives:

Objective 1: Optimization, development and utilization of the most appropriate analytical methods for an accurate analysis of lipid constituents in horse-meat.

In order to achieve this objective, the following specific objectives were set:

1.1. To optimize the fatty acid methyl ester fractionation technique, especially for those fatty acids present at low concentration in horse lipids.

1.2. To apply novel and comprehensive gas chromatography techniques to confirm fatty acid methyl ester and dimethylacetal identifications.

1.3. To develop an accurate method to isolate dimethylacetals from fatty acid methyl esters in horse lipids.

Objective 2: Assessment of the nutritional quality of horse-meat commercialized at both sides of the Cantabrian Mountains in northern Spain.

In order to achieve this objective, the following specific objectives were set:

2.1. To evaluate the effect of the time of the year (season) and region on the fatty acid profile of commercial horse-meat.

2.2. To compare the fatty acid composition of intramuscular and subcutaneous adipose tissues of horse-meat.

2.3. To determine the dimethylacetal content and profile of the intramuscular fat of horse-meat.

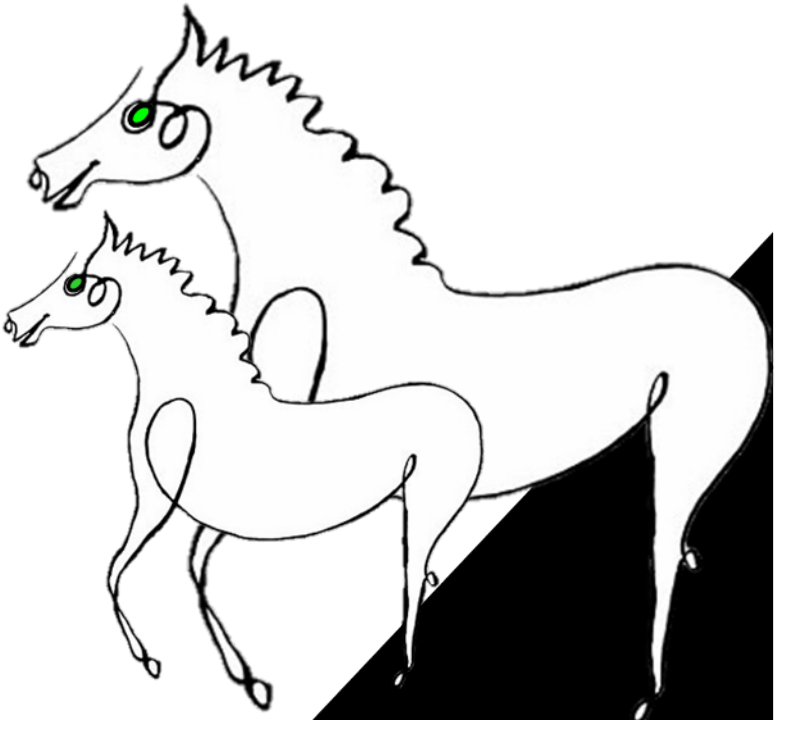
Objective 3: Evaluation of the effect of age, and associated differences in feeding, on the nutritional quality of horse-meat.

In order to achieve this objective, the following specific objectives were set:

3.1. To investigate differences in the fatty acid and dimethylacetal composition of neutral and polar lipid fractions of muscle obtained from suckling and fattened foals.

3.2. To compare the deposition preference between suckling and fattened foals of fatty acids into the muscle neutral and/or polar lipid fractions.

Chapter IV. MATERIAL AND METHODS



Chapter IV. MATERIAL AND METHODS

1. EXPERIMENTAL DESIGN AND SAMPLE COLLECTION

1.1. Experimental design I: Survey

A horse-meat survey was performed which consisted in the collection of horse loin steaks (n=82) from small butcher shops and big grocery stores along the northern Spanish regions, at both sides of the Cantabrian Mountains (Basque, Navarre, Cantabria, Asturias, Galicia and Castile & Leon; Figures IV.1 and IV.2).

	REGIONS	BUTCHER-SHOP	GROCERY
1	Basque	7	3
2	Navarre	5	-
3	Cantabria	7	-
4	Asturias	7	1
5	Galicia	4	-
6	Castile & Leon	7	-

In two time periods
(spring & winter; n = 82)

Figure IV.1. Distribution of horse-meat samples collected in each of the northern Spanish regions surveyed.

Figure IV.2. Horse-meat at retail in a small butcher-shop and a big grocery store.

Horse-meat samples were collected in two different seasons (41 loin samples/season); from April to May of 2013 (spring) and from December of 2013 to January of 2014 (winter). The collections were performed during different seasons in order to encompass all the variability associated to animal management practices, primarily feeding strategies, depending on the time of production. Theoretically, horse-meat collected in spring would likely come from animals that have entered the feedlot in the fall, while meat collected in early winter would likely come from animals that have been suckled and grass-grazed in mountainous regions from spring to late autumn.

Purchased samples were transported to the laboratory in refrigerated coolers. Then, subcutaneous (SC) fat and *Longissimus thoracis et lumborum* (LTL) muscle were dissected for each sample (Figure IV.3), and these were individually weighed, vacuum packed and frozen at -80 ºC for further analysis.

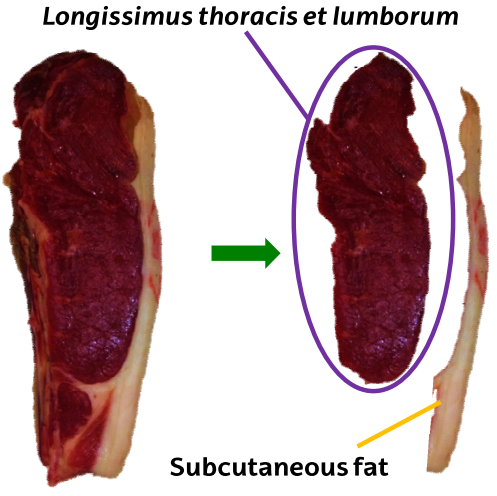


Figure IV.3. Visual diagram of the studied muscle (*Longissimus thoracis et lumborum*) and subcutaneous fat portions.

1.2. Experimental design II: Feeding trial

In the present study 2 groups of crossbred horses were used. The first group was selected from foals that were naturally suckled by their mothers from birth (March-April 2015) to slaughter (July 2015) at 4 months of age (n=8) and consisted of equal numbers from both genders. They were managed under grazing conditions. The second group was selected from crossbred males (n=7) born in April 2015, that after weaning (November 2015) were intensively fattened indoors (Figure IV.4). The amount of commercial concentrate given to foals was gradually increased from 4-5 kg head/day at the start to 10 kg/head/day at the end of the fattening period. Oats and straw were supplied *ad libitum* and they were slaughtered at 12 months of age.

Figure IV.4. Concentrate-based indoors fattening of crossbred foals (Castile & Leon).

The concentrate was composed by barley, wheat bran, soya, alfalfa, sunflower, beet molasses, and a premix of vitamins and trace elements. On dry matter basis, chemical composition of the concentrate was as follows: 18.2 % of crude protein (ISO, 2005), 7.93 % of ash (ISO, 2002), and 2.41 % of ether extract (AOCS, 2008), while the main FAs in the supplement were: 16:0 (20.8 %), 18:0 (2.25 %), 9*c*-18:1 (14.9 %), 18:2n-6 (45.8 %), and 18:3n-3 (5.46 %) (Alves *et al*., 2008).

Foals were slaughtered in a commercial abattoir according to standard procedures. The carcass weight reached an average value of 101 and 272 kg in suckling and fattened foals, respectively (Figure IV.5). The 6^{th} to 8^{th} rib joint of the left half carcasses were transported to the laboratory in refrigerated coolers. Then, the LTL muscle was dissected and two steaks of approximately 3 cm thick were cut. The first steak was used to determine the proximate chemical composition (%) and the second steak was cut into pieces, freeze-dried, ground, vacuum-packaged, and stored at -80 ºC for further lipid analysis.

Figure IV.5. Horse half carcasses hanging at the abattoir cooling room (Castile & León).

2. METHODOLOGY

2.1. Chemical composition of muscle tissue

International standard procedures were used to determine the crude protein (ISO, 2005), ether extract (AOCS, 2008), ash (ISO, 2002), and dry matter (ISO, 1999) contents. Each analysis was performed in duplicate.

2.2. Lipid extraction of muscle tissue

Total lipids were extracted from 1.5 g of freeze-dried LTL muscle tissue using a chloroform/methanol mixture (2:1, v/v; Folch *et al.*, 1957) and improved by Bligh and Dyer (1959). The chloroform layer was collected, and after removal (evaporation) of the solvent the total lipids were gravimetrically weighed. Lipids were then redissolved in 15 mL of chloroform and stored at -80 ºC.

2.3. Total muscle lipid fractionation into neutral and polar lipid classes

Total muscle lipids extracted from experimental design II (n=15) were fractionated into neutral lipid (NL) and polar lipid (PL) classes. Each lipid extract (45-60 mg) was loaded in a solid-phase extraction (SPE) cartridge packed with silica gel (Supelclean^{TM} LC-Si SPE Tubes 1 g/6 mL; Supelco, Bellafonte, PA, USA). After conditioning the cartridges with 2 mL of chloroform, the NL fraction was eluted with 30 mL of chloroform, and then the PL fraction was eluted with 30 mL of methanol as described by Juaneda *et al*. (1985).

The purity of the two fractions was verified using thin layer chromatography (TLC) on silica gel plates (10 cm x 20 cm; Sigma-Aldrich). The developing solvent system was a mixture of hexane/diethyl ether/methanol/acetic acid (90:20:5:2, v/v/v/v). The different bands were identified after spraying the TLC plates with 10 % sulfuric acid in methanol and charred in a hot plate (Figure IV.6).

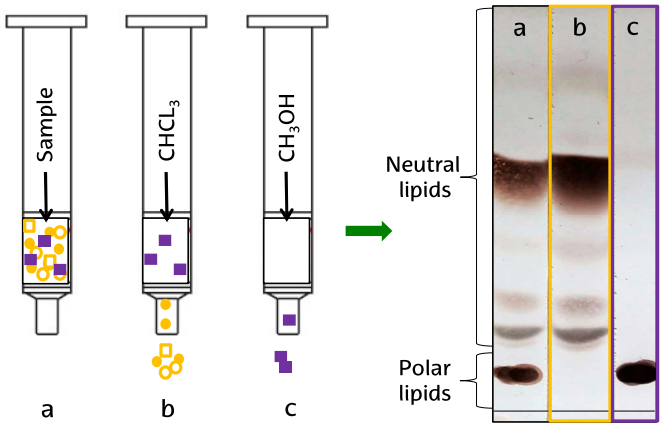


Figure IV.6. Total lipid fractionation and verification by solid-phase extraction and thin layer chromatography techniques, respectively: a) total muscle lipids, b) neutral lipids, and c) polar lipids.

2.4. Lipid derivatization: base and/or acid catalyzed methylations

Subcutaneous adipose tissue samples were weighted (50±1 mg), freeze-dried and derivatized using a base catalyzed transesterification (2 mL of Methanolic-Base, 0.5 N from Supelco). The reaction was carried out at 50 ºC for 15 min (Figure IV.6). Two lipid aliquots of 10 mg each were methylated separately using base (2 mL of Methanolic-Base, 0.5 N from Supelco) and acid (2 mL of sulfuric acid in methanol, 2 %) catalyzed procedures; the later method assured the complete methylation of all type of lipids. Derivatization with methanolic sulfuric acid provided the conversion of acyl and *N*-acyl lipids into fatty acid methyl esters (FAMEs), and the alk-1-enyl ethers of plasmalogenic lipids into dimethylacetals (DMA). On the other hand, the base catalyzed procedure was used to avoid isomerization of conjugated linoleic acids (CLAs) (Aldai *et al.*, 2012a; Kramer *et al.*, 1998). Individual NL and PL fractions were transesterified with sodium methoxide (2 mL; Methanolic-Base, 0.5 N; Supelco) followed by sulfuric acid in methanol (2 %; Figure IV.7).

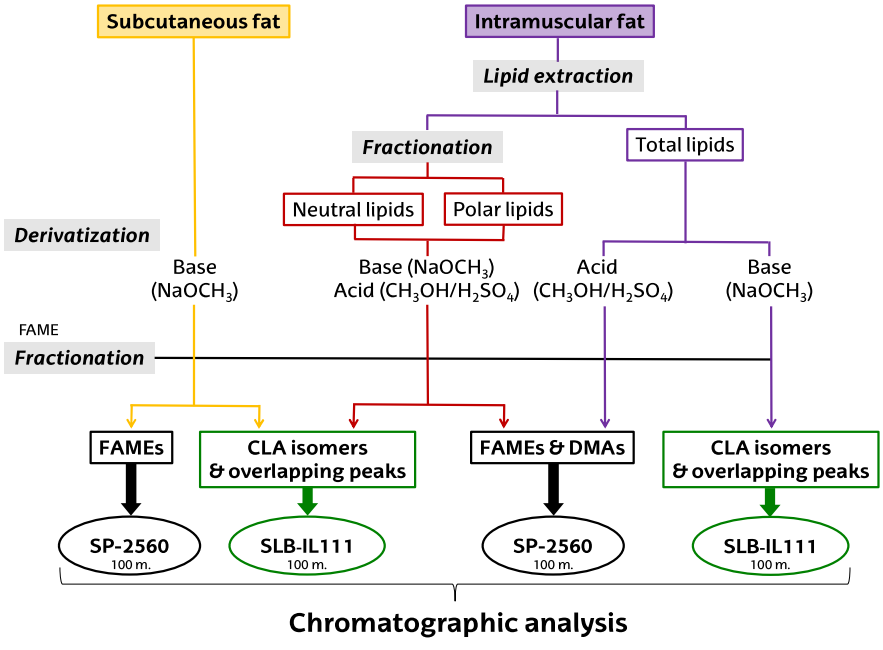


Figure IV.7. Methodological scheme of lipid extraction, fractionation, derivatization, and chromatographic analysis of horse intramuscular and subcutaneous adipose tissues.

FA, fatty acid; FAME, fatty acid methyl ester; CLA, conjugated linoleic acid; DMA, dimethylacetal.

2.5. Silver ion solid-phase extraction

Silver-ion solid phase extraction (Ag^+-SPE) technique was utilized as complementary method in order to ease the identification of several overlapping peaks by differences in chain length and number of double bonds. Ag^+-SPE cartridges available from Sigma-Aldrich (Supelco Discovery, 750 mg/6 mL) were used. After conditioning with 4 mL of acetone followed by other 4 mL of hexane, one mg of FAME was loaded in each cartridge, and 6 mL of the following eight solvents or solvent mixtures (v/v) were added in consecutive order (Kramer *et al.*, 2008) to collect separate FAME fractions: 1) hexane:acetone (99:1) to obtain SFAs and BCFAs; 2) hexane:acetone (96:4) to obtain *trans*-MUFAs plus the *t*/*t*-CLA isomers; 3) hexane:acetone (90:10) to obtain *cis*-MUFA plus *c*/*t*- and *t*/*c*-CLA isomers; 4) hexane:acetone (0:100) to obtain dienes; 5) acetone:acetonitrile (97:3) to obtain trienes; 6) acetone:acetonitrile (94:6) to obtain tetraenes; 7) acetone:acetonitrile (80:20) to obtain pentaenes; and 8) acetone:acetonitrile (0:100) to obtain hexaenes. Collected FAME fractions were reconstituted in hexane and stored at -80 ºC for further analysis.

2.6. Isolation of dimethylacetals from the total fatty acid methyl esters of muscle

Dimethylacetals were isolated from the total FAMEs in samples of both experimental studies. From the survey (experimental design I), having in mind the high n-3 PUFA variability of these samples (1.17-18.9 %), 15 samples were randomly selected from the low (up to 4.5 %), intermediate (4.5 to 11.5 %), and high (over 11.5 %) n-3 PUFA content samples (5 samples from each group; n=15).From the feeding trial (experimental design II), DMAs were isolated from all muscle samples (n=15).

As indicated earlier, derivatization with methanolic sulfuric acid provided the conversion of acyl and *N*-acyl lipids into FAMEs, and the alk-1-enyl ethers of plasmalogenic lipids into DMAs. The isolation of DMAs is based on the different reactivity of acyl esters and alkenyl ethers under base hydrolysis conditions (Figure IV.8). To prove this, derivatization products obtained from muscle lipids (FAMEs

and DMAs) were dried under N_2 and subjected to overnight hydrolysis with 2.85 mL of Ethanol and 150 µL of NaOH solution (15 %) at room temperature. FAMEs were converted into their free FAs while DMAs were stable to base conditions and remained unreactive. DMA fraction was then extracted with 2 mL of hexane and stored at -80 ºC.

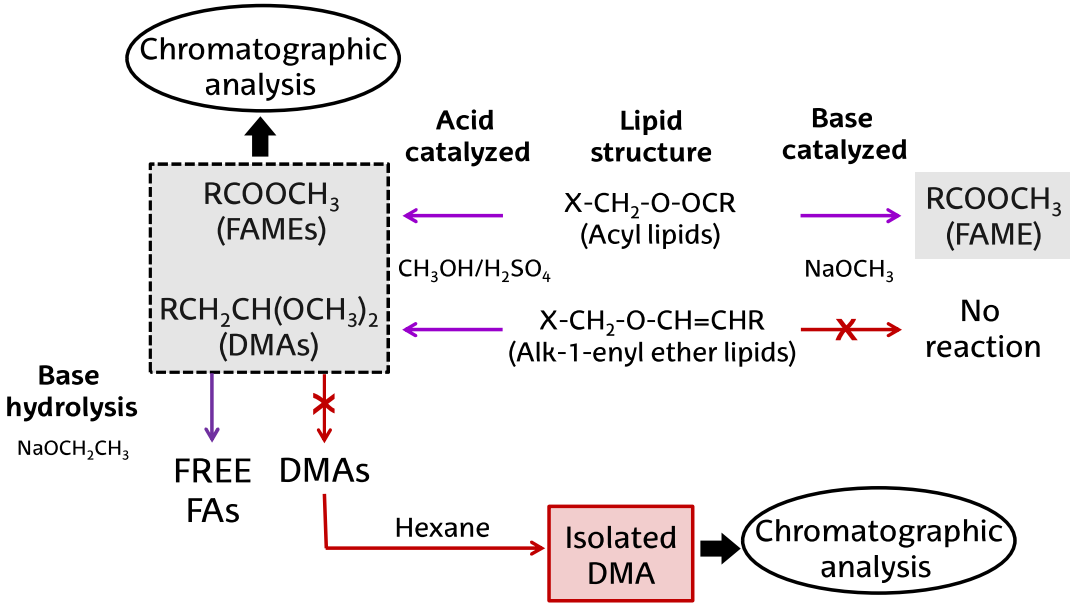


Figure IV.8. Isolation scheme of dimethylacetals from fatty acid methyl esters obtained from total muscle lipids.

FAME, fatty acid methyl ester; FA, fatty acid; DMA, dimethylacetal.

2.7. Chromatographic analysis

2.7.1. Reference standards

For FAME identification purposes several reference standards were used: individual FAMEs (13:0, 21:0, 23:0, 26:0, 28:0) and mixtures such as #463 and #603, #UC-59M CLA obtained from Nu-Check Prep Inc. (Elysian, MN, USA), LA (18:2n-6) and LNA (18:3n-3) isomer mixtures from Sigma-Aldrich (CRM47791 and CRM47792; Supelco, Bellefonte, PA, USA), and a bacterial FAME mixture from Matreya (Pleasant Gap, PA, USA).

2.7.2. Gas chromatography - Flame ionization detector

Gas chromatography – flame ionization detector (GC-FID; Agilent Technologies, Model 7890A, Wilmington, DE, USA) and coupled with an automatic injector (Agilent Technologies, Model 7693) was used to analyze FAMEs plus FAME fractions and DMAs. Samples were injected using a 50:1 split ratio, a Supelco SP-2560 capillary column (100 m x 0.25 mm I.D., 0.2 m coating, Bellefonte, PA, USA) was used, and the temperature program utilized was described by Kramer *et al.* (2008): 4 min at 45 °C, increased at 13 °C/min to 175 °C and held for 27 min, then increased at 4 °C/min to 215 °C and maintained for 41 min. Total GC-FID run of 92 min.

In order to resolve CLA isomers and improve separation of other co-eluting peaks, samples were subjected to a second GC-FID analysis using a SLB-IL111 ionic liquid stationary phase column (100 m x 0.25 mm I.D., 0.2 m coating; Supelco, Bellefonte, PA, USA) using a 50:1 split ratio and a temperature program described by Delmonte *et al.* (2011): 40 min at 168 °C, increased at 6 °C/min to 185 °C and maintained for 35 min. Total GC-FID run of 77.8 min.

In both GC-FID runs, hydrogen was used as a carrier gas at a flow rate of 1 mL/min, the injection volume was 1 µL, and injector and detector ports were set at 250 °C.

In-house validation of the GC-FID method:

The GC-FID analytical method was internally validated using the SP-2560 capillary column and the temperature program described earlier (Kramer *et al.,* 2008), and the GLC #714 mixture (10:0, 12:0, 13:0, 14:0, 16:0, 9*c*-16:1, 17:0, 18:0, 9*c*-18:1, 18:2n-6, 20:0, 11*c*-20:1, 21:0, 22:0, 13*c*-22:1, 20:3n-3, 20:4n-6, 23:0, 24:0, 20:5n-3, 15*c*-24:1, 22:5n-3, and 22:6n-3) obtained from Nu-Check (Elysian, MN, USA).

Six different concentrations ranging from 0.00500 to 0.500 mg/mL of FAMEs from GLC #714 mixture in isooctane were prepared and injected in the GC-FID in triplicate. The mean response factor of each FAME was calculated (peak area relative to the FAME concentration). Coefficients of variation (< 5 %) of the

response factor and linear regressions analysis (R^2 › 0.999) confirmed the linearity of the GC-FID response at the concentration range used (Table IV.1).

The detection and quantification limits of the analytical method were also determined using the mean area of the noise (10 runs of hexane) plus 3 or 10 times the standard deviation of the noise giving values of 0.0006 and 0.001 mg/mL, respectively, for the FAMEs contained in the mixture (Table IV.1).

Table IV.1. Several analytical method validation parameters determined for each of the fatty acid methyl ester included in the GLC #714 mixture.

FAME	rf	SD	CV (%)	EQUATION	SLOPE	R^2	LD (mg/mL)	LQ (mg/mL)
10:0	145	7.56	5.22	y = 128x + 0.292	128	0.998	0.000655	0.00112
12:0	146	6.22	4.25	y = 137x + 0.408	137	0.999	0.000611	0.00104
13:0	150	7.90	5.25	y = 143x + 0.240	143	0.999	0.000587	0.00100
14:0	151	6.15	4.08	y = 146x + 0.175	146	1.000	0.000574	0.000978
16:0	151	7.08	4.69	y = 148x + 0.176	148	1.000	0.000565	0.000962
9*c*- 16:1	151	4.69	3.10	y = 149x + 0.174	149	0.999	0.000562	0.000958
17:0	153	3.32	2.18	y = 149x + 0.160	149	1.000	0.000561	0.000956
18:0	157	8.56	5.46	y = 151x + 0.191	151	0.999	0.000553	0.000942
9*c*- 18:1	152	4.67	3.06	y = 148x + 0.136	148	1.000	0.000565	0.000962
18:2n-6	155	5.83	3.76	y = 150x + 0.157	150	0.999	0.000556	0.000947
20:0	157	4.83	3.08	y = 152x + 0.154	152	0.999	0.000550	0.000937
11*c*- 20:1	154	4.87	3.16	y = 153x + 0.162	153	0.999	0.000547	0.000932
21:0	159	7.91	4.98	y = 153x + 0.167	153	0.999	0.000546	0.000929
22:0	159	2.83	1.78	y = 155x + 0.151	155	0.999	0.000538	0.000917
13*c*- 22:1	158	3.45	2.18	y = 154x + 0.143	154	0.999	0.000543	0.000924
20:3n-3	156	3.96	2.53	y = 152x + 0.171	152	0.999	0.000550	0.000936
20:4n-6	158	4.39	2.78	y = 153x + 0.171	153	0.999	0.000548	0.000933
23:0	179	7.94	4.45	y = 176x + 0.105	176	0.999	0.000476	0.000811
24:0	143	4.70	3.28	y = 142x - 0.0171	142	0.999	0.000591	0.00101
20:5n-3	154	4.73	3.06	y = 150x + 0.395	150	0.999	0.000558	0.000950
15*c*- 24:1	155	7.02	4.53	y = 154x + 0.164	154	0.999	0.000544	0.000927
22:5n-3	155	3.39	2.18	y = 152x + 0.154	152	0.999	0.000551	0.000939
22:6n-3	151	3.06	2.03	y = 148x + 0.146	148	1.000	0.000567	0.000965

FAME, fatty acid methyl ester; rf, response factor; SD, standard deviation; CV, coefficient of variation; R2, coefficient of regression; LD, limit of detection; LQ, limit of quantification, y = peak area; x = FAME concentration (mg/mL); *c, cis*.

2.7.3. Gas chromatography - Online Reduction - Gas chromatography

When required, gas chromatography - online reduction - gas chromatography (GC-OR-GC) equipment was used to achieve a comprehensive analysis of FAMEs and DMAs. This equipment was mounted with a GC-FID (Agilent Technologies, Model 7890B, Wilmington, DE, USA) combined with a Zoex ZX2 dual stage

cryogenic modulator (Houston, TX, USA). A Supelco SP-2560 capillary column (100 m x 0.25 mm I.D., 0.2 m coating, Bellefonte, PA, USA) was used for the separation in the first dimension. A 30 cm x 0.18 mm of 0.05 % Palladium (II) Acetylacetonate capillary reactor (Supelco, Bellefonte, PA, USA) was placed between the first dimension column and the modulator. The cryogenic modulator first loop consisted of a 2 m x 0.25 mm deactivated capillary tube and the modulation (first stage) was set at 25 cm from the beginning of the tube. The second stage of modulation was achieved using part (25 cm) of the second dimension column SLB-IL111 (2.75 m x 0.10 mm I.D., 0.08 m coating; Supelco, Bellefonte, PA, USA) as described by Delmonte *et al.*, 2013 (Figure IV.9).

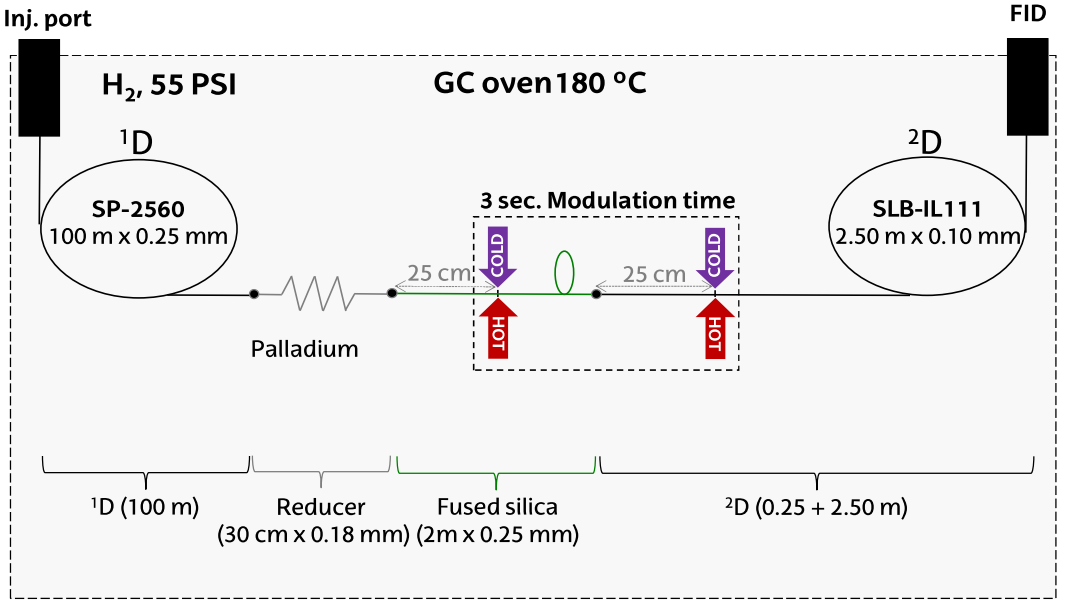


Figure IV.9. Setup scheme of the gas chromatography – online reduction – gas chromatography equipment.

^{1}D, first dimension; ^{2}D, second dimension.

Samples were injected using a 50:1 split ratio, the injection volume was 1 µL, and hydrogen was used as a carrier gas at a flow rate of 3.48 mL/min. The oven temperature was maintained at 180 °C, and injector and detector ports were set at 250 °C. The modulation time was set to 3 seconds, the temperature of the hot jet was set at 350 °C with a pulse of 350 microseconds.

The capillary column coated with palladium and in the presence of H_2 (carrier gas) reduced all the double bonds of unsaturated FAMEs and DMAs to fully hydrogenated carbon skeletons. A two dimensional separation can be easily interpreted based on the principle that all the saturated compounds lie on a straight diagonal, while compounds with the same carbon skeleton but differing in the number/geometric configuration/position of double bonds lie on a parallel to the first dimension axis (time), based on the fact that the reduction process converted them into the same compounds (Figure IV.10.)

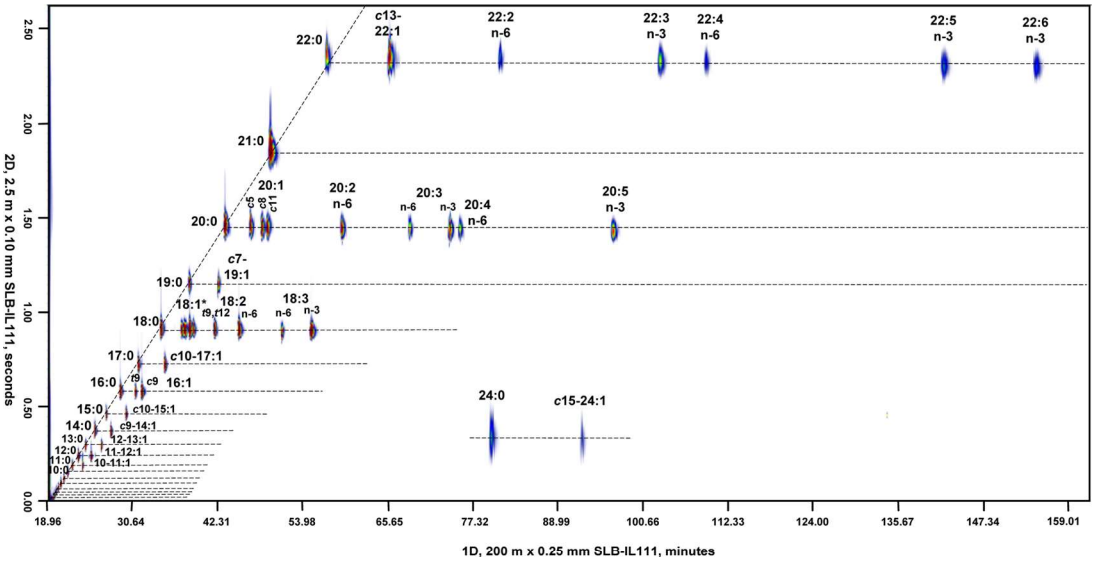


Figure IV.10. An example of the GLC #463 reference standard separation on the gas chromatography – online reduction – gas chromatography equipment. Figure taken from Delmonte *et al.* (2013).

[1]D, first dimension; [2]D, second dimension.

2.7.4. Gas chromatography - Mass spectrometry

Several FAME and DMA identifications were confirmed by high resolution gas chromatography – mass spectrometry (GC-MS) technique. An Agilent 7890B GC was coupled with an accurate-mass quadrupole-time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies, Model 7200A, Wilmington, DE, USA.). Selected samples were injected using a 50:1 split ratio on a Supelco SP-2560 capillary column (100 m x 0.25 mm I.D., 0.2 m coating, Bellefonte, PA) maintained

at 180 ºC. Helium was used as a carrier gas at a flow rate of 1.1 mL/min, the injection volume was 1 µL, and injector and detector ports were set at 250 ºC. Analyses were carried out in chemical ionization (CI^+) mode using isobutane as the ionization gas. The source was maintained at 300 ºC, the electron energy was 250 eV, the emission current was 30 A, and the CI gas flow was set to 20 %. The molecular ions of FAMEs and DMAs helped to identify the chain length and the number of double bonds (Fardin-Kia *et al.,* 2013).

2.8. Quantification of fatty acid methyl esters and dimethylacetals

Quantification of FAMEs and DMAs was achieved by adding, prior to derivatization, 0.05 mg of each internal standard (13:0 and 23:0 methyl esters; Nu-Chek Prep Inc., Elysian, MN, USA) per 1 mg of total lipids or lipid fractions.

First of all, chromatographic peak areas were corrected according to theoretical response factors (AOCS, 2009), and thereafter the absolute contents (mg) of FAMEs and DMAs were calculated according to the internal standard. FAMEs and DMAs were expressed as mg/100 g of fresh meat and as percentage (% of total quantified).

3. DATA TREATMENT AND STATISTICAL ANALYSIS

Statistical analysis was conducted using IBM SPSS Statistics 22 for windows (SPSS Inc., IBM Corporation, NY, USA). Normality and homoscedasticity were checked by Shapiro-Wilk and Leven's test, respectively.

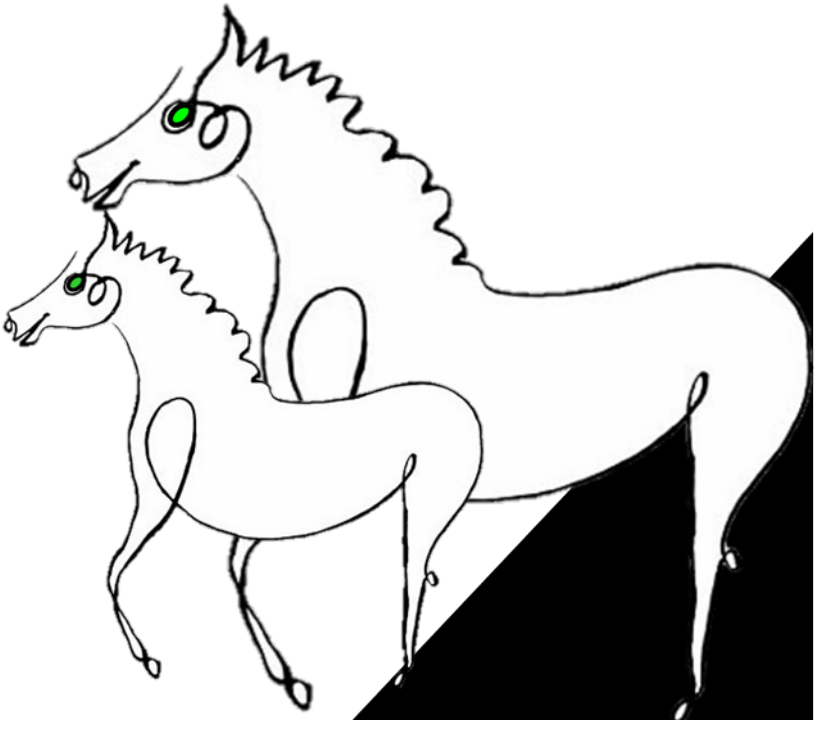
Multifactorial analysis of variance (ANOVA) was applied to study the differences on variables studied (chemical composition and lipid constituents) depending on the factors studied in the survey (season, region, type of tissue; experimental design I). When needed, mean *post-hoc* comparisons were conducted using Tukey HSD procedure to observe differences in studied variables.

A *t*-Student test was applied to compare the chemical composition and lipid constituents (total lipids, NL and PL fractions), as well as the deposition preferences between animal groups from the feeding trial (experimental design II). Deposition preferences within NL or PL fractions of each FA were calculated according to the logarithm of the percentage of an individual FA in the PL fraction divided by the percentage of the same FA in the NL fraction.

Principal component analysis (PCA) as an extraction method was also performed on a correlation matrix of selected variables. Sample distribution plots using the two-dimensional coordinate system defined by the first two principal components (PCs) were used to study the variability in lipid constituents in horse tissues.

Three significant figures were used to express all data and analytical results, while significance was declared at $P \leq 0.05$.

Chapter V. RESULTS AND DISCUSSION



Chapter V. RESULTS AND DISCUSSION

1. METHODOLOGICAL RESULTS

Publication II and **III** describe the problems found and how these were overcame in the effort of fractionating FAMEs obtained from horse-meat lipids with the Ag^+-SPE technique. It was observed that the commercially available Ag^+-SPE cartridges contain appreciable amounts of contaminants that could interfere with the low levels of *trans*-MUFA in horse lipids, as it was evidenced by the GC-FID analysis. Several attempts were done to prewash cartridges and remove interfering impurities before loading them with FAMEs but it was not possible to eliminate impurities. This indicated that commercial Ag^+-SPE cartridges, consisting of strong cation-exchange resins functionalized with Ag^+ loaded in polypropylene tubes, could not be used to isolate *trans*-MUFA in low *trans*-FA samples as horse lipids for GC-FID analysis (Publication II).

After contacting the supplier, custom made glass (instead of polypropylene) cartridges were generously provided which they proved successful to fractionate the *trans*-MUFA in horse and other animal samples with low *trans* content (Publication III).

PUBLICATION II

Limiting of using silver ion solid-phase extraction for animal lipids with a low *trans* content

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Short Communication

Limitation of using silver ion solid-phase extraction for animal lipids with a low *trans* content

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Silver ion solid-phase extraction (Ag^+-SPE) was reported to provide effective separations compared to other Ag^+ techniques but at a fraction of cost and time. Ag^+-SPE cartridges resolved fatty acid methyl esters (FAMEs) with different number and/or geometric configuration of double bonds. Here we attempted to determine the *trans* fatty acids (FA) contained in a low total *trans* FA sample, horse lipids; lamb was used as a control having a markedly higher total *trans* content. Gas chromatographic assessment of the fractions showed a good separation of the *cis* and *trans* monounsaturated FA (MUFA) fractions, but the relative high content of contaminants that coeluted with these FA impaired the identification of the latter in horse lipids. In lamb *trans* MUFA isomers could be identified since their abundance relative to impurities was greater. Several attempts were made to remove the contaminants from the SPE cartridges including an extensive prewash with acetone and hexane, a prewash with solvents that would elute the *cis* MUFA fraction, and a complete prewash of all solvents used in the fractionation, hexane, acetone, and acetonitrile. The prewash using all elution solvents removed most contaminants but subsequently impaired the separation of *trans* and *cis* MUFA fractions. The same samples were subjected to Ag^+-HPLC fractionation that showed no impurities demonstrating that they were derived from the Ag^+-SPE separation. The *trans* MUFA fraction collected from Ag^+-HPLC allowed for the identification of the *trans* 16:1 and 18:1 FA in horse lipids and is recommended for samples with low *trans* levels.

Practical applications: The commercially available silver ion solid-phase extraction (Ag^+-SPE) cartridges contain appreciable amounts of contaminants that can interfere with the subsequent GC-FID elution of low levels of *trans* fatty acid methyl esters (FAMEs), especially when contaminants are leaked by the SPE tube in quantities comparable to the *trans* MUFA content. The contaminants could not be quantitatively removed by prewashing the cartridges with acetone and hexane. Acetonitrile removed most contaminants but altered the Ag^+-SPE tube ability to resolve the *trans* and *cis* MUFA fractions. Ag^+-HPLC fractionation is recommended for isolation of low levels of *trans* MUFA, since the washing and conditioning of the chromatographic column can be extended as needed.

Keywords: Animal fat / GC / Geometric isomers / HPLC / Impurities / Silver-ion / *trans*

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1 Introduction

Separations achieved using silver ion (Ag^+) chromatography to resolve fatty acid methyl esters (FAME) are helpful to identify them based on their number and/or geometric configuration of double bonds. This is particularly valuable when dealing with the incomplete separation of unsaturated FAME using the most polar GC columns (SP-2560, CP-Sil 88, SLB-IL111) that provide the separation of many FAMEs based on their chain length, number of double bonds, and their double bond configuration [1–4]. Animals, and especially ruminant fats, contain complex mixtures of unsaturated fatty acids (FA) positional and geometric isomers, branched-chain FA, and other components. Ag^+ chromatographic separation of these complex FAME

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mixtures prior to GC analysis was shown to simplify their identification of quantification [5]. By applying Ag^+- thin layer chromatography (TLC) [1, 6], Ag^+-high performance liquid chromatography (HPLC) [3, 4, 7], or Ag^+- solid phase extraction (SPE) [2, 5] with various combinations of eluting solvents, complex mixtures of FAMEs with up to six double bonds were successfully resolved based on the number and/or geometry of their double bonds.

Ag^+-SPE separates FAME based on the same principles as Ag^+-TLC or Ag^+-HPLC, and its separation capabilities are sufficient for separating FAME based on the number of unsaturations in most samples. Ag^+-SPE separations are easier to accomplish and faster compared to the later two methods, and do not require expensive instrumentation. Therefore, the Ag^+-SPE technique was applied to separate fractions from total FAMEs obtained from horse and lamb backfat tissues that had been prepared by direct methylation using methanolic base (0.5 N); the elution details were previously described [2]. These two biological samples were selected to test whether Ag^+-SPE could be used to analyze the *trans* MUFA composition of samples with a low content of total *trans* FA as horse lipids [8]. Lamb lipids were used to verify the ability of Ag^+-SPE to effectively fractionate samples with higher total *trans* FA content [9]. The GC analysis of the Ag^+-SPE fractions using a 100 m SP-2560 column did not provide the identification *trans* 16:1 and 18:1 FA from horse lipids because unknown contaminants were present at equal or higher concentrations in the *trans* MUFA fraction. The *cis* MUFA isomers from the horse and lamb lipid fractions also contained these impurities but their relative abundance was smaller causing less interference.

The present study describes efforts to extend Ag^+-SPE to the fractionation of samples containing low levels of *trans* FA, using horse lipids as test matrix. Several prewashing and conditioning procedures of the Ag^+-SPE cartridges were attempted to remove interfering impurities and allow for effective fractionations.

2 Materials and methods

All solvents used for the fractionation were of HPLC grade and purchased from Sigma-Aldrich (acetone, Chromasolv® for HPLC, >99.8%; hexane, Chromasolv® for HPLC, >97.0%; acetonitrile, Chromasolv® Plus for HPLC, >99.9%). Other chemicals of the highest purity grade were also purchased from Sigma-Aldrich. The purity of the solvents used for separations was analyzed by GC to ensure the absence of impurities. Reference standards #463 and #603 and the individual FAMEs 19:0, 21:0, 23:0, and 26:0, together with CLA mixture #UC-59M were purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). A bacterial FAME mixture purchased from Matreya (Pleasant Gap, PA, USA) was used to identify branched-chain FAs. All gases employed for GC (N_2, H_2, air) were of 99.999% purity (Alphagaz B50, Air Liquide, Spain). Ag^+-SPE cartridges used were purchased from Sigma-Aldrich (Supelco Discovery™, 750mg/6mL, 54225-U, Lot. 1744301). Ag^+-SPE cartridges from another lot of production (Lot. 3082501), for comparison, were kindly provided by Sigma-Aldrich.

The cartridges were conditioned with 4 mL of acetone and 4 mL of hexane as recommended [10]. Then a sample (about 1 mg of total FAMEs) was loaded and the fractionation was conducted as previously described [2].

Three different prewash procedures without sample (A, B, C) were tested prior to Ag^+-SPE separation to remove the interfering contaminants. In order to estimate the amount of contaminants present in prewash B and C, an external standard (19:0 methyl ester) was added (0.04 μg/mL) to each and analyzed by GC. All prewash and fractions were analyzed using the same GC column and conditions as previously described [2]; a 100 m SP-2560 column operated using a temperature program with a plateau at 175°C. Several fractions had to be concentrated for GC analysis because of their low content, specifically the *trans* fraction.

Prewash (A). Increase the volumes of the prewash solvents of acetone and hexane from 4 to 8 mL, and insert an extra wash of 12 mL acetone:hexane (60:40, v/v) between the two single solvents.
Prewash (B). Conduct a prewash (blank) that would have included the *cis* fraction: 6 mL of acetone, 6 mL of hexane, 6 mL of hexane:acetone (99:1, v/v; would elute saturated FA), 6 mL of hexane:acetone (96:4, v/v; would elute *trans* FA), and 6 mL of hexane:acetone (90:10, v/v; would elute *cis* monounsaturated FA).
Prewash (C). Conduct a shortened version of the whole fractionation procedure that included all the eluting solvents: 15 mL of acetone, 15 mL of hexane:acetone (90:10, v/v), and 15 mL of acetone:acetonitrile (50:50, v/v).

After prewashing the Ag^+-SPE cartridges using the procedures (A), (B), or (C), the test samples were applied and fractionated as described previously [2].

The same total horse and lamb FAME samples were subsequently fractionated using a Ag^+-HPLC procedure described by Delmonte et al. [3] and analyzed using the same GC column and conditions as described above [2].

3 Results

The GC analysis of the *trans* and *cis* MUFA fractions of both horse and lamb backfat showed the presence of several interfering compounds which did not occur in the unfractionated samples (Fig. 1a & b). The presence of these contaminants affected the identification of the *trans*-18:1 isomers, and jeopardized to identification of the *trans*-16:1

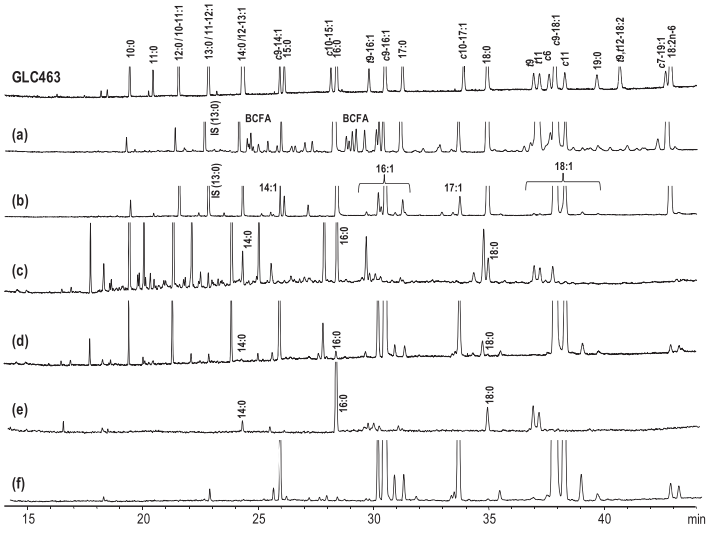

Figure 1. Partial GC chromatograms from 15 min to 18:2 n−6 region of GC reference mixture GLC 463, lamb backfat FAMEs (1a), horse backfat FAMEs (1b), horse *trans* fraction obtained by Ag^+-SPE (1c), horse *cis* fraction obtained by Ag^+-SPE (1d), horse *trans* fraction obtained by Ag^+-HPLC (1e), and horse *cis* fraction obtained by Ag^+-HPLC (1f). GC column and conditions were previously described [2]. BCFA, branched-chain fatty acid; IS, internal standard.

isomers in horse lipids. Prewashes did not resolve the problem, since each prewash provided a different *trans*-16:1 FAME pattern (Fig. 2c–e). The saturated FAME fractions from horse and lamb were generally free of contaminants (not shown), but several prominent impurities including X1 and X2 were evident in all *trans* MUFA fractions irrespective of the originating sample (Fig. 1c, 2c–g). The problem of the interfering compounds was more acute for the horse samples that contained lower total *trans* FA than lamb samples. For the same reason contaminants affected more markedly the separation of 16:1 than the 18:1 isomers from horse lipids.

Since the contaminants eluted mainly in the *trans* and *cis* MUFA fractions, it was attempted to remove them from the Ag^+-SPE cartridges by including the solvents used to elute the *trans* and *cis* MUFA fractions in the prewash procedure (B). Prewash (B) removed about 77 μg of contaminant from each Ag^+-SPE cartridge (GC separation shown in Fig. 2a), but the GC analysis of the subsequent horse lipid fractionation did not show any improvement in the identification of *trans* MUFA (comparison of Fig. 2c & d). After prewash (B) the *trans* fraction from the subsequent lamb fractionation could be used to identify the *trans*-18:1, but not the *trans*-16:1 isomers (not shown). It was of interest to note that the

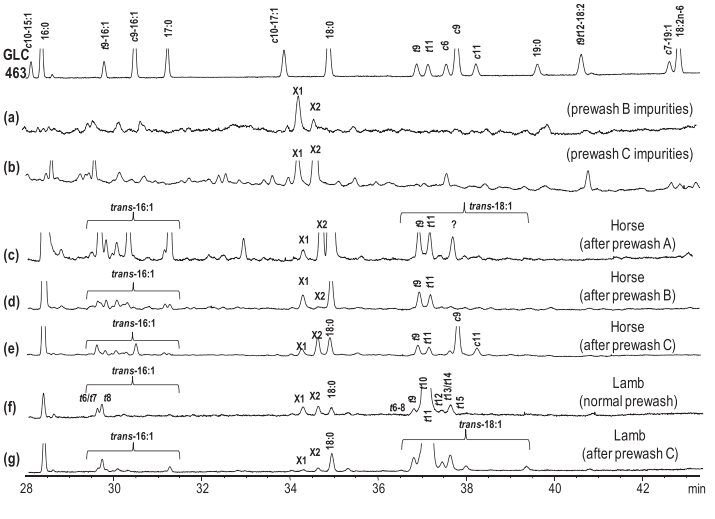

Figure 2. Partial GC chromatograms of the 16:0 to 18:2n−6 region of GC reference GLC 463, prewash B (2a), prewash C (2b), the *trans* fractions from total FAMEs of horse backfat (2c, 2d, 2e) and lamb backfat (2f, 2g) after prewash (normal, A, B, and C). GC column and conditions as previously described [2].

condition of prewash B did not affect the capability of the Ag^+-SPE cartridges of resolving the *trans* from *cis* MUFA from horse lipids.

A third prewash procedure was therefore undertaken in which all three eluting solvents were used in the Ag^+-SPE fractionation. The total prewash (C) of the Ag^+-SPE tube removed 135 μg of contaminants per cartridge (Fig. 2b). Prewash (C) appeared to remove most contaminants; however, it compromised the ability of the stationary phase to resolve the *trans* and *cis* MUFA fractions. This was evidenced by the coelution of the *trans* and *cis* isomers of 16:1 and 18:1 FAMEs (Fig. 2e). Prewash (C) was therefore ineffective for the characterization of the *trans*-16:1 and *trans*-18:1 fractions from horse lipids.

To eliminate the possibility that these impurities were originally present in the lipid extracts from horse and lamb, even though they were not evident in the total lipid extracts (Fig. 1a & b), the same samples were fractionated using Ag^+-HPLC according to the method previously described [3] and analyzed using a 100 m SP 2560 column and same GC condition described above [2]. Both the *trans* and *cis* fractions from Ag^+-HPLC were free from impurities demonstrating that these were not present in the original horse lipid extract. Furthermore, even though the total *trans*-16:1 and *trans*-18:1 contents in horse lipids were low, these isomers could be identified after Ag^+-HPLC fractionation.

4 Discussion

The results of this study show that commercial Ag^+-SPE cartridges, consisting of strong cation-exchange resins functionalized with Ag^+ loaded in polypropylene tubes could not be used to isolate *trans* MUFA in low *trans* FA samples as horse lipids for GC-FID analysis. *Trans* MUFA isolated from lamb lipids could just be analyzed because of their higher relative abundance compared to the contaminants released from the SPE cartridges (Fig. 2). The two prominent contaminants (X1 and X2) did not interfere with the quantification of the *trans* and *cis* FAMEs. Their origin of contaminants could have been derived from the resin or the polypropylene housing. This was not investigated, nor was it of interest in this study, since these commercial Ag^+-SPE cartridges are sold as a unit and recommended for the separation of FAMEs based on unsaturation. Contaminants could be reduced by pre-washing the SPE cartridges by increasing the elution strength of the pre-washing solvents. Prewash (A) provided little benefit for the identification of *trans*-16:1 and *trans*-18:1 FAMEs in horse lipids. Prewash (B) provided some improvement, but the *trans* MUFA fraction was still markedly contaminated, particularly for the horse lipid sample. Prewash (C) was effective in removing most contaminants; however, the subsequent separation of the FAME samples showed an extensive loss of the phase selectivity for FAMEs differing in the number and/or geometry of double bonds. Acetonitrile, a strong elution solvent for Ag^+ loaded resins, might have eluted part of the Ag^+ that resulted in a lack of separation of the geometric MUFA isomer. In prewash (A), we attempted to use larger volumes of the non-polar solvents to remove contaminants without affecting the Ag^+ content of the cartridges, but it proved ineffective. Combining the *trans* MUFA from several Ag^+-SPE fractionation, i.e, applying prewash (B) is no solution, since the contaminants would concentrate along with the *trans* MUFA.

Based on these results it is clear that the commercial Ag^+-SPE cartridges are effective to resolve FAMEs differing in the number and geometric configuration of double bonds, but one cannot extensively concentrate these fractions to analyze components present at low concentration by GC. In such cases the contaminants eluted from the Ag^+-SPE cartridges may mask the FAMEs of interest as evidenced in the analysis of *trans* FAs from horse lipids. This limitation was not observed using an Ag^+-HPLC separation, which makes the latter method suitable as a viable alternative to analyze FAMEs present at low concentration.

5 Conclusions

The presence of contaminants in Ag^+-SPE cartridges that coelute with the eluting solvents in the separation of FAMEs with increasing unsaturation is a limitation to their use, particularly when dealing with fractions present at low concentrations, such as the *trans* FAMEs present in horse lipids. It made it impossible to definitively identify the *trans*-16:1 and *trans*-18:1 composition in horse backfat. Moderate cleanup procedures were not successful to remove contaminants, while a more drastic cleanup procedure was successful to remove them, but rendered the Ag^+-SPE cartridges ineffective to resolve the *trans* and *cis* FAMEs in a mixture. Therefore, caution is necessary to identify the *trans*-16:1 and *trans*-18:1 isomers from samples with a low content of total *trans* isomers after Ag^+ fractionation using commercial Ag^+-SPE cartridges.

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The authors have declared no conflict of interest.

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PUBLICATION III

Silver ion solid-phase extraction cartridges employing glass housing overcome the limitations observed in the GC analysis of animal lipids with low *trans* fatty acid content

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Short Communication

Silver ion solid-phase extraction cartridges employing glass housings overcome the limitations observed in the GC analysis of animal lipids with low *trans* fatty acid content

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Custom made silver ion solid-phase extraction (Ag^+-SPE) cartridges with glass housing were evaluated to see whether they could be used to analyze the *trans* fatty acids (TFA) in animal products when present at low concentration. Previous attempts were not successful to identify and characterize the TFA using commercial Ag^+-SPE cartridges, because of the coelution of artifacts from these cartridges. The glass cartridges proved successful to identify the low levels of TFA present in horse and wild boar meat. It is recommended that the supplier make available Ag^+-SPE cartridges in glass housing to avoid the problem of interferences.

Practical applications: It was previously shown that commercially available silver ion solid-phase extraction (Ag^+-SPE) cartridges could not be used to analyze biological samples with low levels of TFAs, because of interfering contaminants that eluted from the polypropylene housing. Custom made glass cartridges were generously provided by the supplier, and they proved successful to fractionate the geometric isomers of horse and wild boar lipids both of which are known to contain low levels of TFAs. Subsequent gas chromatographic analysis made it possible to identify the *trans* and *cis* isomers without interference of contaminants. The unsaturated fatty acid fractions from these glass cartridges were also free of contaminants.

Keywords: Animal fat / GC / Geometric isomers / Impurities / Silver-ion / *Trans*

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1 Introduction

Commercial silver ion solid-phase extraction (Ag^+-SPE) cartridges have proven to be effective to resolve unsaturated fatty acid methyl esters (FAMEs) based on their number and/or geometric configuration of double bonds [1, 2]. They have often been used to provide separations that could not be achieved using gas chromatography (GC) alone, even with long and high polarity columns [3–5]. However, when we tried to use these cartridges to identify the *trans* fatty acid (TFA) content and isomer distribution in horse meat [6], we were unable to do so because the cartridges leaked impurities that obscured the small level of TFA isomers present. Several attempts to remove impurities from the Ag^+-SPE cartridges using a combination of different solvents were not successful, but instead led to a partial loss of resolution of many unsaturated FAMEs after the additional washes. It was suspected that the impurities came from the polypropylene housing tubes rather than the cation-exchange resin, since the impurity peaks were not present when the same sample of horse total FAMEs was analyzed using Ag^+-high performance liquid chromatography (Ag^+-HPLC) [6, 7]. The supplier (Supelco Inc, Bellefonte, PA) was contacted and they provided us with custom made glass cartridges for evaluation filled with the same

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Abbreviations: ***ai*,** anteiso; **DMA,** dimethyl acetal; **FA,** fatty acids; **FAME,** fatty acid methyl ester; **GC,** gas chromatography; **HPLC,** high performance liquid chromatography; ***i*,** iso; **MUFA,** monounsaturated fatty acids; **PUFA,** polyunsaturated fatty acids; **SFA,** saturated fatty acids; **SPE,** solid-phase extraction; **TFA,** *trans* fatty acids

amount of Ag^+-loaded cation-exchange resin. In the present communication, the FAMEs prepared from horse and wild boar lipids were resolved using the custom made Ag^+-SPE cartridges in glass housing to see whether the TFA isomers can be identified in these animal lipids that are known to be present at low concentrations.

2 Materials and methods

High purity grade reagents and solvents were used to avoid any contamination of the fractions. GC reference standard #463 was used spiked with 19:0, 21:0, 23:0, 26:0, and CLA mixture (UC-59M) FAMEs, all obtained from Nu–Chek Prep Inc. (Elysian, MN, USA). Branched-chain fatty acids (FA) were identified using a bacterial FAME mixture purchased from Matreya Inc. (Pleasant Gap, PA, USA). The test sample of horse backfat was methylated using a base catalyst [3], and the wild boar meat was methylated using a base followed by an acid catalyst [8].

Two lots of Ag^+-SPE cartridges were used. One was the commercial product available from Sigma–Aldrich (Supelco Discovery^{TM}, 750 mg/6 mL). The other was the same amount of Ag^+-loaded cation-exchange resin packed into custom made glass cartridges prepared by Supelco Inc. (Bellefonte, PA). The glass cartridges had slightly different dimensions (i.d. 1.4 cm; length 7.6 cm) than the commercial cartridges (i.d. 1.3 cm; length 6.7 cm), but the thickness of the resin was the same (1.7 cm). The Ag^+-SPE cartridges were then conditioned with 12 mL acetone and 6 mL hexane before addition of the test sample containing about 1 mg of total FAMEs in hexane. After the sample solution was allowed to seep into the resin, 6 mL of each of the following eight solvent mixtures was added in consecutive order as previously described [3]. The solvents used were mixtures of hexane and acetone 99:1 (1, saturated FA, SFA); 96:4 (2, *trans*-monounsaturated FA, MUFA); 90:10 (3, *cis*-MUFA); 0:100 (4, dienoic FA); and acetone and acetonitrile 97:3 (5, trienoic FA); 94:6 (6, tetraenoic FA); 80:20 (7, pentaenoic FA); 0:100 (8, hexaenoic FA). All fractions were analyzed using a GC equipped with a flame ionization detector and an autosampler. The FAMEs from horse were analyzed using an Agilent Model 7890A GC, and the FAMEs from wild boar were analyzed using a Shimadzu Model GC-2100 Plus, and in both cases a 100 m SP2560 column (Sigma–Aldrich, Corp., St Louis, MO) was used operated using the temperature program with a plateau at 175°C, hydrogen as a carrier gas, and the spit mode depended on the amount of sample and its concentration [3]. The FAMEs were identified using a GC reference mixture #463 from Nu–Chek Prep Inc., and by comparison to previously reported assignments [3, 5, 8–10].

3 Results and discussion

For an accurate determination of the *trans* and *cis* monounsaturated FAMEs present in horse tissue, we

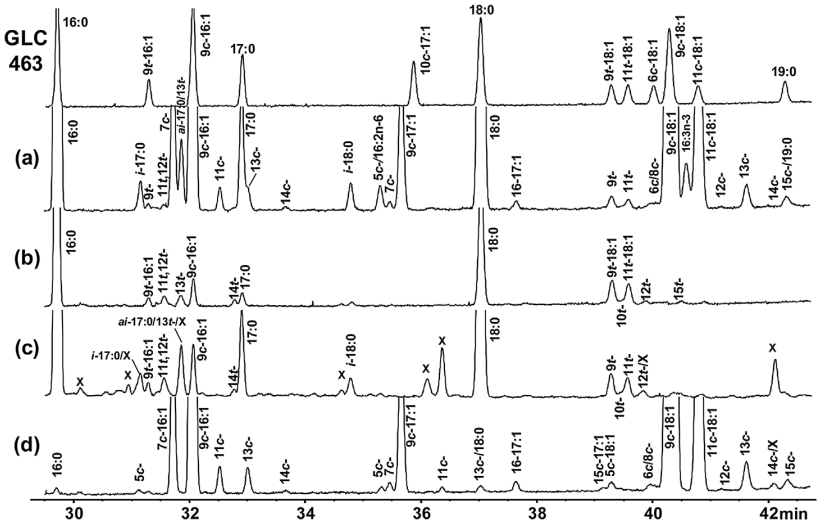

Figure 1. Partial GC chromatogram from 16:0 to 19:0 of the FAMEs prepared from total horse backfat (a), as well as the *trans* (b) and *cis* fraction (d) obtained from total horse backfat FAMEs using the custom made Ag^+-SPE cartridges in glass housing. The *trans* fraction obtained using a commercial Ag^+-SPE cartridge is shown in panel (c). For the GC analysis hydrogen was used as carrier gas, and a temperature program with a plateau at 175°C [3]. GLC reference #463 from Nu–Chek Prep Inc. is shown in the top panel. *i*, iso branched-chain FA; *ai*, anteiso branched-chain FA; X, impurity.

complemented the separation of the FAMEs using a 100 m SP2560 GC column with a prior silver ion chromatographic separation using Ag^+-SPE cartridges. The overlap of geometric and positional isomers together with SFAs, as well as that of polyunsaturated FAs (PUFA) was easily resolved by combining these two techniques. Commercial Ag^+-SPE cartridges available from Sigma–Aldrich were successfully used in a number of applications [3–5, 8–10]. They effectively resolved the geometric isomers of MUFAs, and unsaturated FAMEs based on the number of double bonds [4]; some minor overlap occurs between fractions when the concentration of FAMEs was high. After this prior separation, the positional isomers and FAMEs with different chain length were resolved and identified by GC [4]. That is why it came as a surprise that we were unable to identify the TFA isomers in horse lipids using these cartridges [6]. Impurities that eluted from the cartridges obscured the identification of the small amounts of TFA isomers in horse lipids. Some of the impurities were recognized by their elution in a region where no identifiable FAMEs eluted [3, 5, 8–10], while other were recognized by comparison to the results of Ag^+-HPLC, which did not show these impurities. Using Ag^+-HPLC also helped us identify the TFA isomers in horse lipids and confirmed our suspicion that the impurities came from the polyethylene tubes. We approached Supelco Inc. and they kindly provided us with custom made glass cartridges filled with the same amount of Ag^+-loaded cation-exchange resin.

Figure 1 shows the separation of the total FAMEs prepared from the backfat of horse (Fig. 1a), as well as the *trans* (Fig. 1b) and *cis* (Fig. 1d) fractions obtained using these new custom made glass cartridges. The *trans* fraction obtained using the commercial Ag^+-SPE cartridges is included for comparison (Fig. 1c). To provide a comparison between the two *trans* fractions (Fig. 1b and c), the GC graphs were adjusted so that the two major *trans*-18:1 isomers 9*t*- and 11*t*-18:1, common and visible in both graphs, are at about the same size in both GC graphs. Any SFA or *cis*-MUFA could not be used as reference to compare the content of impurities, since their content in the *trans* fraction differs dependent on slight differences in affinity of the cation-exchange resin (generally due to differences in moisture). It was evident from the results of the new glass cartridge that the TFA isomers of 16:1 (9*t*-, trace amounts 10*t*-, 11*t*-/12*t*-, 13*t*-, and 14*t*-16:1), and 18:1 (9*t*-, trace amounts 10*t*-, 11*t*-, 12*t*-, and trace amounts of 15*t*-18:1) could now be identified. In addition, these results agreed with the identification of these FAME resolved and identified using the ionic liquid GC column SLB-IL111 [7, 11]. This identification was not possible using the commercial Ag^+-SPE cartridges because of the many additional peaks (labeled as X) in the *trans* fraction, and impurities that co-eluted with some of the *trans*-MUFA isomers, specifically in the *trans*-16:1 region (Fig. 1c).

As reported in our previous publication [6], a number of impurities were also present among the PUFAs that made their identification difficult (results not shown). These extra peaks were not present when the new glass cartridges were used. The *trans* fraction did however, contain residual amounts of the major saturated FAMEs because of the high relative concentration of the saturated FAMEs and the sample load. Applying slightly less sample load (total

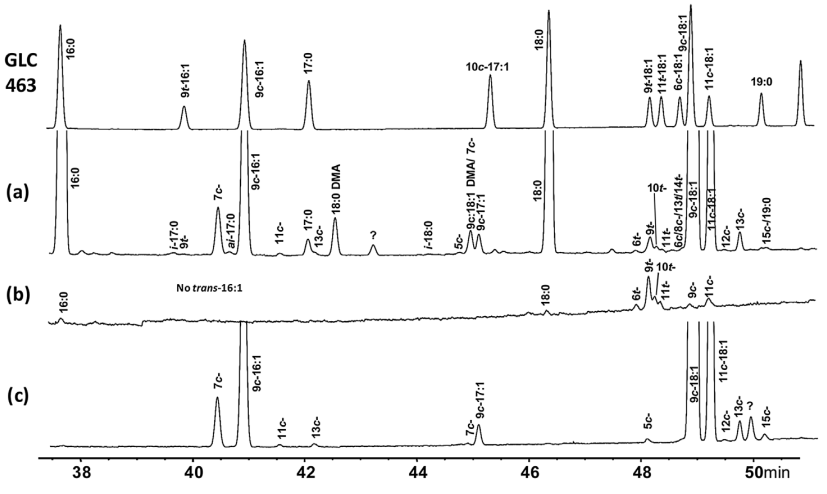

Figure 2. Partial GC chromatogram from 16:0 to 19:0 of the FAMEs prepared from total wild boar muscle (*longissimus dorsi*) (a), as well as the *trans* (b) and *cis* fraction (c) obtained from this FAME mixture using the custom made Ag^+-SPE cartridges in glass housing. For the GC analysis helium was used as carrier gas, and a temperature program with a plateau at 175°C [3]. GLC reference #463 from Nu–Chek Prep Inc. is shown in the top panel. DMA, dimethyl acetal; *i*, iso branched-chain FA; *ai*, anteiso branched-chain FA; X, impurity.

FAMEs) would have resulted in a more complete separation of the fractions, but that would have made the identification of the low levels of TFA even more challenging. The *cis* fraction was included to show the success of the Ag^+-SPE cartridges to resolve and identify all the *cis* isomers of 16:1, 17:1, and 18:1 in horse lipids.

To further test the potential of using the new custom made glass cartridges, we chose to resolve the lipids from wild boar meat that are also known to contain low levels of TFAs. Figure 2 shows the separation of the total FAMEs prepared from the lipids extracted from wild boar muscle (Fig. 2a), as well as the *trans* (Fig. 2b) and *cis* (Fig. 2c) fractions obtained using these new custom made glass Ag^+-SPE cartridges. No *trans* 16:1 isomers were found to be present in wild boar lipids, and the *trans*-18:1 isomer distribution from 6*t*- to 12*t*-18:1 appears to be random with 9*t*-18:1 being the most abundant isomer. The *cis* fraction from wild boar meat showed fewer isomers of 16:1, 17:1, and 18:1 compared to horse lipids. The dimethyl acetals (DMA) are products of methylation of the alk-1-enyl ether moiety from plasmalogenic lipids [12] which were evident in the lipids of wild boar meat. The new custom made glass cartridges were found to be an excellent method to identify the *trans* and *cis* isomers in wild boar meat.

We also observed that the PUFA fractions from the custom made glass cartridges had few if any interfering peaks, which greatly improved the identification of the PUFAs. In this silver ion fractionation, we slightly modified the elution solvents for fractions 7 and 8 to more completely elute the PUFAs with five and six double bonds (fractions not shown).

4 Conclusions

The custom made Ag^+-SPE cartridges in glass housing were found to be successful to resolve and identify the *trans* isomers even when present at low concentration in a biological sample such as horse backfat and wild boar meat. We recommend that the supplier make these glass Ag^+-SPE cartridges available to researchers who wish to use this improved Ag^+-SPE technique in their analyses of the geometric and positional isomers of MUFA and PUFA.

X. Belaunzaran and L. Bravo-Lamas wish to thank the Basque Government for their fellowships. N. Aldai wishes to thank the Spanish Ministry of Economy & Competitiveness and UPV/EHU for the "Ramón y Cajal (RYC-2011-08593)" contract. The authors wish to thank Sigma–Aldrich for generously providing Ag^+-SPE tubes in glass cartridges.

The authors have declared no conflict of interest.

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2. BIOLOGICAL RESULTS

2.1. An assessment of the fatty acid composition of horse-meat available at retail level in northern Spain: experimental design I.

Publication IV evaluates the variability of the nutritional quality of commercial horse-meat surveyed at retail level in the northern Spain. In this survey, the effect of time of the year (season) and region have been investigated, and emphasis has been made on the differences in FA profile of SC and intramuscular (IM) fat tissues. Results from this study complement considerably previously published data on the FA characterization of horse tissues, especially regarding minor components (*i.e.*, DMA, BCFA, *trans*-FA, CLA) that are most of the times neglected in the literature.

PUBLICATION IV

An assessment of the fatty acid composition of horse-meat available at retail level in northern Spain

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An assessment of the fatty acid composition of horse-meat available at the retail level in northern Spain

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Subcutaneous fat

ABSTRACT

The objective of the present study was to assess the fatty acid composition of horse-meat available at the retail market in northern Spain. Horse steaks (*Longissimus thoracis et lumborum* muscle; $n = 82$) were purchased from butcher-shops and large grocery stores throughout six northern regions of Spain in two different seasons. Fat content differed significantly among regions (1.12 to 2.77%). Samples with higher intramuscular fat content presented the highest percentages of total monounsaturated fatty acids and the lowest contents of dimethylacetal and polyunsaturated fatty acids (PUFA), while the opposite was found in the leanest samples. A high variability was observed in the muscle and subcutaneous n-3 PUFA content. Overall, total n-3 PUFA content ranged between 1.17% and 18.9% in muscle fat and between 1.52% and 27.9% in backfat. Interestingly, almost 5% of surveyed loins from horse carcasses (4 out of 82) contained over 300 mg of linolenic acid per 100 g of meat which could have been marketed as a "source" of n-3 FAs according to Commission Regulation (EU) No 116/2010.

1. Introduction

Horses, as non-ruminant herbivores and hindgut fermenters, are adapted to ingest large quantities of forage in a continuous fashion. Dietary lipids are primarily digested by endogenous enzymes and 90–95% of total digested material is absorbed in the small intestine (Santos, Rodriguez, Bessa, Ferreira, & Martin-Rosset, 2011). Generally, the fatty acids (FA) are absorbed before being submitted to microbial metabolism occurring in the hindgut (cecum and colon), and therefore, biohydrogenation activity is low compared to ruminants. The digestive physiology of equines has been associated with high deposition of polyunsaturated fatty acids (PUFA) (Clauss, Grum, & Hatt, 2009) along with very low levels of less investigated *trans*-FAs (TFA) (Hartam, Shorland, & Moir, 1956) and conjugated linoleic acids (CLA) (Cicognini, Rossi, Sigolo, Gallo, & Prandini, 2014). Moreover, none of the scientific literature reviewed described the branched-chain FAs (BCFA) in horse tissues, even though Santos et al. (2011) observed considerable amounts of these FAs in the equine hindgut bacterial biomass.

In the nineteen-fifties, Shorland, Bruce, and Jessop (1952) already confirmed high levels of linolenic acid (LNA; 17%) and moderate levels of linoleic acid (LA; 4%) in grass-fed horse adipose tissue. In recent years, a higher accumulation of LNA was reported in horses managed under extensive grazing (23.9–24.3%) compared to animals finished with concentrates (0.90–8.90%; Guil-Guerrero, Rincón-Cervera, Venegas-Venegas, Ramos-Bueno, & Suárez-Medina, 2013; Lorenzo, Fuciños, Purriños, & Franco, 2010). Guil-Guerrero et al. (2013) even suggested that horse backfat could be a relevant source of energy and n-3 PUFAs for humans in unique environments. Horse-meat has been recently described as "healthier" in terms of its high n-3 PUFA content, it's almost absence of TFA, and its significantly lower footprint of greenhouse gas emission compared to ruminants (Belaunzaran et al., 2015; Lorenzo, 2013). In spite of its advantageous and nutritional qualities, horse-meat consumption is still scarce in many countries because of socio-cultural perceptions and the reoccurring mislabeling scandals. The objective of the present study was to detail the chemical and FA composition of horse muscle and adipose tissues available at the retail level in northern Spain, where equine production has become an interesting option in less favored mountain areas where human population is very low. Special emphasis was put on the PUFA content and other minor and less studied TFA, BCFA and CLA. Such data are relevant to provide "farm to fork" stakeholders with accurate information about the nutritional characteristics, and to improve consumer's perception and acceptance of this undervalued but edible product.

2. Material and methods

2.1. Sample collection

Horse steaks ($n = 82$) were purchased from butcher-shops (90%) and large grocery stores (10%) throughout six northern regions of Spain

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localized at both sides of the Cantabrian Mountains (Basque, BA; Navarre, NA; Cantabria, CA; Asturias, AS; Galicia, GA; Castile & Leon, CL); see map indicating sampling locations (Fig. 1). In these regions, horse-meat may be produced locally from animals grazed in valley or mountain areas and occasionally supplemented with moderate amounts of concentrates or imported, which is more likely when sold in large grocery stores. According to the limited information obtained from butchers and labels, most samples were obtained from animals that were managed and slaughtered locally. From 4 to 10 steaks per region were collected in two different seasons (spring and winter); from April to May of 2013 ($n = 41$) and from December of 2013 to January of 2014 ($n = 41$). The unequal number of samples per region were due to the fact that samples from three grocery chains were collected in the Basque and one in Asturias region. Horse-meat from grocery chains were not sampled in the other regions because the meat came from the same supplier. The two seasonal collections were initiated because of possible variations in meat composition related to different management practices linked to the time of year. Horse-meat collected in spring would likely come from animals that have entered the feedlot in the fall, while horse-meat collected in winter would likely come from animals that have been breastfed and grazed in mountain areas from spring to late autumn. However, precise details of production systems for most of the horse samples collected are unknown, and the information provided on labels or obtained from retailers was limited, but recorded in order to provide a possible explanation for the findings. Samples were transported to the laboratory located in the Lascaray Research Center (Vitoria-Gasteiz, Spain) in refrigerated coolers. Then, subcutaneous (SC) fat and *Longissimus thoracis et lumborum* (LTL) muscle were separated, vacuum packed and frozen at −80 °C for further analysis.

2.2. Chemical composition of muscle

Samples of LTL muscle were analyzed as described in Aldai, Lavín, Kramer, Jaroso, and Mantecón (2012b). Standard procedures were used for dry matter (ISO, 1999), crude protein (ISO, 2005), ether extract (AOCS, 2008) and ash (ISO, 2002) determinations.

2.3. Fatty acid analysis of adipose and muscle tissues

Seventy four SC fat samples were analyzed since 8 steaks were virtually devoid of external fat (butchers sometimes tend to remove the fat covering the rib joint). Fat samples were weighted (50 ± 1 mg), freeze-dried and directly methylated with sodium methoxide (Methanolic-Base, 0.5 N; Supelco). From the muscle tissue, lipids were extracted from 1.5 g of freeze-dried sample using chloroform-methanol (2:1, v/v; Folch, Lees, & Sloane-Stanley, 1957). Lipid aliquots (10 mg) from each steak were then separately methylated using base (Methanolic-Base, 0.5 N; Supelco) and acid (Methanolic HCl, 3 N; Supelco) methylations to ensure complete derivatization of total lipids and avoid CLA isomerization (Aldai et al., 2012a; Kramer et al., 1998; Kramer, Hernandez, Cruz-Hernandez, Kraft, & Dugan, 2008). For quantitative purposes, 1 mL of internal standard (IS; mixture of 0.5 mg/mL of 13:0 and 0.5 mg/mL of 23:0 methyl esters from Nu-Chek Prep Inc., Elysian, MN, USA) was added prior to methylation. Fatty acid methyl esters (FAME) were analyzed using a gas chromatograph equipped with a flame ionization detector (GC/FID; Agilent Technologies, Model 7890A, Wilmington, DE, USA) and automatic injector (Agilent Technologies, Model 7693). Samples were injected using a 50:1 split ratio, a Supelco SP2560 capillary column (100 m × 0.25 mm I.D., 0.2 μm coating, Bellefonte, PA, USA), and temperature programs previously described by Kramer et al. (2008). To resolve CLA isomers and some other overlapping FAMEs, samples were subjected to a second GC/FID analysis using a SLB-IL111 ionic liquid stationary phase column (100 m × 0.25 mm I.D., 0.2 μm coating; Supelco, Bellefonte, PA, USA) using a temperature program described by Delmonte et al. (2011). In both GC runs, hydrogen was used as a carrier gas at a flow rate of 1 mL/min, the injection volume was 1 μL, and injector and detector ports were set at 250 °C. For identification purposes, #463 and #603, individual FAME (21:0, 23:0, 26:0,

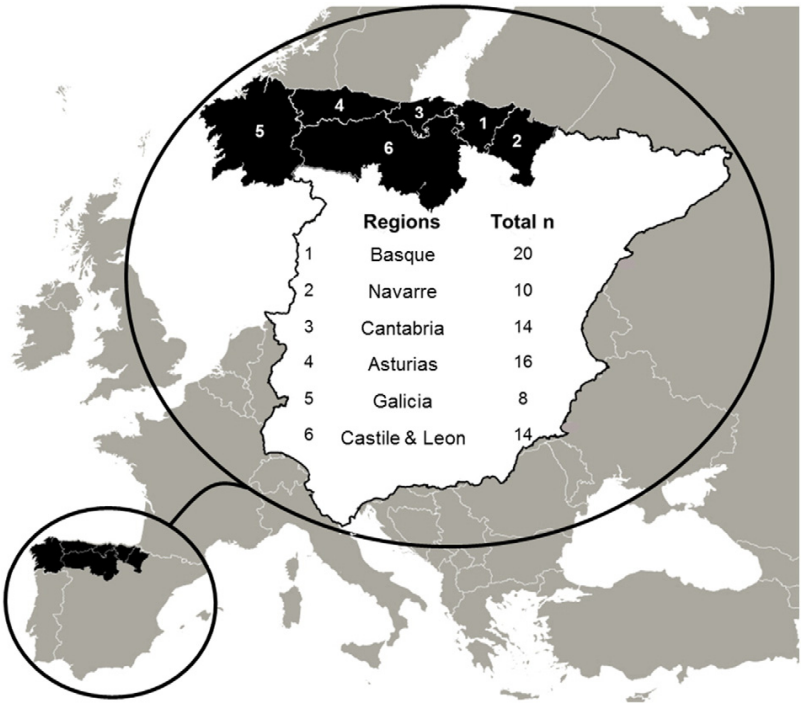

Fig. 1. A map showing sampling regions and the total number of samples collected in northern Spain.

28:0) and #UC-59 M CLA mixture reference standards obtained from Nu-Check (Elysian, MN, USA) were analyzed. Additionally, LA (18:2n-6) and LNA (18:3n-3) isomer mixtures from Sigma-Aldrich (#47,791 and #47,792; Supelco, Bellefonte, PA, USA) and a bacterial FAME mixture from Matreya (Pleasant Gap, PA, USA) were used. Many of the TFA, CLA isomers, non-conjugated (NC) dienes, trienes and long chain (LC)-PUFA not included in the standard references were identified by performing FAME fractionation using silver-ion solid phase extraction (Ag^+-SPE) cartridges (Belaunzaran, Bravo-Lamas, Kramer, & Aldai, 2014; Kraft et al., 2014; Kramer et al., 2008), and retention times and elution orders reported previously in the literature (Alves & Bessa, 2009; Alves, Santos-Silva, Cabrita, Fonseca, & Bessa, 2013; Cruz-Hernandez et al., 2004; Cruz-Hernandez et al., 2006; Delmonte et al., 2012; Rego et al., 2009; Santercole, Delmonte, & Kramer, 2012). The whole horse samples were also analyzed by high resolution GC-Mass Spectrometry (GC-MS) using a Q-TOF mass spectrometer (Agilent Technologies, Model 7200A, Wilmington, DE, USA) coupled with an Agilent 7890B GC (Fardin-Kia et al., 2013) to confirm the identity of all FAME and dimethylacetals (DMA) by their molecular ion. These runs were carried out in the chemical ionization (CI^+) mode using isobutane as ionization gas. The source was maintained at 300 °C, the electron energy was 250 eV, the emission current 30 μA, and the CI gas flow was set to 20%.

For quantitative purposes, only chromatographic peak areas that had a signal to noise ratio of 3 to 1 were considered, and these peaks were then corrected according to theoretical response factors (Wolff, Bayard, & Fabien, 1995). Thereafter the amount of FAMEs (mg) was calculated using the IS added to the sample. Fatty acids were expressed as mg/100 g of fresh meat (intramuscular; IM) and as percentage (%) of total FAME quantified (IM and SC). In general, FAME representing <0.03% were excluded to reduce the size of the tables, except for minor FAMEs of particular interest (BCFA, *trans*-18:1 and CLA isomers, LC n-6 and n-3 PUFA).

2.4. Data treatment and statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics 22 for windows (SPSS Inc., IBM Corporation, NY, USA). The following linear model $Y_{ijk} = \mu + S_i + R_j + (S^*R)_{ij} + e_{ijk}$ was used for analysis of variance (ANOVA), including season (S) and region (R) as fixed effects. In order to compare adipose tissues, IM and SC FA profile data were also submitted to ANOVA. In this case, the same linear model, but including type of adipose tissue (T) as fixed effect together with all binary interactions between main factors, was used. Tukey HSD post hoc test was applied for multiple comparison of means.

Principal component analysis (PCA) was performed on main individual and groups of FAs, in order to establish relationships among them. The model included main saturated FA (SFA; 10:0, 11:0, 12:0, 14:0, 15:0, 16:0 and 18:0), MUFA (9*c*-14:1, 9*c*-16:1, 9*c*-18:1 and 11*c*-18:1), PUFA (18:2n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, 18:3n-3, 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3) and several groups (SFA, BCFA, monounsaturated FA (MUFA), *trans*-MUFA, PUFA, NC-dienes, trienes, and CLA). Sample distribution plots using the two-dimensional coordinate system defined by the first two principal components (PC) were used to study the variability in FA composition derived primarily from the type of adipose tissue.

Three significant figures were used to express the data. Significance was declared at $P \leq 0.05$.

3. Results and discussion

3.1. Chemical composition of muscle

The chemical composition of LTL muscle for both collection periods is presented in Table 1. In general terms, the composition values of horse-meat were quite similar to the meat of other species as reviewed by Lorenzo et al. (2014). Moisture was affected by the interaction of season and region effects (average value of 73.4%) where samples collected in spring had higher moisture in all regions except in Asturias. The explanation of these values was not immediately apparent but could be linked to post slaughter handling of carcasses and meat (i.e., conservation, packaging). No differences were observed in total protein content (average value of 23.4%), while fat content differed significantly among regions ranging from 1.12 to 2.77% ($P = 0.001$). In this regard, samples with higher IM fat content were collected in Navarre and Castile & Leon while the leanest samples were collected in Asturias and Galicia regions, mainly from butcher-shops. Considerable variability in the IM fat content was also reported in the scientific literature related to such factors as breed, gender, age at slaughter and/or production system, and they ranged from 0.500% in extensively reared horses (Guil-Guerrero et al., 2013; Lorenzo et al., 2010) to 12.7% in concentrate-fed horses (He, Ishikawa, & Hidari, 2005); see review by Belaunzaran et al. (2015). Ash content was higher in samples collected in spring compared to samples collected in winter (1.63 and 1.31%, respectively; $P < 0.001$), which could be related to the age of animals and/or the mineral concentration of their diet (Doyle, 1980; Pannier et al., 2014). In the literature, ash values ranging from 0.98 to 4.03% have been reported (Badiani, Nanni, Gatta, Tolomelli, & Manfredini, 1997; Sarriés & Beriain, 2005) and it is known that the mineral content of grasses could be influenced by differences in plant species, physiological status, type of soil, as well as seasonal factors, while the mineral content of commercial supplements if provided is, in most cases, standardized (McDonald, Edwards, Greenhalgh, & Morgan, 2002).

Table 1
Chemical (%) and fatty acid (mg/100 g of fresh meat) composition of horse *Longissimus thoracis et lumborum* muscle from spring and winter collections.

Composition	Season		SEM	*P* values		
	Spring $n = 41$	Winter $n = 41$		S	R	S*R
Chemical						
Moisture	73.9	72.9	0.181	0.007	0.068	0.045
Crude Protein	23.2	23.7	0.181	0.427	0.935	0.131
Fat (ether extract)	1.99	1.97	0.136	0.832	0.001	0.764
Ash	1.63	1.31	0.0352	<0.001	0.376	0.380
Fatty acid						
Total FAME	1950	2000	115	0.864	0.001	0.768
SFA	717	745	45.5	0.818	<0.001	0.737
BCFA	7.32	6.88	0.356	0.592	0.661	0.764
DMA	55.0	57.4	0.912	0.271	0.028	0.644
MUFA	731	732	59.9	0.950	<0.001	0.837
cis-MUFA	727	729	59.7	0.953	<0.001	0.838
trans-MUFA	3.96	3.50	0.201	0.179	0.017	0.503
CLA (18:2)	1.85	1.61	0.122	0.276	0.012	0.929
NC-dienes (18:2)	1.50	1.49	0.0796	0.842	0.059	0.434
Trienes (18:3)	1.90	1.98	0.0849	0.647	0.306	0.774
PUFA	412	451	15.1	0.184	0.049	0.541
n-6	280	300	10.1	0.286	<0.001	0.414
n-3	132	152	10.8	0.387	0.642	0.924

SEM, standard error of the mean; S, season; R, region; SFA, saturated fatty acid (FA); BCFA, branched-chain FA; DMA, dimethylacetal; CLA, conjugated linoleic acid; NC, non-conjugated; PUFA, polyunsaturated FA.

3.2. Fatty acid composition of muscle

The season and region effect on the muscle FA composition of horses in absolute and percentage basis are represented in Table 1 and Tables 2 to 4, respectively. The total muscle FA content averaged 1970 mg/100 g of meat, similar to the total fat content obtained by extraction with ether (1.98%) and it was significantly affected by the region (Table 1; Fig. 2). Region effect was significant for most of the FAs reported and these differences were suspected to be associated with the type of breed and feeding, which are known to affect the muscle fat content. Unfortunately, this information could not be provided by the retailers. However, a few general comments could be made. The samples with

Table 2
Saturated fatty acid composition (%) of horse *Longissimus thoracis et lumborum* muscle from spring and winter collections.

Fatty acid	Season		SEM	*P* values		
	Spring $n = 41$	Winter $n = 41$		S	R	S*R
SFA	37.0	36.5	0.228	0.243	0.275	0.108
10:0	0.118	0.156	0.0103	0.029	0.009	0.490
12:0	0.475	0.583	0.0421	0.163	0.021	0.869
14:0	3.34	3.43	0.0911	0.622	0.243	0.911
15:0	0.249	0.235	0.00534	0.321	0.129	0.390
16:0	26.0	25.4	0.289	0.244	<0.001	0.299
17:0	0.358	0.311	0.00805	0.008	0.057	0.664
18:0[a]	6.21	6.09	0.154	0.595	<0.001	0.445
19:0	0.0458	0.0484	0.00232	0.552	<0.001	0.525
20:0	0.0958	0.103	0.00354	0.215	<0.001	0.818
21:0	0.00864	0.00901	0.000418	0.903	0.039	0.122
22:0	0.0414	0.0448	0.00185	0.311	<0.001	0.632
24:0	0.0533	0.0636	0.00292	0.036	<0.001	0.566
BCFA	0.405	0.396	0.0170	0.958	<0.001	0.751
i-14:0	0.0409	0.0443	0.00234	0.127	<0.001	0.054
i-15:0	0.0209	0.0178	0.000958	0.119	<0.001	0.676
ai-15:0	0.0272	0.0269	0.00138	0.872	0.041	0.905
i-16:0	0.117	0.116	0.00452	0.794	0.003	0.665
i-17:0	0.0396	0.0344	0.00160	0.094	<0.001	0.693
ai-17:0	0.0933	0.0903	0.00476	0.802	<0.001	0.890
i-18:0	0.0528	0.0500	0.00218	0.558	<0.001	0.885
DMA	3.31	3.71	0.172	0.257	<0.001	0.264

SEM, standard error of the mean; S, season; R, region; SFA, saturated fatty acid (FA); BCFA, branched-chain FA; *ai*, *anteiso*; *i*, *iso*; DMA, dimethylacetal.
[a] Coeluted with 16:2n-3.

higher IM fat content were collected in Castile & Leon, Navarre and Basque regions. These samples presented the highest percentages of total MUFA (NA > CL > BA > CA > GA > AS) and the lowest of total DMA, BCFA, and PUFA ($P < 0.001$). The samples with higher fat content also presented highest contents (%) of palmitic, palmitoleic and oleic acids ($P < 0.001$). In contrast, the leanest samples were collected in Asturias and Galicia regions which are, in general terms, characterized by leaner breeds produced on mountain grazing. These samples provided the highest percentages of DMA, BCFA, and n-6 (AS > CA > GA > BA > NA > CL), total n-3 and n-3 LC-PUFA contents ($P < 0.001$; Fig. 2). On an absolute basis, the highest contents of n-3 LC-PUFAs (eicosapentaenoic acid, EPA; docosapentaenoic acid, DPA, and docosahexaenoic acid, DHA; $P < 0.001$) were also found in leanest samples. It is well known that muscle FA composition can be influenced by the relative proportion of its neutral and polar lipid fractions, and these can be affected by genetics and dietary factors as well as animal fatness (De Smet, Raes, & Demeyer, 2004; Kraft, Kramer, Schoene, Chambers, & Jahreis, 2008). Accordingly, a higher fat content was associated with a lower PL/TG ratio and a dilution of those FA located in the polar lipid fraction which includes DMA and LC-PUFA (De Smet et al., 2004; Horrocks, 1972).

Table 3
Monounsaturated fatty acid composition (%) of horse *Longissimus thoracis et lumborum* muscle from spring and winter collections.

Fatty acid	Season		SEM	*P* values		
	Spring $n = 41$	Winter $n = 41$		S	R	S*R
MUFA	35.4	32.3	0.976	0.049	<0.001	0.736
cis-MUFA	35.2	32.1	0.977	0.052	<0.001	0.740
9*c*-14:1	0.303	0.325	0.0150	0.514	<0.001	0.877
cis-16:1	5.90	5.90	0.231	0.955	<0.001	0.670
7*c*-16:1	0.294	0.253	0.0100	0.121	0.067	0.419
9*c*-16:1	5.54	5.56	0.233	0.996	<0.001	0.668
11*c*-16:1	0.0429	0.0451	0.00150	0.526	0.188	0.986
13*c*-16:1	0.0198	0.0287	0.00204	0.053	0.202	0.950
9*c*-17:1	0.392	0.368	0.0118	0.393	<0.001	0.551
cis-18:1	27.8	24.9	0.762	0.021	<0.001	0.805
6-8*c*-18:1	0.0268	0.0290	0.000974	0.595	0.038	0.083
9*c*-18:1	25.8	22.9	0.755	0.022	<0.001	0.801
11*c*-18:1	1.92	1.87	0.0230	0.485	0.089	0.868
13*c*-18:1	0.0892	0.0867	0.00209	0.444	0.047	0.709
11*c*-19:1	0.0521	0.0475	0.00118	0.069	0.504	0.801
7*c*-20:1	0.0232	0.0232	0.000836	0.988	<0.001	0.991
9*c*-20:1	0.0389	0.0344	0.000729	<0.001	0.161	0.219
11*c*-20:1	0.319	0.277	0.00868	0.008	<0.001	0.738
13*c*-22:1	0.0283	0.0231	0.000695	<0.001	0.015	0.765
13*c*-24:1	0.0427	0.0183	0.00421	<0.001	0.002	0.035
15*c*-24:1	0.127	0.112	0.00697	0.078	<0.001	0.203
17*c*-24:1	0.0880	0.0358	0.00750	<0.001	0.002	0.032
trans-MUFA	0.212	0.180	0.00507	<0.001	0.007	0.224
11*t*/12*t*-16:1	0.0371	0.0397	0.00149	0.202	0.002	0.657
trans-18:1	0.149	0.114	0.00589	<0.001	0.075	0.113
6-8*t*-18:1	0.0163	0.00607	0.00317	0.030	0.002	0.161
9*t*-18:1	0.0847	0.0706	0.00240	<0.001	<0.001	0.252
10*t*-18:1	0.0116	0.00616	0.000897	0.003	0.173	0.323
11*t*-18:1	0.0298	0.0249	0.00115	0.016	0.016	0.106

SEM, standard error of the mean; S, season; R, region; MUFA, monounsaturated fatty acid.

Table 4
Polyunsaturated fatty acid composition (%) of horse *Longissimus thoracis et lumborum* muscle from spring and winter collections.

Fatty acid	Season		SEM	*P* values		
	Spring $n = 41$	Winter $n = 41$		S	R	S*R
CLA (18:2)	0.0941	0.0768	0.00306	0.002	0.033	0.810
9*c*,11t-	0.0378	0.0321	0.00152	0.060	0.042	0.880
9*t*,11*c*-	0.00556	0.00332	0.000517	0.010	<0.001	0.995
10*t*,12*c*-	0.00999	0.0118	0.000552	0.040	0.030	0.230
11*t*,13*t*-	0.0102	0.00896	0.000309	0.055	0.507	0.786
7*t*,9*t*-10*t*,12*t*-	0.0262	0.0173	0.00217	0.010	0.002	0.451
NC-dienes (18:2)	0.0810	0.0773	0.00224	0.353	0.018	0.534
9*c*,13*t*/8*t*,12*c*-	0.00783	0.00688	0.000501	0.351	0.088	0.544
9*c*,12*t*-	0.0360	0.0312	0.000998	0.002	<0.001	0.104
9*t*,12*c*-	0.0191	0.0185	0.000623	0.509	0.004	0.587
11*t*,15*c*-	0.00527	0.00611	0.000633	0.261	0.092	0.246
9*c*, 15*c*-	0.0119	0.0144	0.00102	0.338	0.647	0.966
Trienes (18:3)	0.110	0.124	0.00614	0.159	<0.001	0.390
9*c*,12*c*,15*t*-/9*t*,12*t*,15*c*-	0.0500	0.0618	0.00424	0.103	<0.001	0.703
9*c*,11*t*,15*c*-	0.0106	0.00733	0.00146	0.494	0.833	0.043
PUFA	23.4	26.6	0.924	0.033	<0.001	0.513
n-6	15.8	17.2	0.512	0.122	<0.001	0.369
16:2n-6	0.0384	0.0367	0.00140	0.693	0.006	0.441
18:2n-6	13.5	14.7	0.405	0.109	<0.001	0.236
18:3n-6	0.0305	0.0340	0.00140	0.271	<0.001	0.208
20:2n-6	0.245	0.250	0.00764	0.766	0.019	0.964
20:3n-6	0.368	0.454	0.0236	0.039	<0.001	0.242
20:4n-6	1.44	1.61	0.0865	0.306	<0.001	0.911
22:4n-6	0.0889	0.0910	0.00334	0.802	0.649	0.989
22:5n-6	0.0159	0.0172	0.00106	0.499	<0.001	0.992
n-3	7.64	9.35	0.593	0.088	<0.001	0.849
16:3n-3	0.0492	0.0554	0.00363	0.298	0.006	0.849
18:3n-3	5.78	6.87	0.481	0.179	0.010	0.857
18:4n-3	0.0133	0.0139	0.00111	0.842	0.017	0.972
20:3n-3	0.274	0.346	0.0219	0.060	<0.001	0.881
20:4n-3	0.0568	0.0730	0.00534	0.093	<0.001	0.777
20:5n-3	0.317	0.491	0.0374	0.005	<0.001	0.090
22:3n-3	0.0328	0.0484	0.00228	<0.001	<0.001	<0.001
22:4n-3	0.0363	0.0425	0.00240	0.159	<0.001	0.675
21:5n-3	0.0363	0.0368	0.00162	0.986	0.784	0.902
22:5n-3	0.804	1.06	0.0633	0.014	<0.001	0.318
22:6n-3	0.219	0.290	0.0191	0.020	<0.001	0.291
n-6/n-3	3.03	2.86	0.239	0.674	0.020	0.993
P/S	0.640	0.739	0.0283	0.031	<0.001	0.355

SEM, standard error of the mean; S, season; R, region; PUFA, polyunsaturated fatty acid (FA); P/S, polyunsaturated FA/saturated FA; CLA, conjugated linoleic acid; NC, non-conjugated.

3.2.1. Saturated and branched-chain fatty acids

Expressing the data on percentage basis, shows that the interaction between season and region was significant for few minor FA (Tables 2 to 4) while for SFAs season effect was very weak (10:0, 17:0, 24:0). Total SFA content was comparable to the values given in the scientific

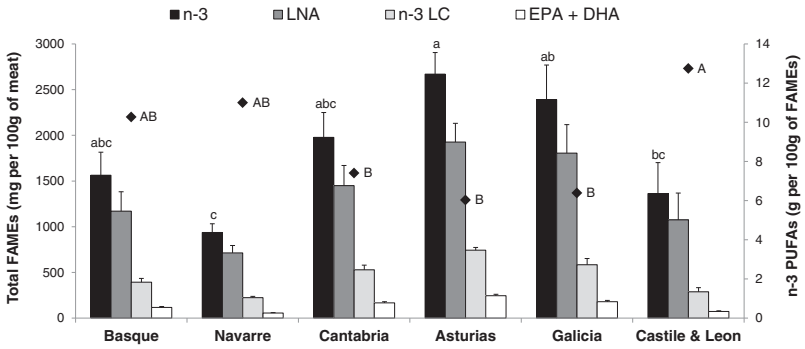

Fig. 2. Total fatty acid methyl ester (FAME) and n-3 polyunsaturated fatty acid (PUFA) content of horse-meat. The primary axis (left) represents the total FAME content (♦) and the secondary axis (right) represents the total n-3 PUFAs, linolenic acid (LNA), n-3 long-chain (LC) PUFAs, and the sum of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in horse-meat. $^{AB\ or\ abc}$ Means with different superscripts are significantly different at $P \leq 0.05$.

literature for horse-meat (34.2–47.8%; Belaunzaran et al., 2015) but this is the first report of the DMA and BCFA content in horse tissues. BCFAs are mainly present in products of ruminant origin, and occur also in tissues of other herbivores with microbial fermentation in the hindgut (Vlaeminck, Fievez, Cabrita, Fonseca, & Dewhurst, 2006; Leiber et al., 2008). Results from the present study show the relatively low content of BCFA in horse-meat (<0.500%) could be due to the absorption of dietary FA prior to modifications by microorganisms present in the hindgut (Clauss et al., 2009). Despite the low content of BCFA, there is an increasing interest in the analysis of these compounds considering their potential antitumor activity (Wongtangtintharn, Oku, Iwasaki, & Toda, 2004; Yang et al., 2000). The content of *iso*-16:0 (0.117%) is the highest among the BCFAs in horse and rabbit tissues (Leiber et al., 2008; Papadomichelakis, Karagiannidou, Anastasopoulos, & Fegeros, 2010) while *iso*- and *anteiso*-15:0 and 17:0 predominate in ruminants (Aldai, Dugan, Rolland, & Kramer, 2009; Vlaeminck et al., 2006). These differences could be induced by dietary variations, as well as different metabolic mechanisms attributable to the diversity of rumen/gut microbiota (Kaneda, 1967; Lourenço, Van Ranst, De Smet, Raes, & Fievez, 2007; Vlaeminck, Fievez, Van Laar, & Demeyer, 2004).

3.2.2. Monounsaturated fatty acids

The total MUFA content was significantly higher in horse-meat samples collected in spring than collected in winter (35.4 and 32.3%, respectively; $P < 0.05$; Table 3), and this was mainly influenced by the major *cis*- and *trans*-MUFA. Overall, *cis*-MUFA represented 99.4% of the total MUFA content, with oleic (24.5%), palmitoleic (9*c*-16:1, 5.55%) and asclepic (11*c*-18:1; 1.89%) acids being the most abundant. Previous studies reported MUFA values ranging from 16.4% in extensively reared horses to 50.2% for concentrate-fed horses (Belaunzaran et al., 2015) which suggests that samples collected in spring might come from animals with less forage/grass in their diet. The total *trans*-MUFA content was also significantly higher in samples collected in spring than in winter season (0.212 and 0.180%, respectively; $P < 0.001$), which was mostly attributed to high levels of *trans*-18:1. Elaidic acid (9*t*-18:1; 0.0776%) was the major *trans*-18:1 isomer in horse-meat followed by vaccenic acid (11*t*-18:1; 0.0273%). On the other hand, the 10*t*-18:1 isomer, which has been associated with negative health effects in animal models (Bauchart et al., 2007; Roy et al., 2007) represented very small proportion. Previous studies have also indicated that 9 *t*- and 11*t*-18:1 are the major *trans*-18:1 isomers in horse tissues (Lanza, Landi, Scerra, Galofaro, & Pennisi, 2009; Sarriés, Murray, Troy, & Beriain, 2006), but no mention was made regarding the 10*t*-18:1 content. These three *trans*-18:1 isomers (9*t*-, 10*t*-, and 11*t*-18:1) are difficult to resolve unless specific GC columns and conditions are used as in this study. It was evident from these results that the total content and isomeric profile of *trans*-18:1 in horse-meat is very different compared to ruminant species where 10*t*- and 11*t*-18:1 acids are normally the two predominant isomers depending on feeding (Bessa, Alves, & Santos-Silva, 2015; Bessa, Portugal, Mendes, & Santos-Silva, 2005; Aldai et al., 2011; Aldai et al., 2009).

3.2.3. Conjugated fatty acids and other dienes and trienes

In the present survey, the total CLA content was slightly higher in samples collected in spring than in winter season (0.0941 and 0.0768%, respectively; $P < 0.01$), which was mostly attributed to *t,t*- isomers. Nevertheless, the CLA content of horse-meat was, in general, very low (<0.100%; Table 4). The total accumulation of other dienes and trienes was also low compared to ruminants (Aldai et al., 2012b; Shingfield, Bonnet, & Scollan, 2013). The absence of a rumen and the transformation of dietary lipids in the post absorptive part of the horse gut (caecum/colon) are associated with low absorption of biohydrogenation intermediates and, therefore, a low deposition of these type of metabolites in horse tissues (Clauss et al., 2009; Hartam et al., 1956).

3.2.4. Polyunsaturated fatty acids

The total PUFA content was significantly higher in samples collected in winter than in spring season (26.6 and 23.4%, respectively; $P < 0.05$; Table 4), mainly influenced by 20:3n-6 (dihomo gamma linolenic acid; DHGLA), 20:5n-3 (EPA), 22:5n-3 (DPA) and 22:6n-3 (DHA). Consequently, a more favorable P/S ratio was provided by winter than spring samples. The most abundant n-6 PUFAs were LA (18:2n-6; 14.1%) and arachidonic acid (ArA; 20:4n-6, 1.53%), whereas LNA (18:3n-3; 6.33%) and DPA (22:5n-3; 0.932%) predominated in the n-3 PUFAs. Considerable deposition of n-3 PUFAs in horse tissues was also reported in the literature from animals managed under extensive conditions (grazing) (Lorenzo et al., 2010; Badiani et al., 1997; Lorenzo, 2013; Gupta & Hilditch, 1951; Guil-Guerrero et al., 2013). The higher n-3 LC-PUFA levels in samples collected in early winter could be associated with animals that were presumably reared under mountain grazing conditions until late fall. When the average content of n-3 PUFA in horse was compared to meats from other species (chicken, rabbit, beef, pork; Fig. 3), a higher n-3 content, mainly influenced by LNA, was observed in horse-meat. In other species, however, the LC-PUFA to LNA ratio was greater (Fig. 3) indicating a more efficient LNA conversion to other n-3 LC-PUFAs.

3.3. Differences in tissue fatty acid composition

Fatty acid composition of IM and SC adipose tissues (percentages) is compared in Table 5. The effect of interaction between the two main factors (tissue and season) was significant essentially for those LC-PUFA (20:2n-6, DHGLA, EPA, DPA and DHA) whose content was higher

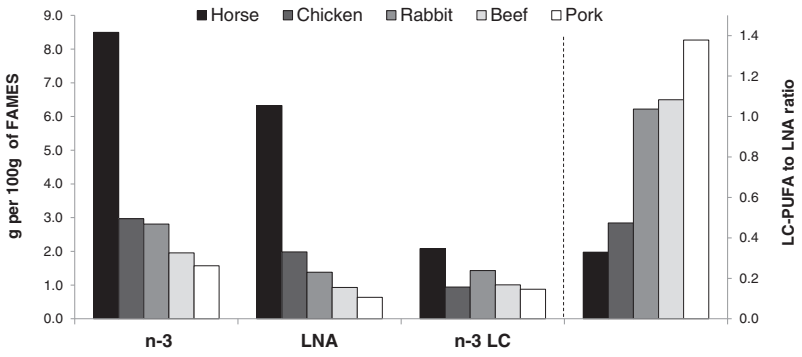

Fig. 3. Content of n-3 polyunsaturated fatty acids (PUFA) in the meat of several species (horse, chicken, rabbit, beef, pork). The primary axis (left) represents the total n-3, linolenic acid (LNA) and n-3 long-chain (LC) PUFA percentages. The secondary axis (right) represents the ratio of n-3 LC-PUFA (>20C) to LNA for each species. Data were obtained from the present study (horse); Ribeiro et al. (2013) for chicken; Kouba, Benatmane, Blochet, and Mourot (2008) and Papadomichelakis et al. (2010) for rabbit; Aldai et al. (2009, 2012b) and Kraft et al. (2008) for beef; and Alonso et al. (2012), Enser, Hallett, Hewitt, Fursey, and Wood (1996), and Turner et al. (2014) for pork. FAMEs, fatty acid methyl esters.

in the winter than spring muscle samples. This compares with the FA composition of SC fat that was not influenced by season (data not shown), which could be explained by the minor content of LC-PUFA in backfat compared to muscle tissue. Other possible binary interactions (T*R, S*R) did not prevail and were, therefore, not discussed.

Tissue effect was not significant for the total SFA but the content of some individual short- and medium-chain SFAs were higher in SC compared to IM fat ($P < 0.001$). Conversely, a higher stearic acid (18:0) content was observed in muscle fat ($P < 0.001$; data not shown). A higher content of SFA in horse backfat compared to muscle was observed by some (Guil-Guerrero et al., 2013; Juárez et al., 2009) but not others (He et al., 2005; Sarriés et al., 2006; Tateo, De Palo, Ceci, & Centoducati, 2008). No tissue effect was found for the total MUFA. The lower temperature of body near the surface could be related to the higher expression and activity of stearoyl Co-A desaturase (SCD) enzyme responsible for the conversion of SFA into *cis* Δ9 MUFA in mammalian tissues (Kouba, Hermier, & Le Dividich, 1999). In general, studies have reported higher percentages of MUFA in horse SC compared to muscle fat (Guil-Guerrero et al., 2013; Juárez et al., 2009; Sarriés et al., 2006; Tateo et al., 2008). By contrast, the contents of *trans* containing MUFA and NC-dienes in horse tissues were very low, and higher in muscle compared to SC fat ($P < 0.001$). The total CLA content in horse tissues was also low, and rumenic acid (RA; 9*c*,11*t*-18:2) predominated and represented about 42% and 70% in muscle and SC fat, respectively (data not shown). Previous studies in ruminant species showed higher levels of RA in SC than in muscle tissue indicating that CLA is predominantly deposited in the neutral lipid fraction (Aldai, Nájera, Dugan, Celaya, & Osoro, 2007; Jerónimo et al., 2011). While the total PUFA content was similar in both adipose tissues, significant differences were observed in the total and individual contents of the n-6 and n-3 PUFA (Table 5; $P < 0.001$). In general, LA and most of the LC n-6 and n-3 PUFAs were significantly higher in muscle, except LNA that was higher in SC fat ($P < 0.001$). This resulted in a significantly higher ratio of LA/LNA in muscle in comparison to SC fat ($P < 0.05$), while the ratios of n-6/n-3 and P/S were not significantly different between the adipose tissues. A lower n-6/n-3 than LA/LNA ratio in muscle fat suggests a higher affinity for desaturation and elongation of n-3 over n-6 FAs, which is consistent with other studies (Holman, 1998). The end products of LA and LNA desaturation and elongation pathways in horse muscle fat were ArA and DPA, the major LC-PUFA from the n-6 and n-3 series, respectively. Within the n-6 group, LA is desaturated to 18:3n-6 by Δ6-desaturase, elongated to 20:3n-6 and converted to ArA by Δ5-desaturase. Likewise, within the n-3 group, the formation of DPA involves desaturation (Δ6) of LNA to 18:4n-3, elongation to 20:4n-3, and additional desaturation (Δ5) to 20:5n-3 and elongation to DPA (Sprecher, Luthria, Mohammed, & Baykousheva, 1995). Even though, ArA and DPA are generally recognized to be quantitatively the most abundant n-6 and n-3 LC-PUFA, respectively, considerable variations are reported on their content in horse tissues; see review by Belaunzaran et al. (2015). For instance, in horse SC fat tissue, 20:2n-6 and 20:3n-3 were the main metabolites of LA and LNA pathways, respectively, but there is evidence that they can be further desaturated to 20:3n-6 and 20:4n-3 via Δ8-desaturase, respectively (Park, Kothapalli, Lawrence, Tyburczy, & Brenna, 2009). However, the content of these metabolites was minimal in backfat suggesting that LC-PUFA was primarily esterified in muscle phospholipids. Results from previous

Table 5
Differences in the fatty acid composition (%) of intramuscular and subcutaneous adipose tissues of horses.

Fatty acid	Adipose tissue		SEM	*P* values		
	Intramuscular $n = 74$	Subcutaneous $n = 74$		T	S	T*S
SFA	36.9	37.8	0.270	0.081	0.794	0.085
BCFA	0.391	0.533	0.0231	<0.001	0.193	0.377
MUFA	35.5	36.8	0.861	0.209	0.043	0.361
cis-MUFA	35.3	36.6	0.861	0.194	0.046	0.364
cis-16:1	6.19	6.70	0.194	0.006	0.279	0.186
cis-18:1	27.2	28.4	0.751	0.250	0.013	0.529
trans-MUFA	0.202	0.161	0.00631	<0.001	<0.001	0.379
trans-16:1	0.0617	0.0318	0.00218	<0.001	0.719	0.894
trans-18:1	0.134	0.0988	0.00576	<0.001	<0.001	0.300
CLA	0.0869	0.0878	0.00383	0.960	<0.001	0.148
NC-dienes	0.0785	0.0624	0.00233	<0.001	0.158	0.824
Trienes	0.110	0.113	0.00720	0.525	0.156	0.744
trans-FA	0.331	0.328	0.01053	0.725	0.057	0.576
PUFA	23.9	24.5	0.841	0.454	0.056	0.190
n-6	15.8	12.1	0.516	<0.001	0.597	0.058
18:2n-6	13.6	11.6	0.439	<0.001	0.681	0.071
20:2n-6	0.238	0.248	0.00850	0.807	0.215	0.033
20:3n-6	0.377	0.0465	0.0240	<0.001	0.038	0.020
20:4n-6	1.38	0.0875	0.0914	<0.001	0.250	0.179
22:4n-6	0.0876	0.0230	0.00447	<0.001	0.889	0.224
n-3	8.07	12.4	0.895	<0.001	0.136	0.892
18:3n-3	6.14	11.6	0.850	<0.001	0.201	0.884
20:3n-3	0.290	0.340	0.0240	0.070	0.154	0.345
20:5n-3	0.363	0.0349	0.0317	<0.001	0.002	0.003
22:5n-3	0.857	0.172	0.0586	<0.001	0.008	0.010
22:6n-3	0.228	0.0394	0.0168	<0.001	0.006	0.005
LA/LNA	3.93	2.68	0.299	0.031	0.895	0.922
n-6/n-3	3.06	2.52	0.384	0.158	0.778	0.869
P/S	0.655	0.654	0.0252	0.852	0.080	0.104

Intramuscular and subcutaneous fat tissues ($n = 38$ in spring and $n = 36$ in winter). SEM, standard error of the mean; T, tissue; S, season; SFA, saturated fatty acid (FA); BCFA, branched-chain FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; LA/LNA, linoleic acid/linolenic acid; P/S, polyunsaturated FA/saturated FA; CLA, conjugated linoleic acid; NC, non-conjugated.

studies also reported 20:2n-6 as the most abundant n-6 PUFA in horse SC adipose tissue (He et al., 2005; Sarriés et al., 2006), while not consistent results are given for n-3 LC-PUFA (Guil-Guerrero et al., 2013; Sarriés et al., 2006).

3.3.1. Nutritional assessment of n-3 polyunsaturated fatty acids

A high variability was observed in the content of n-3 PUFA in horse muscle and SC fat tissue. Overall total n-3 PUFA content ranged between 1.17% and 18.9% in muscle fat and between 1.52% and 27.9% in backfat, likely linked to differences in management practices and diet. These values were greater compared to those observed in ruminant (Aldai et al., 2009) and monogastric species (Enser et al., 1996; Raes, De Smet, & Demeyer, 2004; Raj et al., 2010). Rabbit might be the only specie that seems to reach comparable n-3 contents to those reported in this study (Dal Bosco et al., 2014; Leiber et al., 2008). PCA was performed in order to study individual responses and establish relationships among FAs studied. Loading variables and the distribution of horse IM and SC fat samples from both collection periods are represented in the two-dimensional coordinate system defined by PC1 and PC2 (Fig. 4). Differences between muscle and SC fat were evident, while season effect was not, in the PCA where the first two components explained 67.4% of the total variability.

It was of interest to note that almost 5% of surveyed loins from these horses (4 out of 82 steaks) contained over 300 mg of LNA per 100 g of meat and, therefore, these samples could have been marketed as a source of n-3 FA according to the Commission Regulation (EU) No 116/2010 with regard to the list of nutrition claims (EFSA, 2010). The average value of the 82 surveyed horse loins provided about 108 mg of LNA or 142 mg of total n-3 PUFA per 100 g. However, no claim could be made based on the content of LNA (over 300 mg per 100 g of meat) and/or the content of EPA plus DHA (over 40 mg per 100 g of meat). Nevertheless, if 25 g of trimming fat derived from SC had been included with the horse loin they would provide an average value of 2.3 g of LNA or 2.5 g of total n-3 PUFAs, which would meet or exceed the recommended daily intake established by the European Society of Cardiology and the European Atherosclerosis Society (1 g of total n-3 PUFAs/day) and the Japanese Ministry of Health (2.1–2.4 g of total n-3/day; GOED, 2014).

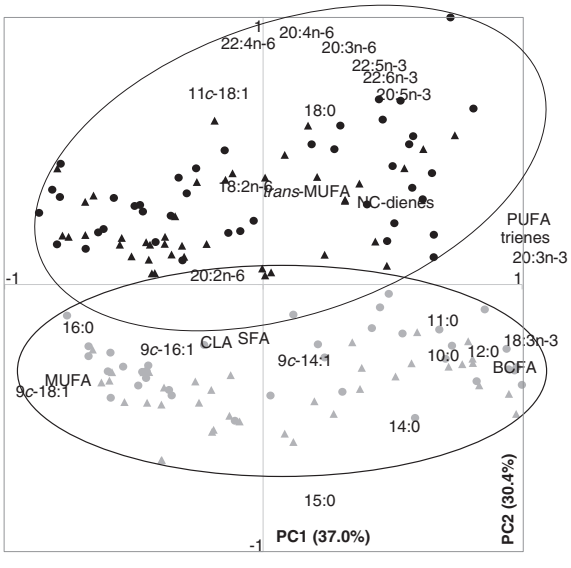

Fig. 4. Loading variables and horse intramuscular (spring ▲, winter ●) and subcutaneous (spring △, winter ○) sample distribution on the two-dimensional coordinate system defined by the principal component (PC) 1 and PC2. SFA, saturated fatty acid (FA); BCFA, branched-chain FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; CLA, conjugated linoleic acid; NC, non-conjugated.

4. Conclusions

From the results of the present study, horse-meat could be defined as lean meat (≤2% fat) with a high content of n-3 PUFA, which would be very suitable as an alternative red meat for consumers desiring low fat products with a favorable FA profile. Seasonal differences in the n-3 PUFA content of horse-meat appear to be associated with management practices, but there is great variability among regions as well as significant differences in the fat content and its FA composition. Higher IM fat level provided a higher content of total MUFA and lower levels of DMA and PUFA. The findings of high contents of LC-PUFA in muscle and LNA in backfat of the horse species suggest that both tissues (muscle and subcutaneous fat) can be combined to provide an important source of n-3 PUFAs, contributing to fulfill the daily n-3 requirements of humans. These results should be valuable to provide a more accurate database for the nutritional assessment of horse-meat, improve consumer acceptance of horse-meat as an edible and healthy food, and provide information for marketing purposes. Further research will be needed to evaluate several factors suspected to influence the FA composition of horse-meat, such as different feeding strategies, dietary ingredients, age and breed of horses.

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2.2. Neutral and polar lipid composition of horse muscle as affected by age and associated feeding practices: experimental design II.

Manuscript V is an investigation aimed at studying the effect of management system where particular emphasis has been placed on not only the influence of age and associated feeding practices on the FAs and the alkenyl ether moieties profile of horse lipids, but also on the selective deposition of these within the NL and PL fractions. To the best of our knowledge, this is the first report of FAs and DMAs distribution in NL and PL fractions in horse muscle. A thorough knowledge of the NL and PL fractions of horse-meat could establish the basis for a better understanding of this red meat in terms of its self-life at the retail level and its stability storage and/or processing.

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Neutral and polar lipid composition of horse muscle affected by age and associated feeding practices

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Short title: Neutral and polar lipid composition of horse muscle

ABSTRACT

This study was undertaken to provide a thorough analysis of the neutral (NL) and polar lipid (PL) fractions of horse-meat that included the content and distribution of acyl and alkenyl moieties in foals under different rearing conditions. Two groups of crossbred horses were studied; the first group was selected from suckling foals produced under grazing conditions and slaughtered at 4 months of age (n=8), and the second group was selected from concentrate-finished foals and slaughtered at 12 months of age (n=7). There were significant differences related to the age and related feeding practices of foals which affected the intramuscular (IM) fat content and the fatty acid (FA) composition of NL and PL fractions. Samples from suckling foals were leaner and provided the highest content of methylation products from the plasmalogenic lipids, and total and n-3 polyunsaturated FA (PUFA). By contrast, the meat from concentrate-finished foals had a higher IM fat level resulting in a greater accumulation of 16:0 and total monounsaturated FAs in the NL fraction, while the muscle PL fraction retained a similar FA composition between both groups. Linolenic acid was preferentially deposited in the NL fraction, but linoleic acid and the long chain n-3 and n-6 PUFAs were incorporated into the PL fraction where they served as cell membrane constituents and in eicosanoid formation.

Keywords:

Foal, lipid fractions, dimethylacetals, n-3 fatty acids, long chain fatty acids

Implications

A comprehensive lipid analysis showed that foal meat is a rich source of n-3 polyunsaturated fatty acids which is healthy for humans. But, when animals are young (suckling) or reared under extensive conditions is difficult to reach levels of n-3 fatty acids on an absolute basis required for labeling purposes according to EFSA regulation. However, a more thorough knowledge of the neutral and polar lipid fractions of horse-meat could establish the basis for a better understanding

of this red meat in terms of its self-life at the retail level and its stability during storage and/or processing.

Introduction

The intramuscular (IM) fat content and its fatty acid (FA) composition are related to the nutritional value, sensory attributes and technological quality of meat. In addition, meat FA composition is strongly influenced by the level of fatness, as well as genetic and dietary factors (De Smet *et al.*, 2004). In general, horse-meat was found to be lean with a significant content of n-3 polyunsaturated FAs (PUFAs) (Belaunzaran *et al.*, 2017; Lorenzo *et al.*, 2010), and for this reason it has been considered an interesting option from a nutritional point of view. However, the higher PUFA content could also negatively affect its stability during storage and/or processing due to oxidation (Wood *et al.*, 2004). The IM lipids are composed of neutral (NL) and polar lipids (PL). The former consists primarily of triacylglycerols, while the latter mainly of phosphatidylcholine and phosphatidylethanolamine (Larick & Turner, 1989). Triacylglycerols are essential components of mammalian energy homeostasis, and their content in meat is directly related to the total fat level (0.200 - 5.00 % of muscle weight). On the other hand, phospholipids are involved in membrane structure and cell functions (Hornstein *et al.*, 1967), and their content is relatively constant (0.200 - 1.00 % of muscle weight) (De Smet *et al.*, 2004). Triacylglycerols were reported to be the major components in the horse NL fraction (55.7 - 63.2 % of total lipids), while phospholipids represented an average value of 0.695 % of muscle weight, ranging from 12.8 to 23.7 % of total lipids depending on production system (Sarriés *et al.*, 2006).

Meat is also known to contain plasmalogenic lipids in which one of the ester linkages is replaced by a vinyl ether (alk-1-enyl moiety). These compounds are seldom reported in meat, and to our knowledge have never been reported in horse-meat. In mammalian tissues, the plasmalogens are only present in small amounts in the NL fraction, while in the PL fraction they are major components, particularly in phosphatidylethanolamine (Horrocks, 1972), where they have been reported to be important components of membrane phospholipids (Nagan & Zoeller, 2001). The ether bond is generally linked to the sn-1 position while long

chain (LC) PUFA occupy the sn-2 position of the molecule. To date, the alkyl chain length of the alkenyl ethers reported in the mammalian tissues did not exceed C18 FAs (Horrocks, 1972).

A comprehensive analysis of horse lipids that includes the content and localization of acyl and alkenyl moieties in the neutral and polar lipid structures is necessary to have a reliable database to evaluate this meat as an alternate nutritional source. It would also provide valuable information to improve the shelf-life at retail conditions of this meat. Such a complete characterization of horse lipids is not currently available. In this manuscript we wish to provide a complete analysis of both the neutral and polar lipids of horse-meat and how this composition is affected by the age of the horse and associated feeding practices.

Material and methods

Experimental design and sampling

In the present study 2 groups of crossbred horses were used. The first group was selected from foals that were naturally suckled by their mothers from birth (March-April 2015) to slaughter (July 2015) at 4 months of age (n = 8), and consisted of equal numbers from both sexes. They were managed under grazing conditions. The second group was selected from crossbred males (n = 7) born in April 2015, that after weaning (November 2015) were intensively fattened indoors. The amount of commercial concentrate given to foals was gradually increased from 4-5 kg/head/day at the start to 10 kg/head/day at the end of the fattening period, and oats and straw were supplied *ad libitum*. Foals were slaughtered at 12 months of age.

The concentrate was composed by barley, wheat bran, soya, alfalfa, sunflower, beet molasses, and a premix of vitamins and trace elements. On dry matter basis, chemical composition of the concentrate was as follows: 18.2 % of crude protein (ISO, 2005), 7.93 % of ash (ISO, 2002), and 2.41 % of ether extract (AOCS, 2008), while the main FAs were: 16:0 (20.8 %), 18:0 (2.25 %), 9c-18:1 (14.9 %), 18:2n-6 (45.8 %), and 18:3n-3 (5.46 %).

Foals were slaughtered in a commercial abattoir according to standard procedures. The carcass weight reached an average value of 101 and 272 kg in suckling and fattened foals, respectively. The 6th to 8th rib joints of the left half carcasses were transported to the laboratory in refrigerated coolers. Then, the *Longissimus throracis et lumborum* (LTL) muscle was dissected and two steaks of approximately 3 cm thick were cut. The first steak was used to determine the proximate chemical composition (%) using standard procedures described earlier (concentrate samples). The second steak was cut into pieces, freeze-dried, ground, vacuum-packaged, and stored at -80 °C for further lipid analysis.

Muscle lipid extraction and fatty acid esterification

Lipids from 1.5 g of freeze-dried muscle were extracted using chloroform-methanol (2:1, v/v; Folch *et al.*, 1957). Two lipid aliquots of 10 mg were separately methylated using base- (2 mL of Methanolic-Base, 0.5 N from Supelco) and acid- (2 mL of sulfuric acid in methanol, 2 %) catalyzed procedures. The latter method assured the complete methylation of all type of lipids; the conversion of acyl and N-acyl lipids into FAME, and the alk-1-enyl ethers of plasmalogenic lipids into dimethylacetals (DMA). On the other hand, the base-catalyzed procedure was used to avoid isomerization of conjugate linoleic acid (CLA) (Kramer *et al.*, 1998). Prior to derivatization, 0.5 mg of two internal standards (13:0 and 23:0 methyl esters from Nu-Chek Prep Inc., Elysian, MN, USA) was added for quantitative purposes.

Fractionation of neutral and polar lipid classes

Intramuscular lipids (45-60 mg) were fractionated into their NL and PLs using solid-phase extraction (SPE) cartridges packed with silica gel (Supelclean^{TM} LC-Si SPE Tubes 1 g/6 mL; Supelco, Bellafonte, PA, USA). The NLs were eluted with chloroform (30 mL) and the PLs with methanol (30 mL), as described by Juaneda & Rocquelin (1985). To the individual fractions, and prior to derivatization, the internal standard (23:0 methyl ester) was added at approximately 0.05 mg per 1 mg of lipids, and then transesterified using sequential base- and acid-catalyzed procedures.

Gas chromatographic analysis of lipid compounds

Fatty acid methyl esters (FAMEs) and DMAs were analyzed using a gas chromatograph equipped with a flame ionization detector (GC-FID; Agilent Technologies, Model 7890A, Wilmington, DE, USA) and coupled with an automatic injector (Agilent Technologies, Model 7693). Samples were injected using a 50:1 split ratio on a Supelco SP2560 capillary column (100 m x 0.25 mm I.D., 0.2 m coating, Bellefonte, PA) using the 175 ºC temperature program described by Kramer *et al*., (2008). In order to resolve CLA isomers and several other overlapping peaks, the FAMEs were subjected to a second GC analysis using a SLB-IL111 ionic liquid stationary phase column (100 m x 0.25 mm I.D., 0.2 m coating; Supelco, Bellefonte, PA, USA) and using the temperature program described by Delmonte *et al*. (2011). Hydrogen was used as a carrier gas at a flow rate of 1 mL/min, the injection volume was 1 µL, and injector and detector ports were set at 250 ºC.

For identification purposes, reference standards of individual FAMEs (21:0, 23:0, 26:0, 28:0) and mixtures such as #463, #603 and #UC-59M CLA were obtained from Nu-Check (Elysian, MN, USA), linoleic (LA; 18:2n-6) and linolenic acid (LNA; 18:3n-3) isomers were purchased from Sigma-Aldrich (#47791 and #47792, respectively; Supelco, Bellefonte, PA, USA), and a bacterial mixture was obtained from Matreya (Pleasant Gap, PA, USA). Many of *trans*-FA, CLA, non conjugated (NC) dienes, trienes, and LC-PUFA not included in the standard references were identified by retention times and elution orders previously reported in the literature (Alves & Bessa, 2009; Delmonte *et al*., 2012), and by FAME fractions obtained using silver-ion SPE cartridges (Belaunzaran *et al*., 2016; Kramer *et al*., 2008).

For quantitation purposes, chromatographic peak areas were corrected according to theoretical response factors (Bannon *et al*., 1985), and thereafter the amounts (mg) were calculated according to the internal standard added. FAMEs and DMAs were expressed as mg/100 g of fresh meat or as percentage of total FAME quantified (%).

Data analysis

Statistical analysis was conducted using IBM SPSS Statistics 22 for windows (SPSS Inc., IBM Corporation, NY, USA). A *t*-Student test was applied to compare the

chemical composition, fat constituents (FAME, DMA) composition, and deposition preference values in total, neutral and polar lipids between animal groups (suckling and fattened foals). Deposition values of individual FAs in NL and/or PL fraction of horse muscle were calculated as follows:

log (individual FA % in the PL fraction/same FA % the NL fraction)

Principal component analysis (PCA) was conducted on main individual and groups of FAs to establish relationships among them. Sample distribution plots using the two-dimensional coordinate system defined by the two first principal components (PCs) were used to study the variability in composition. Three significant figures were used to express the data. Significance was declared at $P \leq 0.05$.

Results

Chemical composition of muscle

In terms of the chemical composition of the horse-meat (Table 1), the fat content was significantly higher in concentrate-finished (2.53 %) than in suckling foals (0.853 %; $P < 0.01$). Moisture (73.8 %; $P < 0.05$) and protein (23.9 %; $P < 0.01$) percentages were the highest in suckling foals, while no differences in total ash content (1.46 %) were observed between studied groups.

Table 1. Chemical composition (%) of *Longissimus thoracis et lumborum* muscle from suckling and fattened foals.

Chemical composition	Suckling	Fattened	SEM	*P value*
Fat (ether extract)	0.853	2.53	0.277	0.003
Moisture	73.8	72.4	0.312	0.018
Crude protein	23.9	21.9	0.403	0.007
Ash	1.41	1.53	0.0511	0.303

SEM, standard error of the mean.

Fatty acid and plasmalogen composition muscle

Total fatty acid content and composition of LTL muscle from suckling and fattened foals are presented in Table 2 (mg/100g of fresh meat and %). On the absolute basis, total FAME content was significantly higher in concentrate-fed (2854 mg/100 g of meat) than in suckling foals (996 mg/100 g of meat; $P < 0.001$),

and it was influenced by higher levels of saturated FA (SFA; ($P < 0.001$), monounsaturated FA (MUFA; $P < 0.001$) and n-6 PUFA ($P < 0.001$; Table 2), whereas the opposite was true for n-3 PUFA ($P < 0.05$). The total branched-chain FA (BCFA) content was not affected by age and feeding practices, although *i*-16:0 content was slightly higher in concentrate-fed foals ($P < 0.05$). Similarly, DMA, CLA ($P < 0.001$), NC-diene ($P < 0.01$) and *trans*-FA ($P < 0.001$) levels were higher in concentrate-fed foals, while no differences in trienes were found between studied groups.

Looking at the muscle FA profile data on the percentage basis, SFA were not affected by age and feeding (36.1 %), although differences were significant for some individual SFA; the percentage of palmitic acid (16:0) was higher while stearic acid (18:0) was lower in fattened compared to suckling foals ($P < 0.001$; Table 2). The percent of BCFA were higher in suckling (0.534 %) than in fattened foals (0.206 %; $P < 0.001$). Likewise, the total percent of DMA was higher in suckling (5.88 %) compared to fattened foals (2.37 %; $P < 0.001$). When expressed as percentages of total quantified DMA in horse muscle, the saturated DMA represented 76.9 % of the total, with 16:0 (50.6 %) and 18:0 (23.0 %) the most predominated isomers. Monounsaturated represented about 17.4 %, 3.47 % were BCFA and only 2.15 % were PUFA. The percentage of MUFA were significantly higher in concentrate-fed (41.6 %) than in suckling foals (19.9 %; $P < 0.001$), and this was influenced by both the major *cis*- (9*c*-16:1 and 9*c*-18:1) and *trans*-MUFA (9*t*-18:1; $P < 0.001$; Table 2). No differences were found for the total CLA content between studied groups (average value of 0.107 %); however, the percentage of NC-dienes (11*t*,15*c*-/9*c*,15*c*-/12*c*,15*c*-18:2) and trienes (9*c*,11*t*,15*c*-18:3) related to LNA metabolites, and *trans*-FA were significantly higher in suckling compared to intensively fattened foals ($P < 0.01$). Similarly, the PUFA content was higher in samples from suckling (36.6 %) than concentrate-fed foals (19.5 %; $P < 0.001$; Table 2), and it was attributable to all n-3 ($P < 0.001$) and most n-6 PUFA ($P < 0.01$), except 22:5n-6 that was higher in fattened foals. A more favorable P/S ratio was obtained in meat from suckling foals ($P < 0.001$), while the n-6/n-3 ratio was higher in fattened foals ($P < 0.001$).

Table 2. Total fatty acid content (mg/100 g of fresh meat) and composition (mg/100g of fresh meat and %) of *Longissimus thoracis et lumborum* muscle from suckling and fattened foals.

Fatty acid	mg/100 g of meat				%			
	Suckling	Fattened	SEM	*P value*	Suckling	Fattened	SEM	*P value*
Total FAME	996	2854	272	‹0.001				
SFA	363	1029	99.1	‹0.001	36.3	36.0	23.3	0.659
14:0	32.8	95.1	9.68	‹0.001	3.22	3.30	0.389	0.745
16:0	230	761	78.2	‹0.001	23.0	26.5	0.0249	‹0.001
18:0	76.5	144	9.97	‹0.001	7.76	5.09	0.0128	‹0.001
BCFA	5.32	5.78	0.273	0.429	0.534	0.206	0.00162	‹0.001
i-16:0	1.47	1.90	0.0987	0.026	0.148	0.0680	0.00611	‹0.001
ai-17:0	1.15	1.12	0.0634	0.785	0.115	0.0394	0.00693	‹0.001
i-18:0	0.682	0.680	0.0325	0.971	0.0689	0.0242	0.0146	‹0.001
DMA	57.4	65.4	1.64	0.009	5.88	2.37	0.971	‹0.001
MUFA	201	1201	146	‹0.001	19.9	41.6	0.00183	‹0.001
cis-MUFA	199	1192	145	‹0.001	19.7	41.3	0.0111	‹0.001
9*c*-16:1	25.0	196	25.4	‹0.001	2.45	6.75	0.0366	‹0.001
9*c*-18:1	141	883	109	‹0.001	13.9	30.6	0.00249	‹0.001
11*c*-18:1	19.1	60.2	6.10	‹0.001	1.93	2.10	2.07	0.083
trans-MUFA	2.10	8.57	0.922	‹0.001	0.210	0.301	2.54	‹0.001
9*t*-18:1	0.713	4.27	0.515	‹0.001	0.0704	0.149	0.00308	‹0.001
10*t*-18:1	0.255	0.807	0.0818	‹0.001	0.0257	0.0282	0.0114	0.451
11*t*-18:1	0.419	1.13	0.0996	‹0.001	0.0422	0.0408	0.00421	0.756
CLA	1.11	2.90	0.269	‹0.001	0.112	0.102	0.0180	0.065
9*c*,11*t*-	0.407	1.28	0.129	‹0.001	0.0410	0.0448	0.00312	0.018
NC-dienes	1.56	2.76	0.200	0.003	0.159	0.0975	0.0574	‹0.001
Trienes	2.13	2.16	0.0711	0.869	0.216	0.0781	0.00176	‹0.001
trans-FA	4.85	11.9	1.05	‹0.001	0.488	0.420	0.00173	0.009
PUFA	361	542	29.1	‹0.001	36.6	19.5	1.92	‹0.001
n-6	216	427	30.5	‹0.001	22.1	15.4	0.00489	‹0.001
16:2n-6	0.395	0.943	0.0912	‹0.001	0.0392	0.0338	1.59	0.248
18:2n-6	181	374	28.0	‹0.001	18.5	13.4	0.00640	0.002
20:2n-6	3.17	7.78	0.663	‹0.001	0.325	0.278	0.00246	0.071
20:3n-6	7.73	8.12	0.231	0.424	0.791	0.291	0.0215	‹0.001
20:4n-6	22.6	31.2	1.30	‹0.001	2.31	1.14	0.0786	‹0.001
22:4n-6	0.913	2.90	0.273	‹0.001	0.0936	0.106	0.000885	0.267
22:5n-6	0.375	1.36	0.136	‹0.001	0.0385	0.0493	0.0113	0.033
n-3	143	114	7.39	0.042	14.4	4.09	0.00399	‹0.001
16:3n-3	0.526	0.582	0.0376	0.484	0.0521	0.0206	1.83	‹0.001
18:3n-3	91.2	87.1	5.76	0.733	9.03	3.11	0.01002	‹0.001
20:3n-3	5.56	4.01	0.289	0.003	0.563	0.144	0.00247	‹0.001
20:4n-3	1.33	0.765	0.105	0.003	0.133	0.0269	0.0548	‹0.001
20:5n-3	14.5	3.22	1.54	‹0.001	1.48	0.117	0.0192	‹0.001
22:5n-3	20.8	13.8	0.989	‹0.001	2.14	0.502	0.00280	‹0.001
22:6n-3	7.74	3.36	0.591	‹0.001	0.792	0.122	0.226	‹0.001
n-6/n-3	1.55	3.77	0.306	‹0.001				
P/S	1.01	0.545	0.0692	‹0.001				

SEM, standard error of the mean; FAME, fatty acid methyl esters; SFA, saturated fatty acids; BCFA, branched-chain fatty acids; i, iso; ai, anteiso; DMA, dimethylacetal; MUFA, monounsaturated fatty acids; CLA, conjugated linoleic acids; NC, non-conjugated; PUFA, polyunsaturated fatty acids; P/S, polyunsaturated fatty acids/saturated fatty acids.

trans-FA: summatory of all fatty acids with at least one double bond in *trans* configuration except CLA isomers.

Neutral and polar lipid composition of muscle

Total FAME content (mg/100 g of fresh meat) and profile (percentages) of NL and PL fractions are shown in Table 3. The total FAME content in the NL fraction was significantly higher (2449 mg/100g of meat) in fattened compared to suckling foals (588 mg/100g of meat; $P < 0.001$), which was mainly influenced by the increased content of oleic acid (9*c*-18:1; +758 mg/100 g of meat), 16:0 (+532 mg/100g) and palmitoleic acid (9*c*-16:1; +172 mg/100 g).

In fattened foals, the overall FA composition of the NL fraction (Table 3) was fairly similar to that of the total lipids (Table 2), since this fraction represented 86.3 % of total lipids. In contrast, the PL fraction remained fairly constant with an average muscle weight of 0.390 % (Table 3; $P > 0.05$). Overall, the PL represented a higher percentage of the total lipids in suckling (39.9 %) compared to fattened foals (13.7 %). The main FA constituents (representing > 5 %) of the NL fraction were 16:0 (average value of 27.2 %), 9*c*-18:1 (average value of 26.8 %), LNA (average value of 9.52 %), and LA (average value of 7.78 %), which together represented an average of 71.2 % of the total FA. In the PL fraction, LA was the major FA (average value of 35.1 %), followed by 16:0 (average value of 19.7 %), 18:0 (average value of 11.4 %), and 9*c*-18:1 (average value of 7.06 %), which together represented an average of 73.3 % of the total.

In the NL fraction, most of the FA were significantly greater in suckling than fattened foals except for 16:0 ($P < 0.05$), all the *cis*- ($P < 0.001$) and several *trans*-MUFA ($P < 0.05$), rumenic acid (RA; 9*c*,11*t*-18:2, $P < 0.001$) and 22:5n-6 ($P < 0.05$), while most of the remaining FA were greater in fattened foals. In the PL fraction, on the other hand, few differences were observed between studied groups. Some FA were significantly greater in suckling (BCFA, $P < 0.001$; *cis*-MUFA, $P < 0.05$; RA, $P < 0.001$; *trans*-FA, $P < 0.001$; 20:3n-6, $P < 0.01$ and all n-3 PUFA, $P < 0.001$), while others such as 10*t*-18:1 ($P < 0.01$) and the main n-6 PUFA ($P < 0.01$) were greater in fattened foals.

Table 3. Total fatty acid content (mg/100 g of fresh meat) and composition (%) of neutral and polar lipid fractions of *Longissimus thoracis et lumborum* muscle from suckling and fattened foals.

Fatty acid	Neutral Lipids				Polar Lipids			
	Suckling	Fattened	SEM	*P value*	Suckling	Fattened	SEM	*P value*
Total FAME	588	2449	273	‹0.001	390	389	8.57	0.936
SFA	40.9	36.9	0.648	‹0.001	32.2	31.9	0.275	0.549
14:0	5.54	3.80	0.254	‹0.001	0.257	0.1869	0.0118	‹0.001
16:0	26.4	28.0	0.361	0.020	20.0	19.4	0.200	0.189
18:0	5.36	3.94	0.203	‹0.001	11.3	11.6	0.193	0.435
BCFA	0.838	0.251	0.0807	‹0.001	0.291	0.138	0.0214	‹0.001
i-16:0	0.264	0.0877	0.0245	‹0.001	0.0588	0.0366	0.00367	‹0.001
ai-17:0	0.155	0.0438	0.0152	‹0.001	0.0701	0.0263	0.00616	‹0.001
i-18:0	0.0732	0.0258	0.00663	‹0.001	0.0764	0.0203	0.00762	‹0.001
DMA	5.25	1.35	0.627	‹0.001	4.93	5.87	0.493	0.363
MUFA	27.1	47.7	2.83	‹0.001	11.4	10.5	0.203	0.017
cis-MUFA	26.8	47.4	2.83	‹0.001	11.3	10.3	0.204	0.016
9*c*-16:1	3.98	7.88	0.551	‹0.001	0.482	0.449	0.0119	0.176
9*c*-18:1	19.2	35.4	2.24	‹0.001	7.47	6.60	0.183	0.011
11*c*-18:1	1.63	2.07	0.0743	‹0.001	2.36	2.24	0.0546	0.310
trans-MUFA	0.291	0.327	0.00801	0.019	0.169	0.182	0.00699	0.376
9*t*-18:1	0.104	0.173	0.00964	‹0.001	0.0445	0.0480	0.00108	0.109
10*t*-18:1	0.0317	0.0296	0.00137	0.446	0.0167	0.0230	0.00120	0.003
11*t*-18:1	0.0456	0.0341	0.00248	0.014	0.0470	0.0437	0.00141	0.266
CLA	0.101	0.105	0.00213	0.366	0.0956	0.0993	0.00310	0.568
9*c*,11*t*-	0.0404	0.0532	0.00200	‹0.001	0.0318	0.0205	0.00172	‹0.001
NC-dienes	0.149	0.0797	0.0102	‹0.001	0.144	0.144	0.00346	0.987
Trienes	0.186	0.0534	0.0183	‹0.001	0.270	0.269	0.00959	0.972
trans-FA	0.578	0.441	0.0219	‹0.001	0.411	0.341	0.0135	0.005
PUFA	24.7	13.3	1.65	‹0.001	50.3	50.7	0.352	0.546
n-6	7.83	9.18	0.434	0.126	39.3	46.8	1.06	‹0.001
16:2n-6	0.0583	0.0368	0.00390	0.002	0.00857	0.00989	0.000560	0.254
18:2n-6	7.05	8.61	0.426	0.064	32.2	38.3	0.871	‹0.001
20:2n-6	0.180	0.194	0.00775	0.372	0.485	0.675	0.0285	‹0.001
20:3n-6	0.165	0.0860	0.0123	‹0.001	1.57	1.32	0.0491	0.004
20:4n-6	0.311	0.175	0.0227	‹0.001	4.70	5.77	0.206	0.004
22:4n-6	0.0343	0.0379	0.00212	0.425	0.163	0.446	0.0393	‹0.001
22:5n-6	0.00923	0.0131	0.00081	0.020	0.0714	0.225	0.0217	‹0.001
n-3	16.8	4.15	1.72	‹0.001	10.8	3.82	0.939	‹0.001
16:3n-3	0.0720	0.0105	0.00856	‹0.001	-	-		
18:3n-3	14.7	3.64	1.50	‹0.001	1.65	0.343	0.176	‹0.001
20:3n-3	0.499	0.137	0.0497	‹0.001	0.586	0.164	0.0573	‹0.001
20:4n-3	0.102	0.0265	0.0104	‹0.001	0.168	0.0241	0.0200	‹0.001
20:5n-3	0.243	0.0319	0.0291	‹0.001	2.91	0.530	0.327	‹0.001
22:5n-3	0.692	0.183	0.0738	‹0.001	3.87	2.13	0.235	‹0.001
22:6n-3	0.207	0.0360	0.0243	‹0.001	1.44	0.532	0.123	‹0.001
n-6/n-3	0.467	2.23	0.241	‹0.001	3.65	12.3	1.18	‹0.001
P/S	0.606	0.365	0.0383	‹0.001	1.56	1.59	0.0152	0.299

SEM, standard error of the mean; FAME, fatty acid methyl esters; SFA, saturated fatty acids; BCFA, branched-chain fatty acids; i, iso; ai, anteiso; DMA, dimethylacetal; MUFA, monounsaturated fatty acids; CLA, conjugated linoleic acids; NC, non-conjugated; PUFA, polyunsaturated fatty acids; P/S, polyunsaturated fatty acids/saturated fatty acids.

trans-FA: summatory of all fatty acids with at least one double bond in *trans* configuration except CLA isomers.

The major FA present in the NL fraction of suckling and fattened foals were clearly differentiated, while the major FA in the PL fraction from both groups of animals showed a very close relationship (Figure 1).

The NL fraction of suckling foals showed positive relationships with short-chain SFA (‹15C), BCFA, unresolved NC-dienes (9*c*,13*t*-/8*t*,12*c*) and trienes (9*c*,12*c*,15*t*-/9*t*,12*t*,15*c*-), *trans*-FA and some individual n-3 PUFA (16:3 and 18:3; Figure 1), while the NL fraction of fattened foals was related to 16:0, the main *cis*- and *trans*-MUFA and RA that showed negative deposition preference values (Figure 2). Deposition of *i*-18:0 and 11*t*-18:1 was about equally distributed between the NL and PL fractions (deposition preference close to zero). There was no clear trend observed for the 9*c*,11*t*,15*c*-18:3 isomer, which in suckling foals deposited primarily in the NL fraction and in fattened foals deposited mainly in the PL fraction (Figure 2). Meanwhile, positive deposition preference values were observed for 18:0, 18:1 (11*c*-/12*c*-/15*c*-), some NC-dienes (9*c*,12*t*-/9*t*,12*c*-18:2), 18:2n-6 and all LC-PUFA (n-6 and n-3), indicative of a favored deposition of them in the PL fraction (Figure 2).

Even though deposition preferences were affected by the age and feeding of foals, it did not appear to modify the preferential pattern of incorporation into one or the other fraction. Overall, the higher and positive deposition preference values of LC-PUFA in the PL fraction of fattened foals could be due to the low content of these LC-PUFAs in NL fraction of concentrate-fed foals, while it was relatively constant in PL fraction of both groups of animals (Figure 2).

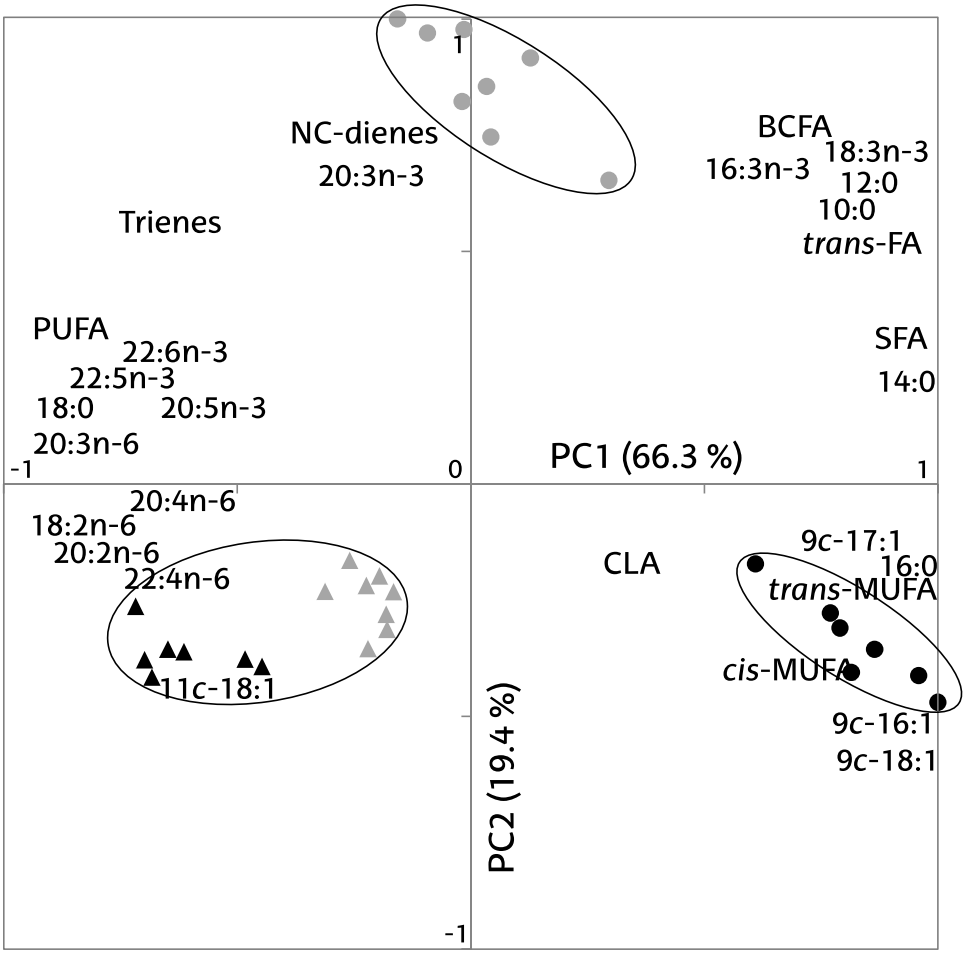

Figure 1. Loading variables and horse muscle neutral (suckling ●, fattened ●) and polar (suckling ▲, fattened ▲) lipids distribution on the two-dimensional coordinate system defined by principal component (PC) 1 and PC2.

FA, fatty acids; SFA, saturated fatty acids; BCFA, branched-chain fatty acids; MUFA, monounsaturated fatty acids; CLA, conjugated linoleic acids; NC, non-conjugated; PUFA, polyunsaturated fatty acids; *c*, *cis*.

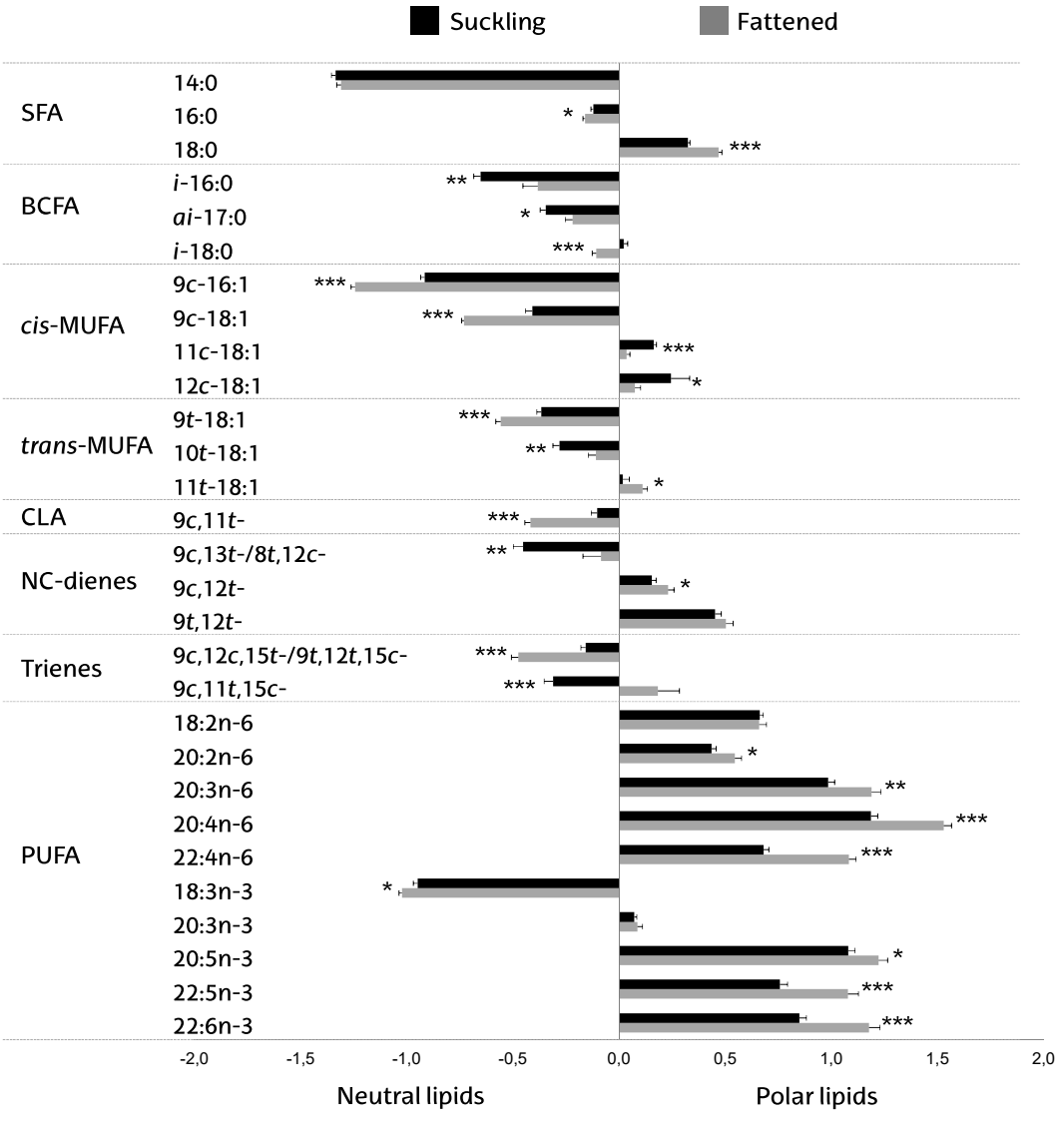

Figure 2. Deposition preference of main fatty acids into neutral (negative values) or polar lipid (positive values) fraction of *Longissimus thoracis et lumborum* muscle from suckling and fattened foals. Deposition preferences were calculated according to the logarithm of (individual fatty acid in percentage basis in the polar lipid fraction divided by the same fatty acid in percentage basis in the neutral lipid fraction).

SFA, saturated fatty acids; BCFA, branched-chain fatty acids; MUFA, monounsaturated fatty acids; CLA, conjugated linoleic acids; NC, non-conjugated; PUFA, polyunsaturated fatty acids; *i*, *iso*; *ai*, *anteiso*; *c*, *cis*; *t*, *trans*.

Error bars indicate the standard error of the mean for each group.

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Discussion

As observed by others, differences in IM fat content were most likely related to age (Sarriés & Beriain, 2005) and/or associated feeding (Lorenzo *et al.*, 2010). Our results agree with a higher moisture and protein content reported in extensively reared foals (Lorenzo *et al.*, 2014). The ash content was also in agreement with the values reported in the literature, which ranged from 0.98 % in 6-10 year-old horses to 4.03 % in foals slaughtered at 16 months of age (Sarriés *et al.*, 2005).

In terms of lipid constituents, data obtained from a survey of horse loin steaks (n = 82) collected in the northern Spain showed that the highest MUFA, and the lowest DMA, BCFA, and PUFA content (%) were found in samples with higher content of IM fat (Belaunzaran *et al.*, 2017). Higher values of 16:0 and lower values of 18:0 were reported in tissues of concentrate-fed horses and higher MUFA values (50.2 %) were shown to be associated with concentrate-based diets (Lorenzo *et al.*, 2010). We did not conduct gene expression of stearoyl-CoA desaturase, but the higher percentages of all *cis*-9 MUFAs, (14:1, 16:1, 17:1, 18:1 and 20:1; $P < 0.001$) found in the fattened foals (Table 2) suggest a higher 9-desaturase activity in this group of animals, also reported by others (Daniel *et al.*, 2004). The present study provides the first complete DMA composition of horse-meat. Although acid-catalyzed transesterification procedures were used to methylate horse lipids (Lorenzo *et al.*, 2010; Sarriés *et al.*, 2006), the expected DMA content was not reported. Very few studies report the plasmalogen content and composition in any meat (Aldai *et al.*, 2011; Kraft *et al.*, 2008), even though there is increased interest of these compounds considering their important biological activities and their implication in the development of several human diseases (Nagan & Zoeller, 2001). Ether lipids represented an average value of 4.401 % of the total horse lipids and the plasmalogen composition could be valuable information from its nutritional point of view. We found that the DMA content was relatively independent of the IM fat level between the two groups at approximately 0.0611 % by weight in horse-meat. This meant that the relative proportion of DMA in the total fat of the foals would be diluted with the higher levels of IM fat level in fattened foals. The alkenyl chain composition was reported to consist mainly of saturated and monounsaturated

moieties with 16:0, 18:0 and 9*c*-18:1 predominating (Horrocks, 1972). However, in the current study we found small amounts of polyunsaturated alkenyl moieties of 18:2 and 18:3 in horse plasmalogens that will be reported separately.

The formation and deposition of BCFA, CLA isomers and other biohydrogenation intermediates in horses is low largely due to minimal microbial metabolism of amino acids and PUFA in the small intestine. Microbial metabolites formed in the hindgut are generally poorly absorbed (Clauss *et al.*, 2009). Nevertheless, we did find several metabolites which are believed to be linked to LNA biohydrogenation. These differences could be influenced by the higher availability of LNA and its metabolites in grass-fed mares' milk (Malacarne *et al.*, 2002) than in LA-rich (45.8 %) concentrate-fed foals. On the other hand, the higher accumulation of RA in fattened foals was potentially related to the active 9-desaturase activity in IM fat, which converted vaccenic acid (11*t*-18:1) to RA, and is responsible for the formation of other 9*c*-containing FA in animal tissues (Vahmani *et al.*, 2016). Deposition of n-3 PUFAs in horse tissues was previously found to be significant under grazing conditions (Lorenzo *et al.*, 2010), while the higher 22:5n-6 percentage observed in fattened foals could be associated with a compensatory accumulation of this FA due to a reduction of 22:6n-3 in this group of animals with low n-3 PUFA in their diet (Galli *et al.*, 1974).

Regarding lipid fractions, present results agree with the general knowledge that fat accumulation occurs within the NL fraction while the PL content stays relatively constant (De Smet *et al.*, 2004; Figure 1). Furthermore, the FA composition of the NL fraction will be largely influenced by the diet, while the FA composition of the PL will be less affected. A knowledge of the FA composition of both horse lipid fraction is helpful to assess the nutritional value of the meat product and how it will impact its shelf-life. The higher content of 18:0 found in PL fraction was consistent with the high content also found by others in horse IM fat compared to subcutaneous (SC) adipose tissue that consists primarily of triacylglycerols and is low in LC-PUFA (Belaunzaran *et al.*, 2017; Sarriés *et al.*, 2006). Differences in DMA content between sucking and fattened foals were only observed in the NL fraction (Table 3). Neutral plasmalogens have been found in small amounts in mammalian tissues and in high amounts in some marine species

(Hayashi *et al.*, 1983). Interestingly, the DMA content located in the NL fraction of suckling foals was higher than values observed in mammalian tissues (Horrocks, 1972).

Most of the MUFA were preferentially deposited in the NL fraction, except for some minor *cis*-18:1 isomers (*i.e.*, 11*c*-, 12*c*-, and 15*c*-) that were mainly found in the PL fraction. It is of interest to note that Jerónimo *et al.* (2011) also showed a favored esterification of these particular isomers in the PL fraction of sheep muscle fat, and Emken *et al.* (1980) showed that human plasma lipids have a selective preference to incorporate 12*c*-18:1 compared to 9*c*-18:1. The function of the *cis*-18:1 isomers in the PL fraction remains unclear. However, contrary to the general principle of higher levels of PUFA found in PL compared to NL, we found that hexadecatrienoic acid (16:3n-3) was only detected in the NL fraction. The reason could be that 16:3n-3 originates from dietary galactolipids that is transferred from grass-fed mares' milk to the suckling foals. Galactolipids are the main components of chloroplasts and thylakoid membranes of botanical species (Siebertz *et al.*, 1979) and 16:3n-3 is present in high proportion at the sn-2 position of monogalactosyldiagylclycerol (Amara *et al.*, 2010). Interestingly, the LNA content was found to be higher in NL compared to PL fraction in agreement with the higher content found in SC adipose tissue than in the IM fat of horse (Table 3) (Belaunzaran *et al.*, 2017; Sarriés *et al.*, 2006). By contrast, LNA content in the PL fraction was lower due to the active conversion of LNA to LC n-3 PUFAs as membrane bound phospholipids reviewed by Pugh & Kates, (1979), which was also observed in ruminant species (Wood *et al.*, 2004). The LC-PUFAs are known to be essential membrane components in the cell regulating fluidity and metabolic functions (Spector & Yorek, 1985). The higher affinity for desaturation and elongation of LNA over LA has been previously described by others (Emken *et al.* 1992).

Conclusions

To our knowledge, this is the first report detailing the FA composition and their preference in the NL and PL fractions of horse muscle. It also provided for the first time the content of plasmalogenic lipids in horse-meat. There were significant effects related to the age and associated feeding practice of foals which affected the IM fat content and the FA composition of NL and PL fractions. Samples from suckling foals were the leanest, and provided the highest content of DMA, PUFA and n-3 PUFA both as % and as mg/100g of fresh meat. By contrast, the meat from concentrate-finished foals had a higher IM fat level resulting in a greater accumulation of 16:0 and total MUFA in the NL fraction while muscle PL fraction retained a similar FA composition between these two animal groups. This study provided evidence that LNA was preferentially deposited in the NL fraction, but LC n-3 PUFAs were incorporated into the PL fraction. On the other hand, LA was preferentially incorporated in the PL fraction. Even though the LA percentages were higher in both lipid fractions in fattened foals, the leaner meat from suckling foals presented higher proportion of PL and, thus, provided higher percentage of LA located mainly in this lipid fraction. LC-PUFA deposition occurred primarily in the PL fraction suggesting their biological role in cell membrane structures and eicosanoid formation. High PUFA content of horse-meat, LNA in NL and LC n-3 PUFAs in PL fraction, could represent an alternative product to ruminant meat, even though the n-3 content was not sufficiently high to receive a label as a source of n-3 FA. Further research will be needed to evaluate the influence of a higher content of PUFA on meat stability during storage and/or processing.

Acknowledgements

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2.3. Identification and quantification of dimethylacetals from horse-meat: experimental design I and II.

Meat is known to contain plasmalogenic lipids. In this type of lipids one of the ester linkages is replaced by a vinyl ether (alk-1-enyl moiety) which is generally linked to the sn-1 position of the molecule. These compounds are seldom reported in meat, and to our knowledge have never been reported in horse-meat. In mammalian tissues, the plasmalogens are only present in small amounts in the neutral lipid fraction, while they are major components of the polar lipid fraction. They are particularly found in phosphatidylethanolamine, which is an important structural component of the cell membranes in animals (Horrocks, 1972; Nagan *et al.*, 2011).

For identification and visualization purposes, a two dimensional separation of horse-meat lipids have been depicted in Figure V.1. This can be easily interpreted based on the principle that all the saturated FAMEs lie on the straight diagonal line, while the FAMEs with the same carbon skeleton but differing in the number/geometric configuration/position of double bonds lie on parallel lines to the ^{1}D time axis. The reduced FAMEs and DMAs were placed on separate lines.

A more comprehensive identification of FAMEs and DMAs of horse meat lipids have been provided in the partial chromatograms from 10:0 to 18:0 (Figure V.2) and from 18:0 to 18:3n-3 (Figure V.3) of GC-MS and GC-OR-GC analyses performed at isothermal conditions (180 °C). The total ion current (black line) and the extracted ion current of DMAs (red line) have been presented in the top section, and the two dimensional GC-OR-GC chromatogram in the bottom section. As evidenced in the GC-MS figure, DMAs coeluted with FAMEs when analyzed isothermally. However, GC-OR-GC resolved the DMAs from FAMEs in two well differentiated planes making possible their identification.

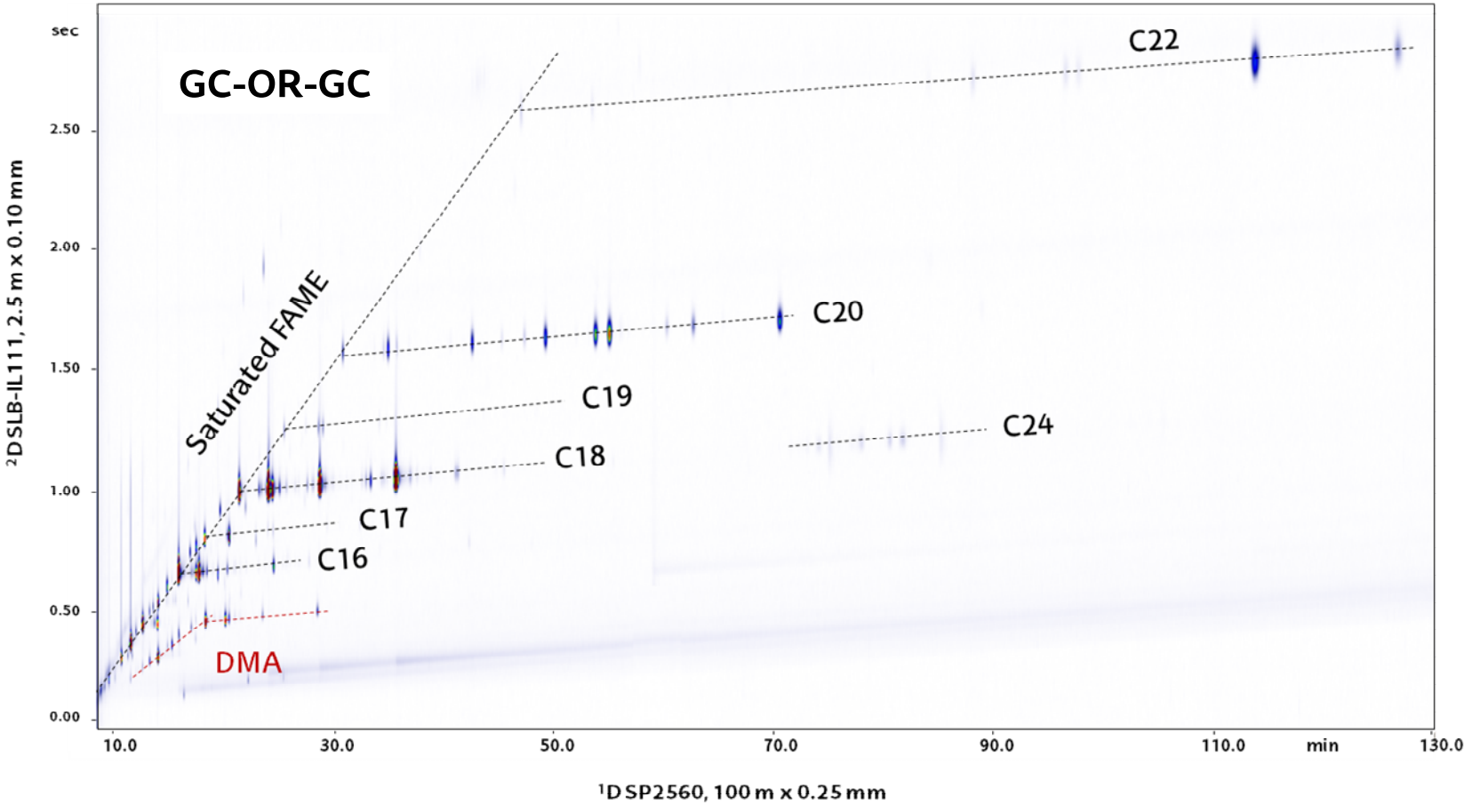


Figure V.1. The complete two dimensional GC-OR-GC chromatogram of horse meat lipids.

GC-OR-GC, gas chromatography – online reduction – gas chromatography; ^{1}D, first dimension; ^{2}D, second dimension; FAME, fatty acid methyl ester; DMA, dimethylacetal.

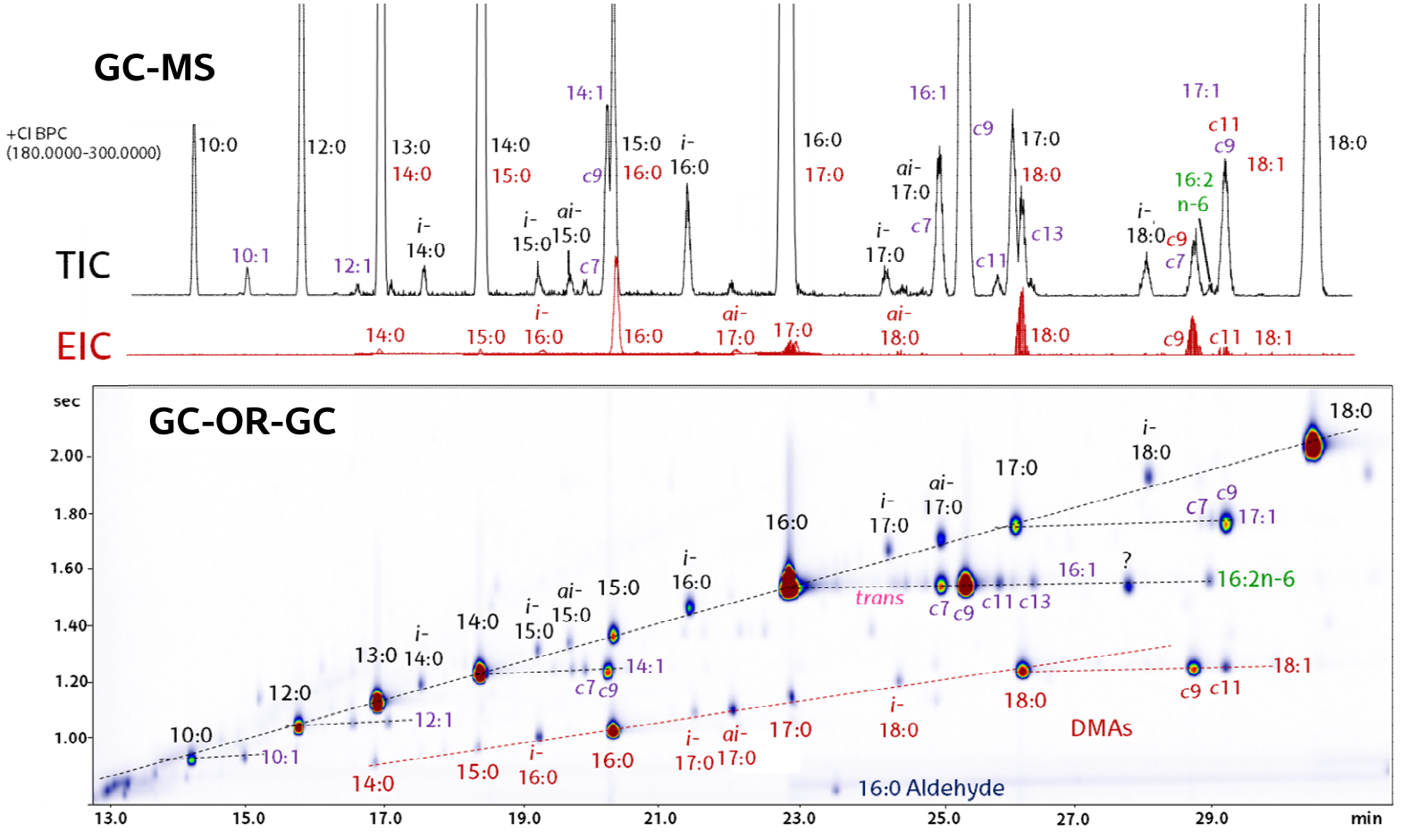


Figure V.2. Partial GC-MS (top) and GC-OR-GC (bottom) chromatograms from 10:0 to 18:0 of horse meat lipids.

GC-MS, Gas chromatography - mass spectrometry; GC-OR-GC, gas chromatography - online reduction - gas chromatography; TIC: total ion current (mass range 180.0000-300.0000; EIC: extracted ion current; DMA, dimethylacetal; *c, cis*; *i, iso*; *ai, anteiso*.

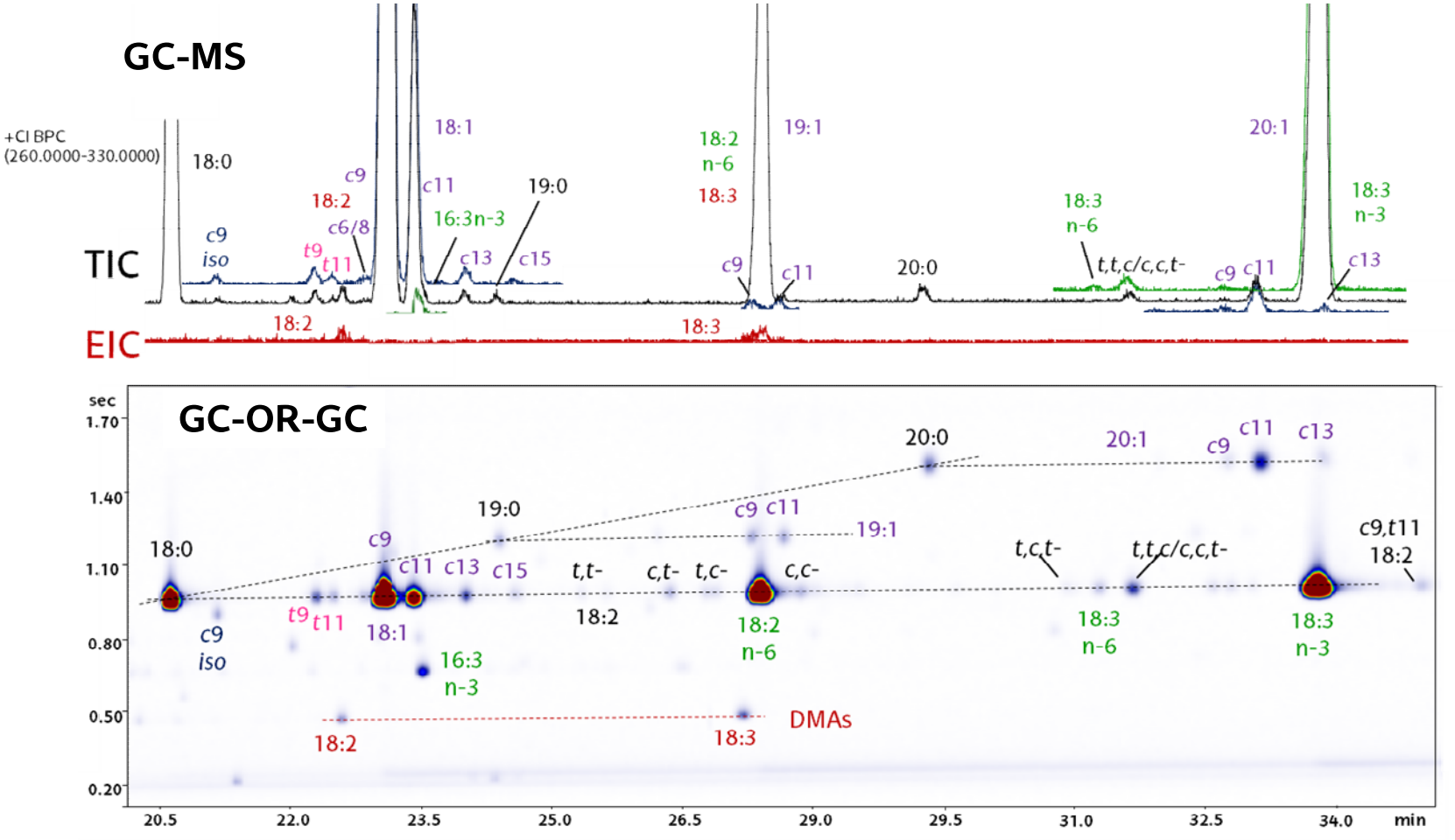


Figure V.3. Partial GC-MS (top) and GC-OR-GC (bottom) chromatograms from 18:0 to 18:3n-3 of horse meat lipids.

GC-MS, Gas chromatography - mass spectrometry; GC-OR-GC, gas chromatography - online reduction - gas chromatography; TIC: total ion current (mass range 260.0000-330.0000; EIC: extracted ion current; DMA, dimethylacetal; *c, cis*; *t, trans*.

The composition of the alkenyl ether chain is generally considered simple and it consisted mainly of saturated and monounsaturated moieties (Horrocks, 1972). However, as observed in Figure V.3, the GC-OR-GC analysis allowed the identification of polyunsaturated DMAs with 2 and 3 double bonds (18:2 and 18:3) which were also confirmed with the extracted ion current chromatogram obtained by GC-MS.

For quantification purposes, DMAs were, first of all, isolated from the total FAMEs, of horse-meat samples in both experimental studies, and then, identified and quantified as described in Chapter IV. From the experimental design I, 15 surveyed samples were used for this purpose. Due to the high n-3 PUFA variability of the samples (1.17-18.9 %), 5 samples were randomly selected from the low (below 4.5 %), 5 samples were randomly selected from the intermediate (4.5 to 11.5 %), and 5 samples were randomly selected from the high (over 11.5 %) n-3 PUFA content groups. Additionally, DMA analysis was performed in all muscle samples from the feeding trial (experimental design II; n=15). Partial chromatograms from 10:0 to 18:0 and from 18:0 to 18:3n-3 of GC-FID analysis of total lipids of horse-meat and isolated DMA fraction (red chromatogram) are shown in Figure V.4 and Figure V.5, respectively. The separation of DMAs from FAMEs was improved using the temperature program plateauing at 175 ºC (Kramer *et al.*, 2008). But, when analyzed together, still several DMAs (*i*-18:0, 9*c*-18:1, 18:2n-6, and 18:3n-3) coeluted with the prominent FAMEs (11/12*t*-16:1, 7*c*-17:1, 12*t*-18:1, and 18:2n-6, respectively). Therefore, they could only be accurately quantified after their isolation from FAMEs and the subsequent GC-FID analysis.

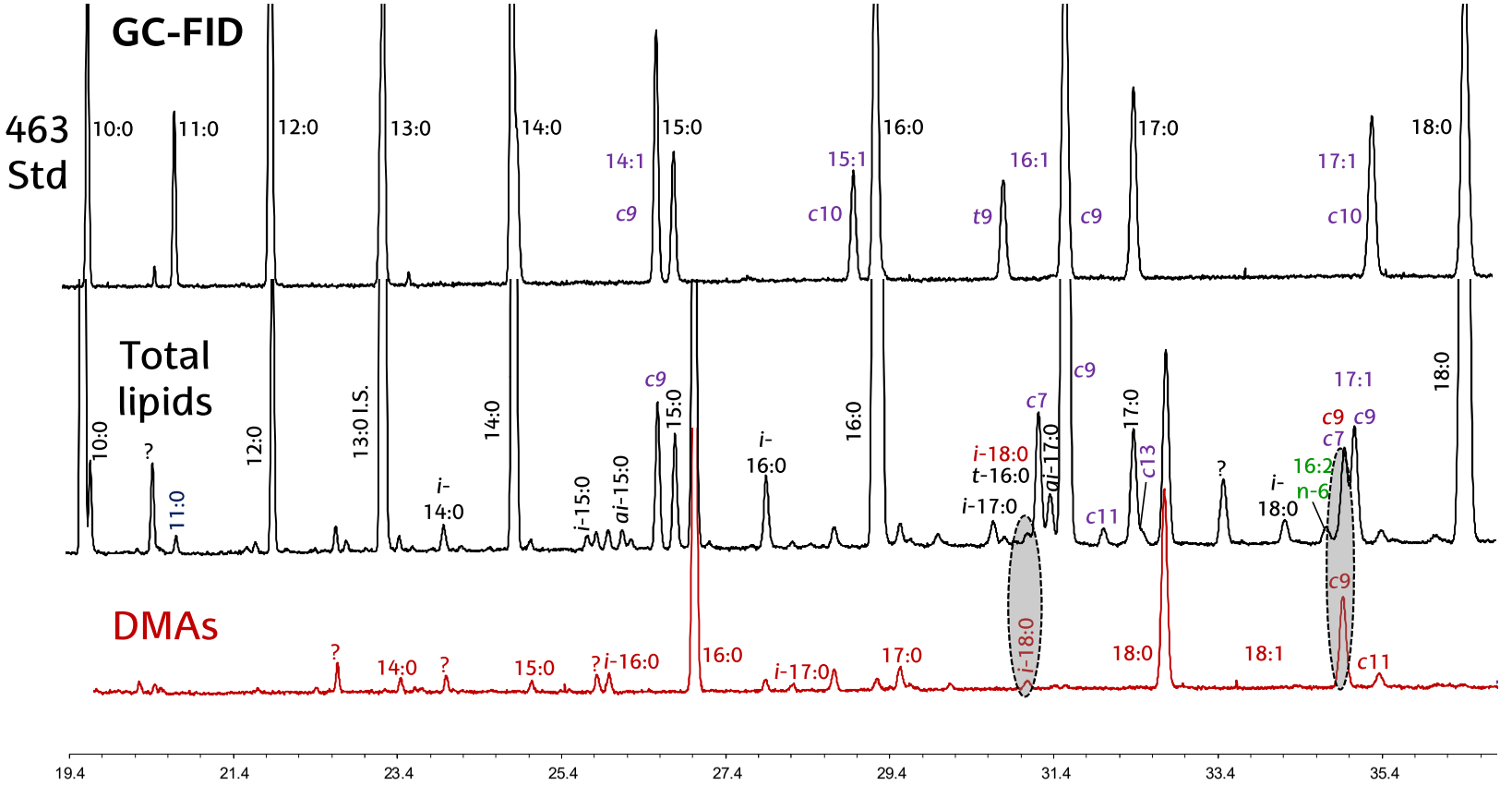


Figure V.4. Partial GC-FID chromatogram from 10:0 to 18:0 of total lipids and isolated DMA fraction (red) of horse-meat, and the #463 GC standard.

GC-FID, gas chromatography - flame ionization detector; DMA, dimethylacetal; *c, cis*; *t, trans*; *i, iso*; *ai, anteiso*; std, standard.

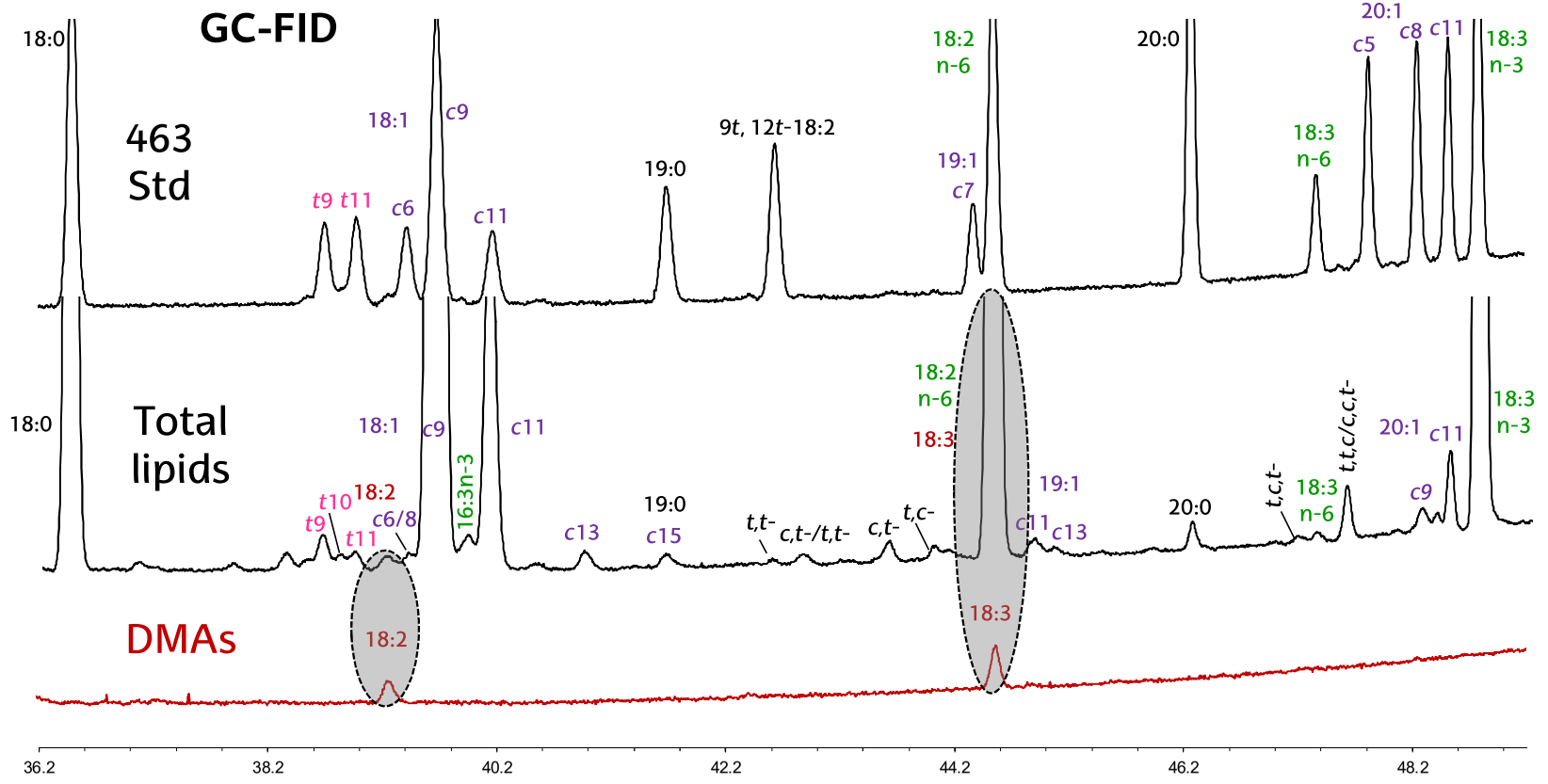


Figure V.5. Partial GC-FID chromatogram from 18:0 to 18:3n-3 of total lipids and isolated DMA fraction (red) of horse-meat, and the #463 GC standard.

GC-FID, gas chromatography - flame ionization detector; DMA, dimethylacetal; *c, cis*; *t, trans;* std, standard.

Total DMA content (mg/100 g of fresh meat) and composition (%) of horse-meat from experimental design I and II have been compared in Table V.1. Considerable variability in the total IM fat content was observed among studied samples. Highest content was found in fattened (2854 mg/100 g) and lowest in suckling foals (996 mg/100 g), while surveyed samples presented intermediate values (2067 mg/100 g). Leanest samples (suckling foals) showed highest DMA content (5.88 %) compared to the other two groups ($P < 0.001$) which was influenced by higher percentages of saturated ($P < 0.001$), branched-chain ($P < 0.001$), monounsaturated ($P < 0.001$) and 18:3 polyunsaturated ($P < 0.05$) DMAs.

Table V.1. Total fatty acid methyl ester and dimetylacetal content (mg/100 g of fresh meat) and dimetylacetal composition (%) of horse-meat from experimental design I (survey; n=15) and II (suckling and fattened foals; n=15).

Composition	Survey	Suckling	Fattened	SEM	*P value*
FAME & DMA (mg/100g)	2067^{ab}	996^{b}	2854^{a}	224	0.007
DMA (%)	3.39^{b}	5.88^{a}	2.37^{b}	0.358	<0.001
Saturated	2.56^{b}	4.58^{a}	1.8^{b}	0.282	<0.001
14:0	0.0310^{b}	0.0734^{a}	0.0147^{b}	0.00533	<0.001
15:0	0.0284^{a}	0.0344^{a}	0.0116^{b}	0.00225	<0.001
16:0	1.69^{b}	3.06^{a}	1.16^{b}	0.193	<0.001
17:0	0.0619^{b}	0.112^{a}	0.0476^{b}	0.00647	<0.001
18:0	0.754^{b}	1.31^{a}	0.562^{b}	0.0771	<0.001
Branched-chain	0.158^{b}	0.246^{a}	0.0655^{c}	0.0176	<0.001
i-16:0	0.0516^{a}	0.0694^{a}	0.0212^{b}	0.00498	<0.001
i-17:0	0.0118^{b}	0.0282^{a}	0.00474^{b}	0.00218	<0.001
ai-17:0	0.0586^{b}	0.0992^{a}	0.0281^{b}	0.00718	<0.001
i-18:0	0.0266^{b}	0.049^{a}	0.0115^{b}	0.00358	<0.001
Monounsaturated	0.571^{b}	0.955^{a}	0.442^{b}	0.0540	<0.001
9*c*-18:1	0.518^{b}	0.841^{a}	0.406^{b}	0.0470	<0.001
11*c*-18:1	0.0521^{b}	0.115^{a}	0.0362^{b}	0.00736	<0.001
Polyunsaturated	0.102	0.101	0.0614	0.00823	0.120
18:2n-6	0.0424	0.0416	0.0404	0.00277	0.961
18:3n-3	0.0593^{a}	0.0592^{a}	0.021^{b}	0.00590	0.017

SEM, standard error of the mean; FAME, fatty acid methyl ester; DMA, dimethylacetal; *i, iso; ai, anteiso; c, cis.*

From our samples, ether lipids represented an average value of 3.88 % of the total horse lipids and the plasmalogen composition could be a valuable information from the nutritional point of view. We found that the DMA content of horse-meat was relatively constant at approximately 0.0588 % of muscle weight. But the relative proportion of DMA would be diluted with the higher IM fat level of fattened foals.

In the scientific literature, the alkenyl ether chain composition is reported to be composed mainly of saturated and monounsaturated moieties with 16:0, 18:0 and 9*c*-18:1 being predominant (Horrocks, 1972). However, in the current study, small amounts of polyunsaturated alkenyl ether moieties of 18:2 and 18:3 were found in horse plasmalogens. In general, even though differences in DMA percentages were observed among studied groups (Table V.1), the profile pattern was very similar. Saturated DMAs represented the main group at an average value of 76.4 % of the total DMA quantified, with 16:0 (average value of 50.5 %) and 18:0 (average value of 22.4 %) being the major moieties. Monounsaturated represented an average value of 16.9 %, followed by branched-chain (average value of 4.21 %), and polyunsaturated (average value of 2.48 %) alkenyl ether moieties.

The present results provide the first complete DMA profile of horse-meat. Others have also performed acid-catalyzed derivatization procedures in horse lipid samples (He *et al.*, 2005; Lorenzo *et al.*, 2010; Sarriés *et al.*, 2006), however, DMA content and profile were not reported. Only very few previous studies have reported plasmalogen content and composition in any meat (Aldai *et al.*, 2011; Kraft *et al.*, 2008), even though there is increased interest of these compounds considering their important biological activities and their implication in the development of several human diseases (Nagan *et al.*, 2001).

3. GENERAL DISCUSSION

Global protein needs driven by the increased population could be at least partially complemented with horse-meat. However, there is a clear lack of information about quality horse-meat production for human consumption and on top of that, in recent years horse-meat has become the subject of discussions pertaining to its nutritional value, acceptability and labeling fraud. The objective of the present Ph. D. thesis was to assess the nutritional quality of horse-meat by providing a thorough characterization of the horse-meat lipids by applying a number of lipidomic techniques. The regularly used GC-FIC technique was complemented by other more comprehensive techniques like GC-MS and GC-OR-GC for the identification of all methylated products from the acyl and alkenyl ether lipids. The structure of the unsaturated FAMEs and their geometric and positional isomers was confirmed using Ag^+- chromatographic separations. These improved analytical techniques were first used to analyze the horse-meat collected in a survey to examine the variation of this product on the retail market, and in a subsequent feeding trial to see whether diet would affect the nutritional profile of the meat.

Therefore, the first objective of the present Ph. D thesis was to optimize, develop and utilize the most appropriate analytical methodology in order to achieve an accurate, detailed and complete analysis of lipid constituents in horse-meat. In the process, the FAME fractionation technique was optimized, especially for those FAMEs present at low concentration in horse lipids. The present work confirmed the low *trans*-FA content of horse-meat (‹1 %). However, when the commercial Ag^+-SPE cartridges were employed to accurately determine the *trans*- and *cis*-MUFA fractions present, several impurities were found to interfere in the identification of the small amounts of *trans* isomers present in horse lipids (Publication II). These impurities were suspected to elute from the polypropylene housing of the cartridges. The supplier was then contacted to prepare the same amount of Ag^+-loaded cation-exchange resin in custom made glass cartridges. The results confirmed the finding that the impurities had indeed come from the

polypropylene housing and new Ag^+-SPE cartridges were then used in all subsequent analyses to quantitate the minor *trans* isomers in horse-meat. The improved methodology with Ag^+-SPE glass cartridges was recommended for future studies (Publication III).

The second objective was to assess the nutritional quality of horse-meat at retail level available in butcher-shops and grocery stores at both sides of the Cantabrian Mountains in northern Spain and during two different time periods of the year (spring and winter). The results from the survey study demonstrated the unique *trans*-18:1 content and composition of horse-meat compared to ruminant species where 10*t*- and 11*t*-18:1 are the two predominant isomers depending considerably on the type of feeding (Aldai *et al*., 2009; Aldai *et al.,* 2011; Bessa *et al*., 2005; Bessa *et al*., 2015). This compares to horse tissues, where 9*t*-18:1 was the main isomer. The total accumulation of other dienes, trienes or CLA was also low compared to ruminants (Aldai *et al.,* 2012b; Shingfield *et al*., 2013). The low absorption and deposition of biohydrogenation intermediates in horse tissues is due to the fact that the transformation of dietary lipids occurs in the post absorptive part of the horse gut (caecum/colon) (Clauss *et al.,* 2009; Hartam *et al*., 1956). At retail level, there was a significant variability in the IM fat content and its FAME profile among regions revealing differences in management practices where type of breed, feeding and age at slaughter might be the most relevant factors. The data obtained showed the highest percentages of total MUFA and the lowest of total DMA, BCFA and PUFA in samples with higher IM fat content. Furthermore, high overall variability was observed in the n-3 PUFA content ranging from 1.17 % to 18.9 % in muscle tissue, and from 1.52 % to 27.9 % in subcutaneous fat. The n-3 PUFA level was higher in samples collected in early winter, presumably since animals were reared under mountain grazing conditions until late fall. The average content of n-3 PUFA in horse was higher compared to other species (*i.e.*, poultry, rabbit, beef, pork), which was mainly related to the high content of LNA in horse-meat (Publication IV).

The third objective was to evaluate the effect of age, and associated differences in feeding, on the nutritional quality of horse-meat. A semi-controlled experiment was undertaken and two groups of crossbred horses were studied; suckling and

fattened foals slaughtered at 4 months and 12 months of age, respectively. In the present study, NL and PL fractions of horse-meat were separated and the FAME profile of each were compared in order to assess their deposition preference into the NL and/or PL fraction as affected by age and associated feeding. The meat obtained from suckling foals was the leanest and provided a healthier profile with higher dimethylacetal, linolenic acid, and all (n-3 and n-6) long chain polyunsaturated fatty acid content. Meat obtained from concentrate-finished foals, however, presented a significantly higher IM fat content which mainly affected the relative FAME composition of the neutral lipid fractions. It is known that muscle FAME composition can be influenced by the relative proportion of its NL and PL fractions, and these can be affected by genetics and dietary factors as well as animal fatness (De Smet *et al.*, 2004). Higher IM fat content was associated with a lower PL/NL ratio and dilution of those FAMEs deposited in the PL fraction which includes DMAs and LC-PUFAs. However, due to the preferential deposition of LNA (18:3n-3) in the NL fraction results in a significant accumulation in muscle and subcutaneous fat, making horse-meat a good option to be marketed as a source of n-3 (Manuscript V).

Meat is also known to contain plasmalogenic lipids where one of the ester linkages is replaced by a vinyl ether (alk-1-enyl) moiety. Upon acid-catalyzed transesterification, these vinyl ethers produce DMAs and the present study provides the first complete DMA composition of horse-meat. Even though acid-catalyzed transesterification procedures were previously used to methylate horse lipids (He *et al.*, 2005; Lorenzo *et al.*, 2010; Sarriés *et al.*, 2006), the expected DMA content were never reported. Very few studies report the plasmalogen content and composition in any meat (Aldai *et al.*, 2011; Kraft *et al.*, 2008), even though there is increased interest of these compounds considering their important biological activities and their implication in the development of several human diseases (Nagan & Zoeller, 2001). In mammalian tissues, the plasmalogens are preferentially found in phosphatidylethanolamine, which is an important structural component of the cell membranes in animals. The composition of the alkenyl ether chain was reported to consist mainly of saturated and monounsaturated moieties (Horrocks, 1972). However, by applying advanced GC

techniques (*i.e.*, GC-OR-GC, GC-MS) during the Ph. D. investigations, small amounts of DMA were identified in which the alk-1-enyl ether moiety contained 2 and 3 double bonds (18:2 and 18:3). The improved method to analyze DMA also involved a modification of the extraction and methylation procedure, which was developed in co-operation with researchers at the US FDA and to be published separately. The application of this improved method proved successful to comprehensively quantify horse lipids (*i.e.*, methylation products of acyl ester and alkenyl ether moieties). It was of interest to note that the horse-meat samples analyzed in all experimental studies proved to be relatively constant at approximately 0.0588 % of muscle weight. The real impact and nutritional significance of the plasmalogenic content and composition remains to be determined.

In order to compile and put into common all the experimental results obtained in the present work, sample distribution plot using the two-dimensional coordinate system defined by the first two PCs was used to study the variability in FAME composition of horse muscle (IM) and backfat (SC) adipose tissues collected throughout the experiments (experimental design I, n=82, and experimental design II, n=15; Figure V.6.). Differences between IM and SC fat were evident in the PCA where the first two components explained 66.9 % of the total variability. The IM and SC fat tissues from suckling foals were associated with the variables with positive loading in PC1. Fat tissues of suckling foals were well differentiated based on their characteristic composition (▲ and ●). On the other hand, fat tissues from concentrate-finished foals were associated with the variables with negative loading in PC1. In this case, IM and SC fat tissues were also well differentiated (▲ and ●), but much closer compared to fat samples from suckling foals due to their similarity in composition. In terms of total muscle lipid fractions, NLs represented 60.1 % in samples obtained from suckling foals and 86.3 % in samples obtained from fattened foals. Because SC fat tissue consists primarily of NLs, and the NL content is higher in the IM fat of concentrate-finished foals, the FAME profile of SC and IM fat of fattened foals was closer to each other.

Overall, as it was observed, all samples collected from concentrate-fed foals were related to 16:0 and *cis*-MUFAs. On the other hand, SC samples from suckling foals displayed positive relationships with variables such as short-chain SFA (‹15 atoms of carbon), BCFA, NC-dienes, trienes, *trans*-FAs, and some individual n-3 PUFAs (16:3, 18:3 and 20:3) that showed a favored esterification in the NL fraction. Muscle samples from suckling foals, conversely, were positively associated with 18:0, 11*c*-18:1, LA, and all LC-PUFAs (n-6 and n-3) which showed a preferential deposition in the PL fraction that represented 39.9 % of total lipids, while PLs represented only 13.7 % of total lipids in fattened foals.

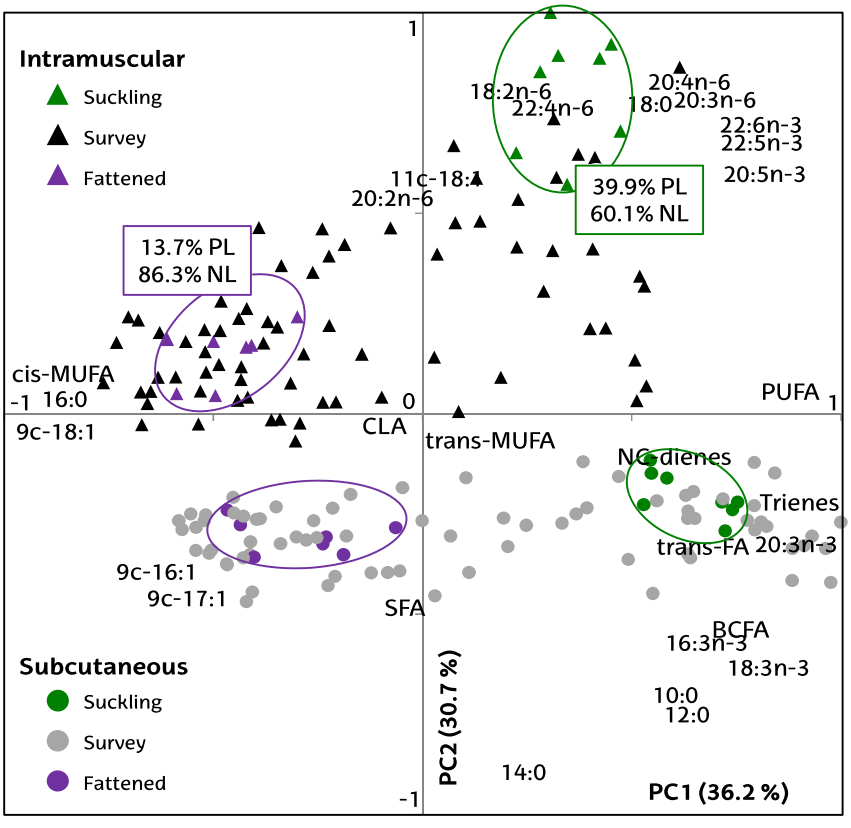


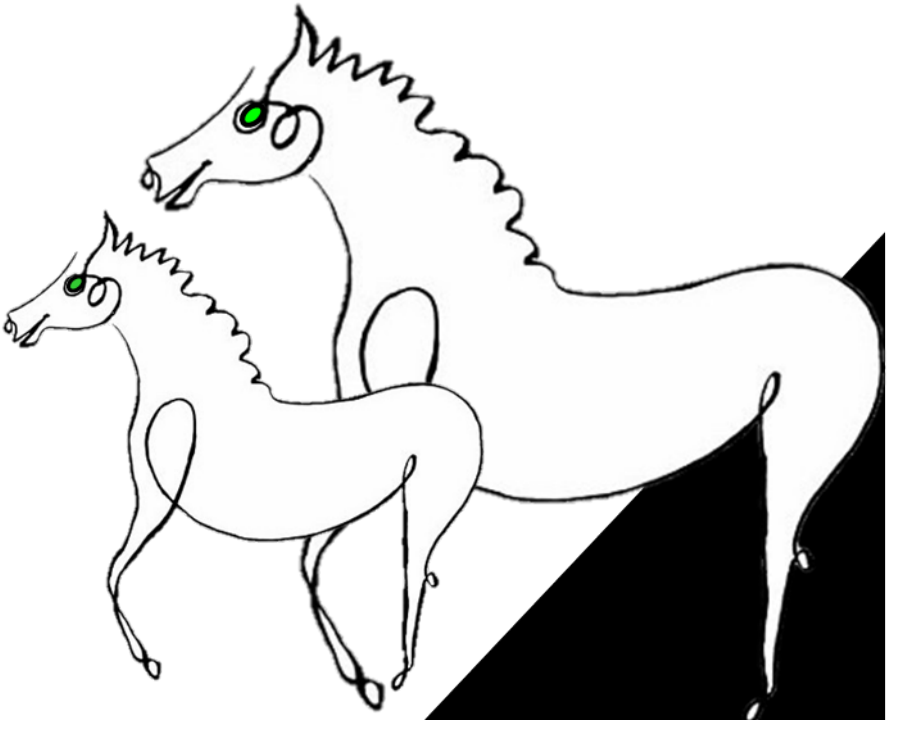
Figure V.6. Loading variables and horse intramuscular (△) and subcutaneous (○) sample distribution on the two-dimensional coordinate system defined by the principal component 1 and 2.

FA, fatty acid; SFA, saturated fatty acids; BCFA, branched-chain fatty acids; MUFA, monounsaturated fatty acids; CLA, conjugated linoleic acids; NC, non-conjugated; PUFA, polyunsaturated fatty acids; PL, polar lipids; NL, neutral lipids.

In the figure it can be also observed that many of the IM and SC samples collected during the survey (experimental design I) were located in between the samples obtained from fattened and suckling foals (experimental design II) suggesting that they might come from other breeds, feeding strategies or management systems, and making visible the considerable variation among samples. At the same time, interestingly, 5 % of the surveyed horse muscles (4 out of 82) reached 300 mg of LNA per 100 g of fresh meat which was the minimum level to market this product as a source of n-3 according to the European Commission Regulation 116/2010 with regard to the list of nutrition claims. However, when the animals are young (suckling foals) or reared under extensive conditions it seems very difficult to reach this level of n-3 on an absolute basis required for labeling purposes. But if part of the trimming fat derived from backfat was included to the loin, or fatter meat was utilized, horse-meat could meet or exceed the recommended daily intake established by several European Societies (*i.e.*, 1 g of total n-3 PUFAs per day).

In addition, the present study provides the first report of the presence of hexadecatrienoic acid (16:3n-3) in horse-meat. In fact, the very significant LNA content together with other small amounts of 16:3n-3 found in SC fat and in the NL fraction of muscle fat, could be associated either with pregastric microbial activity and/or with a specific pancreatic lipase related to protein 2 (Amara *et al.*, 2013; Andersson *et al.*, 1996), providing the horse a higher ability to hydrolise dietary galactolipids located in chloroplasts and thylakoid membranes of higher plants and in which LNA and 16:3n-3 are mainly esterified. This enzyme could potentially explain the high content of LNA deposition in horse tissues in comparison to other species.

Chapter VI. CONCLUSIONS



Chapter VI. CONCLUSIONS

From this research conducted on horse lipidomics, the following conclusions were drawn:

1. The regularly used gas chromatography – flame ionization detector technique complemented with other more comprehensive and novel techniques such as gas chromatography – online reduction – gas chromatography facilitates the identification of methylated products of acyl ester and alkenyl ether moieties of horse lipids. In addition, due to the low *trans* fatty acid content of horse-meat, the chemical nature of silver-ion solid phase extraction cartridges is very relevant to avoid interferences of impurities with target compounds and, therefore, glass cartridges are recommended.

2. The method developed to isolate dimethylacetals from fatty acid methyl esters proved to be a successful tool to comprehensively identify and quantify horse lipids. Additionally, dimethylacetals in horse-meat have been reported for the first time which represent from 1.40 to 8.99 % of the total lipids in horse-meat.

3. Along the Cantabrian Mountains, the fattest muscle samples were collected in Navarre and Castile & Leon, while the leanest samples were collected in Asturias and Galicia regions. The variability among regions revealed the differences in management practices where breed, feeding and age at slaughter could be the most relevant factors.

4. At retail level, high variability in the n-3 polyunsaturated fatty acid content was observed (1.17-18.9 % in muscle fat and 1.52-27.9 % in subcutaneous fat), and the higher n-3 polyunsaturated fatty acid levels in samples collected in early winter could be associated with animals that were presumably reared under mountain grazing conditions until late fall.

5. The meat obtained from concentrate-finished foals presented a significantly higher intramuscular fat content (1.56-3.99 %) resulting in a

greater accumulation of palmitic acid and total monounsaturated fatty acids in the neutral lipid fraction that represent 86.3 % of the total lipids.

6. The meat obtained from suckling foals was the leanest (0.565-1.23 %) and provided a healthy profile with higher dimethylacetal, linolenic acid, and all (n-3 and n-6) long chain polyunsaturated fatty acid contents due to their favorable deposition in the polar lipid fraction that represents 39.9 % of the total lipids.

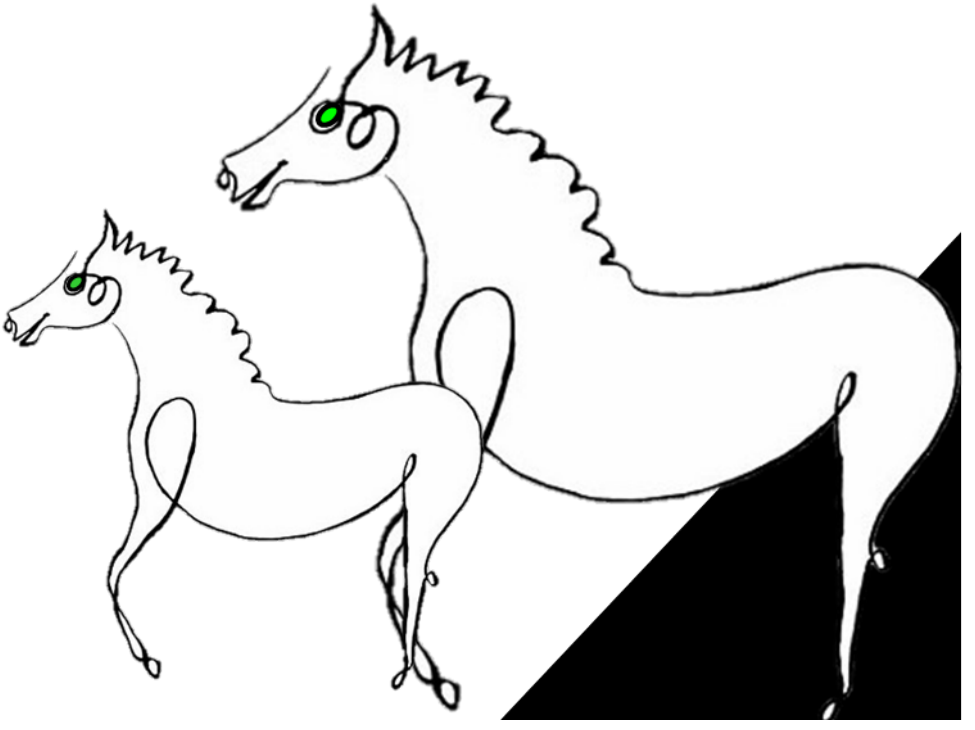
7. The high polyunsaturated fatty acid content of horse-meat due to the high linolenic acid content in subcutaneous fat and long chain n-3 polyunsaturated fatty acid content in muscle tissue, can make horse-meat a good source of n-3 fatty acids in comparison to other species.

8. Five percent of the surveyed horse muscles reached the minimum of 300 mg of linolenic acid per 100 g of fresh meat required for being marketed as a source of n-3 fatty acids according to the European Commission Regulation 116/2010. And if trimming fat derived from subcutaneous fat is included to the loin, horse-meat could meet or exceed the recommended daily intake established by other European Societies (*i.e.*, 1 g of total n-3 polyunsaturated fatty acids per day).

9. These results are remarkably valuable to provide a more accurate database for the nutritional assessment of horse-meat, to promote consumer acceptance of horse-meat as an edible and healthy food, and to contribute in marketing of horse-meat.

10. Lipidomic tools proved to be a useful approach to accurately evaluate the changes occurring in the nutritional quality of horse lipids as affected by management practices.

Chapter VII. LITERATURE CITED



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