PROTEOMA ALDAKETAK ETA APOPTOSI-PROZESUAK BEHI-HARAGIAN: GARAPEN METODOLOGIKOA ETA ANIMALIA MANEIUAREN ERAGINA

CAMBIOS DEL PROTEOMA Y PROCESOS DE APOPTOSIS EN LA CARNE DE VACUNO: DESARROLLO METODOLÓGICO E INFLUENCIA DEL MANEJO ANIMAL

PROTEOME CHANGES AND APOPTOSIS PROCESSES IN BOVINE MEAT: METHODOLOGICAL DEVELOPMENT AND EFFECT OF ANIMAL MANAGEMENT

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Proteoma aldaketak eta apoptosi-prozesuak behi-haragian: garapen metodologikoa eta animalia maneiuaren eragina

Cambios del proteoma y procesos de apoptosis en la carne de vacuno: desarrollo metodológico e influencia del manejo animal

Proteome changes and apoptosis processes in bovine meat: methodological development and effect of animal management

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The last experimental work derived from the present Ph. D. Thesis (Appendix VII) has been sent to *Food Sci Technol Int*.

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LABURPENA

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

Doktorego-tesi honen helburu orokorra haragiaren azken kalitateaz arduratzen diren prozesu biokimikoak, hau da, behi-aziendaren muskulua haragi bihurtzean gertatzen direnak, ebaluatzea izan da, bereziki muskulu-proteomaren aldaketak eta post mortem apoptosia. Halaber, estrategia proteomiko eta entzima-saiakuntza desberdinen erabilgarritasuna frogatzea izan da, muskuluaren post mortem metabolismoari buruzko prozesu horiek zehazki aztertu ahal izateko. Prozesu horiek okela akastunak eragin ditzakete maneiu-praktiken ondorioz. Helburu horiek lortzeak elikadura-katean zehar kalitate handiko behi-okela baldintza jasangarrietan, kontsumitzaileak ekoizpen-praktiken ikuspegitik onartzen dituenak (animalien ongizatea eta ingurumen-kontserbazioa), ekoizteko ekintzak sustatzen lagunduko luke.

Doktorego-tesi hau argi eta garbi bereizitako bi zatitan banatu da, muskulu-proteomaren aldaketak eta apoptosi-prozesuak aztertzeko erabilitako metodologiaren arabera. Hori dela eta, lehenengo blokeak proteina muskularrak aztertzeko hurbilketa proteomikoen garapena eta inplementazioa zein izan den azaltzen du, eta bigarrenak, berriz, hainbat maneiu-baldintzapean ekoiztutako animalien muskuluan post mortem apoptosi-prozesuak ebaluatzeko saiakuntza entzimatikoak optimizatzeko eta erabiltzeko egindako lana azaltzen du.

Proteomika oso tresna baliagarria bihurtu da okela akastunen agerpenarekin lotutako biomarkatzaile proteikoak identifikatzeko, hauek okela normalak baino azken pH handiagoa dutelako. Beraz, lehen helburu espezifikoa estrategia proteomikoak optimizatzea, garatzea eta inplementatzea izan zen, behi-haragiaren proteinei buruzko analisi zehatz eta osoa egiteko post mortem denbora goiztiarretan (24 ordu). Lehen helburu hori lortzeko, erabilitako metodologia analitikoak eboluzionatzen joan ziren, masen espektrometriara akoplatutako kromatografia likido teknika ohikoagoetatik (LC-MS), zeintzuk laginen aldez aurreko zatikapen-etapak erabiltzea eskatzen zuten, LC-MS estrategia sofistikatuagoetara, zeintzuk aldez aurreko zatikapenik gabe laginen analisi zuzena ahalbidetzen zuten.

Lehenengo fase batean, proteinak ingurune likidoan zatikatzean (OFFGEL) oinarritutako analisi-estrategia garatu zen, proteinak lehenik gelean bereizita, eta ondoren, LC-MS bidez

identifikatuta. Honek azken pH normal eta altuak zituzten haragien bereizketa eta sub-proteoma sarkoplasmikoa eta miofibrilarra modu bereizian aztertzea ahalbidetu zituen. Gelean oinarritutako proteinak bereizteko teknikak behi-okelaren proteoma aztertzeko oso erabiliak izan diren arren, hauen erabilera konbinatua, ingurune likidoan aurre-zatikatze metodo batekin, oraindik urria da. Era berean, azken pH altuko haragiak aztertzeko orain arte erabili diren ikuspegi gehienak proteina-aterakin osoaren analisian oinarritu dira, frakzio sarkoplasmikoa eta miofibrilarra bereizi gabe. Oro har, banatze-metodo hori baliagarria izan zen muskulu-proteina nagusiak (bai estraktu sarkoplasmikotik datozenak, bai miofibrilarretik datozenak) karakterizatzeko eta identifikatzeko. Proteina horiek azken pH normal eta altu bat duten haragiak bereizteko gai ziren, eta, beraz, mota horretako akatsen gertaera iragartzeko.

Lortutako emaitzak onak izan arren, gelean oinarritutako proteinak bereizteko metodologiek muga garrantzitsuak dituzte, OFFGELaren aberastea zuzenean LC-MS analisiari egokituko balitzaio gaindi daitezkeenak. Hala ere, gelik gabeko hurbilketa honek LC-MS metodo sendo baten inplementazioa eskatzen zuen datu kualitatibo zein kuantitatiboak automatikoki prozesatzeko. Horregatik, doktorego-tesiaren lehen zati honen bigarren fase batean, gelik gabeko estrategia bat garatzea planteatu zen, zatikatze aurreko etapa batean oinarrituta (OFFGEL) eta LC-MS analisiari zuzenean erantsita, sub-proteoma sarkoplasmikoaren azterketa kualitatibo/kuantitatibo automatizatua egiteko. Honek, sub-proteoma sarkoplasmikoaren analisi kualitatiboak, aztertutako OFFGEL zatikietan zehar banatutako 47 proteinen zalantzarik gabeko identifikazioa ahalbidetu zuen. Aitzitik, horien analisi kuantitatibo automatizatuaren inplementazioa konplexuagoa zela frogatu zen, hainbat balidazio-urrats behar izan baitziren. Lehen baliozkotzean konfiantza-atalase bat ezarri zen, modu automatikoan, zehatzean eta linealtasun-balioekin eta aldakuntza-koefiziente egokiekin ugaritasun ertain-altuko peptido proteotipikoak kuantifikatzeko. Horri esker, aztertutako OFFGEL zatikietan zehar banatutako 20 proteina sarkoplasmikoetatik sortutako 41 peptido zenbatu ahal izan ziren, emaitzak analisi kualitatiboaren bidez aldez aurretik lortutakoekin aldera zitezkeelarik. Azterlan horren emaitzek agerian utzi zuten OFFGEL zatikapenaren eraginkortasuna, proteinak aberasteko eta ondoren kuantifikatzeko estrategia gisa, gelean oinarritutako estrategiarik erabili gabe.

Hala ere, OFFGELaren aberaste-etapa laginaren prozesatzeari lotutako mugetatik salbuetsita dago, eta horrek ziurgabetasunak sortzen ditu peptidoen LC-MS bidezko kuantifikazioan. Beraz, erronka analitiko garrantzitsua da aurre-zatikatze etaparik egin gabe proteinen analisia sinplifikatzea ahalbidetuko duten LC-MS estrategia sentikorrak, sendoak eta espezifikoak ezartzea. Horregatik, doktorego-tesiaren lehen zati honen hirugarren fase batean, gelik gabeko metodologia bat garatzea eta ezartzea planteatu zen, OFFGELaren aurre-zatikapenik gabe LC-MS bidez laginak zuzenean aztertzeko. Helburu hori lortzeko, erreakzio selektiboen jarraipenean (ingelesezko Selected Reaction Monitoring edo SRM) oinarritutako estrategia kuantitatibo bat garatu zen, pH normal eta altudun haragiak bereizteko gai diren sub-proteoma sarkoplasmikoko biomarkatzaile proteikoak kuantifikatzeko helburuarekin. Oro har, SRM bidez egindako analisi kuantitatiboan oinarritutako gelik eta aurretiazko zatikapen

gabeko estrategia proteomikoa baliagarria izan zen behi-okelaren muskulu-proteoman desberdintasun kuantitatiboak aztertzeko. Ildo horretan, 10 proteinak alde nabarmenak erakutsi zituzten entseatutako bi taldeen artean (haragiaren azken pH normala eta altua), eta batipat, azken pH balio altudun laginetan. Gainera, ikusi zen aurrez karakterizatutako eta azken pH altuko haragietan esklusibotzat jotako proteinak (gelezko bereizketan oinarritutako estrategian), pH normaldun hainbat laginetan ere ikusi zirela nahiz eta oso ugaritasun txikian. Horrek agerian uzten du SRM analisiaren erabilgarritasuna, aurretik erabilitako beste kuantifikazio-aukera batzuekin alderatuta. Proteina disolbagarrien estraktuen zuzeneko analisiaren bidez lortutako emaitzen kalitate handiagoa, efizientzia handiagoa eta beharrezko eskulan txikiagoarekin batera, haragiaren kalitateari buruzko ohiko ikerketa-azterlanetan metodologia hori ezartzeko aukera erakutsi zen.

Garatutako hurbilketa proteomikoa alde batera utzita, interesgarria da azpimarratzea azken pH altudun haragietan animalien estresari lotuta asko aztertu diren talka termikoko hainbat proteina (ingeleseko Heat Shock Proteins edo HSP) hauteman zirela. Aurkikuntza interesgarri hau, programatutako heriotza zelularraren edo apoptosiaren prozesua kontsideratuz uler daiteke, hau da, HSPek betetzen duten paper anti-apoptotikoa ulertuta. Prozesu horren aktibazioa (kaspasa izeneko entzima batzuen bidez) hasierako estimuluaren izaeraren araberakoa da, eta estresa da faktore garrantzitsuenetako bat. Nahiz eta ikerketa asko apoptosiaren azterketan eta honek haragiaren heltze-prozesuan duen eraginean oinarritu diren, aurretiko lan horiek ez diote heldu animalien metabolismoan eragina duten hil aurreko faktoreen eta muskulu eskeletikoaren hil osteko apoptosiaren arteko balizko erlazioari. Horregatik, doktorego-tesi honen bigarren zatian, bigarren eta hirugarren helburu espezifikoak proposatu ziren: alde batetik, entzima-saiakuntza bat optimizatzea eta erabiltzea muskulu eskeletikoan 3/7 kaspasen jarduera neurtzeko, eta, bestetik, animalien maneiu-praktikek muskuluko apoptosi-prozesuan duten eragina aztertzea hil osteko denbora goiztiarretan.

Honetarako, fluoroforo baten askapen entzimatikoan oinarritutako saiakuntza fluorogeniko bat optimizatu zen 3/7 kaspasaen jarduera muskulu-estraktuetan neurtzeko substratu espezifiko batetik abiatuta. Estraktuok entzima horien kontzentrazio txikia dute. Oro har, baldintza instrumental eta erreakzio optimizatuei esker eta jarduera-maila edozein izanda ere, 3/7 kaspasen jarduera neurtu ahal izan zen muskulu-estraktu askotan erreproduzigarritasun altuarekin. Gainera, kit komertzial batekin alderatu zen optimizatutako saiakuntza eta linealtasun-emaitza hobeak lortu ziren, bereziki jarduera baxua zuten laginetan.

Azkenik, saiakuntza optimizatua erabili zen 3/7 eta 9 kaspasen jarduera neurtzeko eta maneiu-faktore desberdinek (ekoizpen-sistema eta garraio/ukuiluratze baldintzak) hil osteko denbora goiztiarretan (2, 8 eta 24 ordu), estatuko hiru behi-arrazetatik ('Asturiana de los Valles', 'Retinta' eta 'Rubia Gallega') eratorritako muskuluaren ezaugarri biokimikoetan duten eragina aztertzeko. Lortutako emaitzetatik abiatuta, ikusi zen bi kaspasek post mortem denboran zehar izandako jarduera neurri handi batean arrazaren eta garraio/ukuiluratze-baldintzen araberakoa zela, ekoizpen-sistemaren eragina nabarmen txikiagoa izan zen

bitartean. Arrazaren eragina aztertzean, bi kaspasen portaera desberdina ikusi zen denboran zehar 'Rubia Gallega' arrazan 'Asturiana de los Montes' eta 'Retinta' arrazekin alderatuta. Bi kaspasen jarduerak post mortem denboran zehar 'Rubia Gallega' arrazan murrizten joan ziren bitartean, 'Asturiana de los Valles' eta 'Retinta' arrazetan aldiz jarduerak handitzen joan ziren. Gertaera hau, HSPek estres egoeretan betetzen duten paper anti-apoptotikoa kontuan hartuz azal daiteke, hauek kaspasekin elkarreragiten baitute beraien funtzioa blokeatuz, eta beraz, programatutako heriotza zelularraren prozesua mantsotuz. Garraio/ukuiluratze-baldintzen efektuari dagokionez, 'Asturiana de los Valles' eta 'Retinta' arrazek antzera jokatu zuten (patroi gorakorra denborarekin) eta ez zuten alderik izan nahastutako eta nahastu gabeko animalien artean. Aldiz, oso patroi desberdina ikusi zen 'Rubia Gallega' arrazan; nahasi gabeko animaliek beheranzko jarduera-eredua izan zuten denboran zehar eta nahasitako animaliek, berriz, hil ondorengo 8 orduetan lortu zituzten jarduerarik handienak, eta gero gutxitu egin ziren. Animalia ezezagunen nahasketa faktore estresagarrienetako bat dela kontuan hartuta, honek HSPen sintesia eragin lezake eta apoptosia denboran zehar atzeratu. Oro har, 3/7 eta 9 kaspasen jarduera-neurriari esker, arrazak bereizi ahal izan ziren hil ondorengo 2 eta 24 orduetan, bereziki desberdin jokatu zuten arrazak ('Rubia Gallega' vs 'Asturiana de los Valles' eta 'Retinta'). Gainera, animalia maneiua diskriminatzeko gaitasun hobea erakutsi zuen 9 kaspasak hil ondorengo 24 orduetan, 3/7 kaspasak baino. Horrek adieraziko luke erabilgarriagoa dela, adierazle gisa, maneiu faktoreek eragindako aldeak detektatzeko, hala nola arrazan eta garraio/ukuiluratze-baldintzek okelaren azken kalitatean eragin handia baitute.

Hainbat arrazatan 3/7 eta 9 kaspasen jardueran (bereziki hil ondorengo 24 orduetan) maneiu-faktoreek (ekoizpen eta garraio/ukuiluratze-baldintzek) duten eragina aztertu ondoren, hiru arraza horietatik lortutako pH normal eta altudun laginak bereizteko entzima-saiakuntzek duten gaitasuna ebaluatzea planteatu zen ('Asturiana de los Valles', 'Retinta' eta 'Rubia Gallega') eta baita animalia gurutzatuetan. Oro har, arrazak eta azken pHak eragina izan zuten bi kaspasen jardueran. Arrazak 9 kaspasaren jardueran duen eraginari dagokionez eta aurreko esperimentuan ikusi den bezala, azterlan honen emaitzek agerian utzi zuten 'Asturiana de los Valles' eta 'Retinta' arrazek hil osteko 24 orduetan kaspasa honen jarduera handiagoa zutela, 'Rubia Gallega' arrazarekin alderatuta. 3/7 kaspasari dagokionez, berriz ere, alde nabarmenak hauteman ziren arrazen artean, baina desberdintasun horiek aztertutako azken pHaren araberakoak izan ziren. Interesgarria da nabarmentzea gurutzatutako animaliek jarduera-mailarik baxuenak erakutsi zituztela bi kaspasetan aztertutako pH baliotaldea edozein izanda ere, eta horrek zerikusia izan lezake animalia horien estresarekiko erresilientzia handiagoarekin. Haragiaren azken pHaren efektuari dagokionez, pH altudun haragi laginek bi kaspasen jarduera handiagoa erakutsi zuten pH normaldun laginekin alderatuta. Arestian esandako moduan, hau estres-egoeraren araberako HSPen sintesiarekin lotuta egon liteke, horrek hil osteko apoptosi-prozesua atzeratuz. Emaitza horiek berresten dute 3/7 eta 9 kaspasen jarduerak adierazle onak izan daitezkeela hil osteko lehen 24 orduetan azken pH normal eta altudun muskuluak bereizteko. Hala ere, arrazen arteko

ezberdintasunek ikerketan sakontzen jarraitu beharra adierazten dute, arraza bakoitzaren portaerak kaspasen jardueran nola eragin dezakeen ulertzeko.

Ondorioz, doktorego-tesi honetan lortutako emaitzek berresten dute garatutako tresna proteomikoak eta entzima-saiakuntzak baliagarriak direla muskulua haragi bihurtzean gertatzen diren proteoma-aldaketak eta apoptosi-prozesuak zehaztasunez ebaluatzeko, eta animalien maneiu-praktiken eragina ulertzeko. Tresna hauei esker, pH normal eta altudun haragiak bereizi ahal izan dira, eta beraz, behi-aziendan horrelako akatsek duten eragina aurreikus daiteke. Era berean, lortutako emaitzek datu-base zehatzagoak sortzen lagun dezakete, haragiaren ezaugarri biokimikoak, maneiu-praktikak eta haragiaren kalitate-parametroak erlazionatzeko eta aurrerantzean maneiu-praktika jasangarriagoekin ekoiztutako kalitate handiko haragien ekoizpena sustatzeko.

RESUMEN

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

El objetivo general de esta Tesis Doctoral ha sido evaluar los procesos bioquímicos responsables de la calidad final de la carne que tienen lugar en el músculo del ganado vacuno durante la transformación del mismo en carne, en particular, los cambios en el proteoma muscular y la apoptosis *post mortem.* También ha sido el demostrar la utilidad de diferentes estrategias proteómicas y ensayos enzimáticos para poder estudiar de manera precisa estos procesos sobre el metabolismo *post mortem* del músculo que pueden dar lugar a carnes defectuosas como resultado de las prácticas de manejo. La consecución de estos objetivos contribuiría a promover acciones a lo largo de la cadena alimentaria para la producción de carne de vacuno de alta calidad en condiciones sostenibles y que, al mismo tiempo, sea aceptada por el consumidor desde el punto de vista de las prácticas de producción (bienestar animal y conservación medioambiental).

Esta Tesis Doctoral se ha dividido en dos bloques claramente diferenciados en función de la metodología utilizada para estudiar los cambios en el proteoma muscular y los procesos de apoptosis. Por ello, el primer bloque expone lo que ha sido el desarrollo e implementación de diferentes aproximaciones proteómicas para el estudio de las proteínas musculares, mientras que el segundo expone el trabajo desarrollado en la optimización y utilización de ensayos enzimáticos para evaluar los procesos de apoptosis *post mortem* en el músculo bajo diferentes sistemas de manejo.

La proteómica se ha convertido en una herramienta muy útil para identificar biomarcadores proteicos relacionados con la aparición de carnes defectuosas, caracterizadas por tener un pHu más elevado que la carne normal. Por lo tanto, el primer objetivo específico fue optimizar, desarrollar e implementar estrategias proteómicas para un análisis preciso, detallado y completo de las proteínas de la carne de vacuno a tiempos *post mortem* tempranos (24 horas). Para conseguir este primer objetivo, las metodologías analíticas utilizadas fueron evolucionando desde técnicas de cromatografía líquida acoplada a espectrometría de masas (LC-MS) más convencionales, que requerían del empleo de etapas de fraccionamiento previo

de las muestras, hasta estrategias LC-MS más sofisticadas que permitían un análisis directo de las muestras sin necesidad de fraccionamiento previo.

En una primera etapa se desarrolló una estrategia de análisis basada en el pre-fraccionamiento de proteínas en medio líquido (OFFGEL) combinado con la separación de las mismas en gel y posterior identificación por LC-MS para discriminar entre carnes de pHu normal y pHu elevado mediante el estudio separado del sub-proteoma sarcoplásmico y miofibrilar. Aunque las técnicas de separación de proteínas basadas en gel han sido ampliamente utilizadas para estudiar el proteoma de la carne de vacuno, el uso combinado de éstas con un método de pre-fraccionamiento en medio líquido es todavía escaso. Asimismo, la mayoría de enfoques utilizados hasta el momento para el estudio de las carnes de pHu elevado se han basado en el análisis del extracto de proteína total, sin distinguir entre la fracción sarcoplásmica y miofibrilar. En general, este método de separación demostró ser útil para caracterizar e identificar las principales proteínas musculares (tanto las procedentes del extracto sarcoplásmico como del miofibrilar) capaces de discriminar entre carnes con un pHu normal y elevado, y por tanto, predecir la ocurrencia de este tipo de defecto.

A pesar de los buenos resultados obtenidos, las metodologías de separación de proteínas basadas en gel presentan limitaciones importantes que podrían superarse si el enriquecimiento OFFGEL se acoplara directamente al análisis LC-MS. Sin embargo, esta aproximación libre de gel necesitaba implementar un método LC-MS robusto para el procesamiento automatizado de datos, tanto cualitativos como cuantitativos. Por este motivo, en una segunda etapa de esta primera parte de la Tesis Doctoral se planteó el desarrollar una estrategia libre de geles basada en una etapa de pre-fraccionamiento OFFGEL directamente acoplado al análisis LC-MS para el estudio cualitativo/cuantitativo automatizado del sub-proteoma sarcoplásmico. A este respecto, el análisis cualitativo del sub-proteoma sarcoplásmico permitió la identificación inequívoca de 47 proteínas distribuidas a lo largo de las fracciones OFFGEL estudiadas. Por el contrario, la implementación del análisis cuantitativo automatizado de las mismas demostró ser más compleja, ya que requirió de diferentes pasos de validación. En una primera validación se estableció un umbral de confianza para cuantificar de manera automática, precisa y con valores de linealidad y coeficientes de variación satisfactorios los péptidos proteotípicos de abundancia intermedia-alta. Esto permitió la cuantificación de hasta 41 péptidos procedentes de 20 proteínas sarcoplásmicas distribuidas a lo largo de las fracciones OFFGEL estudiadas, siendo los resultados comparables a los obtenidos previamente mediante el análisis cualitativo. Los resultados de este estudio pusieron de manifiesto la eficacia del fraccionamiento OFFGEL como estrategia de enriquecimiento de proteínas y posterior cuantificación de las mismas sin hacer uso de estrategias basadas en gel.

Sin embargo, la etapa de enriquecimiento OFFGEL tampoco está exenta de limitaciones asociadas al procesamiento de la muestra, lo que introduce incertidumbres en la cuantificación de péptidos mediante la metodología LC-MS. Es por tanto un reto analítico importante el implementar estrategias LC-MS dirigidas sensibles, robustas y específicas que permitan

simplificar el análisis de proteínas sin necesidad de realizar etapas de pre-fraccionamiento. Por ello, en una tercera etapa de esta primera parte de la Tesis Doctoral se planteó el desarrollo e implementación de una metodología libre de gel y, además, sin necesidad de pre-fraccionamiento OFFGEL que permitiera el análisis directo de las muestras por LC-MS. Para la consecución de este objetivo se desarrolló una estrategia cuantitativa basada en el seguimiento de reacciones selectivas (del inglés "*Selected Reaction Monitoring*" o SRM) con el objetivo de cuantificar biomarcadores proteicos del sub-proteoma sarcoplásmico capaces de discriminar entre carnes de pH normal y elevado. En general, la estrategia proteómica libre de gel y de fraccionamiento previo basada en el análisis cuantitativo dirigido por SRM demostró ser útil para estudiar diferencias cuantitativas en el proteoma muscular de carne de vacuno. En este sentido, hasta 17 péptidos de 10 proteínas mostraron diferencias significativas entre los dos grupos ensayados (carne de pHu normal y elevado) con valores generalmente más altos en las muestras de pHu elevado. Además, se observó que proteínas previamente caracterizadas y consideradas exclusivas de las carnes de pHu elevado en base a la estrategia basada en la separación en gel, ahora se observaron en varias muestras de carne de pHu normal, aunque en una abundancia muy baja. Esto evidencia la utilidad del análisis SRM en comparación con otras alternativas de cuantificación utilizadas anteriormente. La mayor calidad de los resultados obtenidos por el análisis directo de extractos de proteínas solubles, unido a la mayor eficiencia y menor trabajo manual, demuestra la posibilidad de implementar esta metodología en estudios de investigación de rutina sobre la calidad de la carne.

Independientemente de la aproximación proteómica desarrollada, es interesante resaltar que en las carnes de pHu elevado se observó una mayor abundancia de varias proteínas de choque térmico (del inglés *"Heat Shock Proteins"* o HSPs) que han sido ampliamente estudiadas en relación al estrés. Este interesante hallazgo puede entenderse considerando el proceso de apoptosis o muerte celular programada, en el cual se ha descrito el papel anti-apoptótico que ejercen las HSPs. La activación de este proceso (por medio de la acción de unas enzimas denominadas caspasas) depende de la naturaleza del estímulo inicial, siendo el estrés uno de los factores más relevantes. A pesar de que muchas investigaciones se han centrado en el estudio de la apoptosis y su influencia en el proceso de maduración de la carne, estos trabajos previos no han abordado la posible relación entre los factores *ante mortem* que influyen en el metabolismo animal y la apoptosis *post mortem* del músculo esquelético. Por ello, en el segundo bloque de esta Tesis Doctoral se propusieron el segundo y el tercer objetivo específico que consistieron, por un lado, en la optimización y utilización de un ensayo enzimático para medir la actividad de las caspasas 3/7 en el músculo esquelético, y por otro, en estudiar el efecto de las prácticas de manejo animal sobre el proceso de apoptosis en el músculo a diferentes tiempos *post mortem*.

Para ello, se llevó a cabo la optimización de un ensayo fluorogénico basado en la liberación enzimática de un fluoróforo a partir de un sustrato específico para la medida de la actividad caspasa 3/7 en extractos musculares, caracterizados por presentar una baja concentración de estas enzimas. En general, las condiciones instrumentales y de reacción optimizadas

permitieron la medida de la actividad caspasa 3/7 en un gran número de extractos musculares con una alta reproducibilidad independientemente de su nivel de actividad. Además, en el estudio comparativo del ensayo optimizado con un kit comercial se obtuvieron mejores resultados de linealidad para la metodología optimizada en el laboratorio, especialmente en aquellas muestras que presentaron una actividad más baja.

Por último, se empleó el ensayo optimizado para medir la actividad de las caspasas 3/7 y 9 y estudiar la influencia de diferentes factores de manejo (sistema de producción y condiciones de transporte/estabulación) en las características bioquímicas del músculo en tres razas bovinas españolas (Asturiana de los Valles, Retinta y Rubia Gallega) a tres tiempos *post mortem* (2, 8 y 24 horas). A partir de los resultados obtenidos se observó que la actividad de ambas caspasas a lo largo del tiempo *post mortem* dependía en gran medida de la raza y las condiciones de transporte/estabulación, mientras que el efecto del sistema de producción fue notablemente menor. Al estudiar el efecto de la raza, se observó un comportamiento diferente de ambas caspasas a lo largo del tiempo en la raza Rubia Gallega con respecto a las razas Asturiana de los Valles y Retinta. Mientras que las actividades de ambas caspasas fueron disminuyendo con el tiempo *post mortem* en la raza Rubia Gallega, las actividades de las razas Asturiana de los Valles y Retinta, sin embargo, fueron en aumento. Este hecho podría explicarse considerando el papel anti-apoptótico que ejercen las HSPs bajo situaciones de estrés, las cuales interaccionan con las caspasas bloqueando su función, y por tanto, ralentizando el proceso de muerte celular programada. En relación al efecto de las condiciones de transporte/estabulación, las razas Asturiana de los Valles y Retinta se comportaron de manera similar (patrón creciente con el tiempo) y sin diferencias entre animales mezclados y no mezclados; en cambio, se observó un patrón muy diferente en la raza Rubia Gallega. En esta última raza, los animales no mezclados mostraron un patrón de actividad decreciente a lo largo del tiempo, mientras que los animales mezclados alcanzaron las actividades más altas a las 8 horas *post mortem* y luego disminuyeron. Teniendo en cuenta que la mezcla de animales desconocidos es uno de los factores más estresantes para el animal, este hecho podría inducir la síntesis de HSPs y retrasar la apoptosis a lo largo del tiempo. En general, la medida de actividad de las caspasas 3/7 y 9 permitió discriminar entre razas a las 2 y 24 horas *post mortem*, especialmente entre las razas que se comportaron de manera diferente (Rubia Gallega *vs* Asturiana de los Valles y Retinta). Además, la mejor capacidad discriminante de la actividad de la caspasa 9 en comparación con la de la caspasa 3/7 a las 24 horas *post mortem* indicaría su uso preferible como indicador para detectar diferencias en los factores de manejo como la raza y el transporte/estabulación, los cuales tienen gran influencia en la calidad final de la carne.

Una vez estudiado el efecto de los factores de manejo (sistema de producción y transporte/estabulación) en la actividad de las caspasas 3/7 y 9 (especialmente a las 24 horas *post mortem*) en varias razas, se planteó evaluar la capacidad de los ensayos enzimáticos para discriminar entre muestras normales y de pHu elevado obtenidas de las mismas tres razas (Asturiana de los Valles, Retinta y Rubia Gallega), así como de animales cruzados a las 24 h *post mortem*. En general, la actividad de ambas caspasas se vio afectada por la raza y el pHu.

Con respecto al efecto de la raza sobre la actividad de la caspasa 9, los resultados de este estudio pusieron de manifiesto que las razas Asturiana de los Valles y Retinta presentaban una actividad caspasa 9 mayor a las 24 h *post mortem* en comparación con la raza Rubia Gallega. Sin embargo, con respecto a la caspasa 3/7, nuevamente, se observaron diferencias significativas entre razas, aunque estas diferencias dependieron del grupo de pHu estudiado. Es interesante resaltar que los animales cruzados mostraron los niveles más bajos de actividad, tanto de la caspasa 3/7 como de la 9 independientemente del grupo de pHu estudiado, lo que podría estar relacionado con una mayor resiliencia al estrés por parte de estos animales. Con respecto al efecto del pHu, las muestras de carne de pHu elevado mostraron una mayor actividad de la caspasas 3/7 y 9 en comparación con las muestras de pHu normal, lo cual podría estar asociado con la síntesis de HSPs bajo situaciones de estrés, retrasando de este modo el proceso de apoptosis en el periodo *post mortem*. Estos resultados confirman que la medida de las actividades de las caspasas 3/7 y 9 pueden ser buenos indicadores para discriminar entre carnes de pHu normal y elevado a las 24 horas *post mortem*. Sin embargo, las diferencias entre las razas indican la necesidad de seguir profundizando en la investigación para entender cómo puede afectar el comportamiento de cada raza a la actividad caspasa.

En conclusión, los resultados obtenidos en esta Tesis Doctoral confirman que las herramientas proteómicas y los ensayos enzimáticos desarrollados han demostrado ser útiles para evaluar con precisión los cambios en el proteoma y los procesos de apoptosis que tienen lugar durante la conversión de músculo en carne y que se ven afectados por las prácticas de manejo. Estas herramientas también han permitido discriminar entre carnes de pH normal y elevado, pudiendo predecir así la incidencia de este tipo de defectos en el ganado vacuno. Asimismo, los resultados obtenidos pueden contribuir a la creación de bases de datos más precisas para relacionar las características bioquímicas de la carne, las prácticas de manejo y los parámetros de calidad de la carne final con el objetivo de promover la producción de carnes de alta calidad bajo prácticas de manejo más sostenibles.

SUMMARY

This Ph. D. Thesis has been conducted in collaboration between 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 Biochemistry of Meat and Meat Products Research Group at the Institute of Agrochemistry and Food Technology of the Spanish Reasearch Council (CSIC), which is focused on the study of the biochemical mechanisms responsible for the conversion of muscle to meat.

The main objective of this Ph. D. Thesis was to evaluate the biochemical processes taking place in bovine muscle during the conversion of muscle to meat and responsible for the final meat quality attributes, specifically changes in the muscle proteome and *post mortem* apoptosis processes. It has also been probed the usefulness of different proteomic approaches and enzymatic assays to accurately monitor these processes in the *post mortem* muscle metabolism that can lead to defective meats as a result of handling practices. This understanding would help to promote actions throughout beef production chain to obtain high quality meats under more sustainable production systems and that simultaneously influences consumer´s final decision on product selection in terms of good production practices (*i.e.*, animal welfare and protection of the environment).

This Ph. D. Thesis was divided in two clearly differentiated parts based on the methodology used to study changes in the muscle proteome and apoptosis processes. Therefore, the first part was the development and implementation of different proteomic approaches for the study of muscle proteins, while the second was the optimization and use of enzymatic assays to evaluate *post mortem* apoptosis processes in muscle under different management practices.

Proteomics has become a powerful tool to unveil reliable protein biomarkers related to the appearance of defective meats, characterized by having higher pHu than normal meat. Thus, the first specific objective was to optimise, develop and implement new proteomic approaches to achieve an accurate, detailed and complete analysis of bovine proteins at early *post mortem* times (24 h). To address this objective, the analytical methodologies used evolved from conventional liquid chromatographic coupled to mass spectrometry (LC-MS) techniques, which required the use of previous fractionation steps of the samples, to sophisticated LC-MS strategies that allowed direct analysis of samples without prior fractionation.

First of all, a proteomic strategy based on liquid isoelectric focusing pre-fractionation (OFFGEL) of proteins combined with their separation in gel and subsequent identification by LC-MS was applied to discriminate between normal and high pHu meats studying the sarcoplasmic and myofibrillar sub-proteomes. Although *gel-based* approaches have been

widely used for the study of muscle sub-proteome of beef cattle, their combination with liquid pre-fractionation methods are still limited. Meanwhile, most proteomic approaches addressing high pHu meats in beef cattle are based on the study of total protein extracts and do not differentiate between sarcoplasmic and myofibrillar sub-proteomes. In general, this separation method proved to be useful to characterize and identify the main muscle proteins (both those from sarcoplasmic and myofibrillar extract) capable to discriminate between normal and high pHu meats, and thus, predict the occurrence of this type of defective meats.

Despite the good results obtained, *gel-based* approaches still have significant limitations that could be overcome if OFFGEL enrichment were directly coupled to LC-MS analysis. However, this *gel-free* approach needed the implementation of a robust LC-MS method for automated qualitative and quantitative data processing. For this reason, in a second step, it was proposed the development of a *gel-free* strategy supported by OFFGEL pre-fractionation directly hyphenated to LC-MS analysis addressing automated qualitative/quantitative research in sarcoplasmic proteome. In this regard, the qualitative analysis of the sarcoplasmic sub-proteome allowed the unequivocal identification of 47 proteins distributed throughout the studied OFFGEL fractions. In contrast, the implementation of an automated quantitative analysis was more complex since it required different validation steps. In a first validation, a confidence threshold was established to automatically and accurately quantify intermediate-high abundant proteotypic peptides with satisfactory linearity values and coefficients of variation. This allowed the quantification of up to 41 peptides from 20 sarcoplasmic proteins distributed throughout the studied OFFGEL fractions, being these results comparable to those previously obtained by qualitative analysis. The obtained results showed the efficacy of OFFGEL fractionation as a protein enrichment strategy as well as quantification tool without using *gel-based* strategies.

However, the OFFGEL enrichment still have some constraints associated with sample processing which introduces uncertainties to the quantification of peptides using LC-MS methodology. It was therefore a major analytical challenge to implement sensitive, robust and specific targeted LC-MS strategies allowing a clear protein analysis without using pre-fractionation steps. Therefore, in a third step of this first part, the development and implementation of a *gel-free* methodology was proposed without a OFFGEL pre-fractionation step, which allowed the direct analysis of the samples, by LC-MS. To achieve this, a quantitative strategy based on *selected reaction monitoring* (SRM) was developed aiming at quantifying protein biomarkers of sarcoplasmic sub-proteome capable to discriminate among normal and high pHu meats. Overall, the *gel-free* proteomic strategy without pre-fractionation and based on SRM quantitative analysis proved to be useful to study quantitative differences in the bovine muscle proteome. In this sense, 17 peptides from 10 proteins showed significant differences between the two assayed groups (normal and high pHu meats) with generally higher values in high pHu compared to normal samples. Interestingly, it was observed that proteins previously characterised and considered exclusive of high pHu meats following the *gel-based* approach were now observed in low abundance in some normal pHu samples. This

indicates the usefulness of SRM analysis compared to less specific LC-MS untargeted alternatives previously used. The higher quality of the results obtained by the direct analysis of soluble protein extracts, together with the higher efficiency and less manual work, demonstrates the possibility to implement this methodology in routine meat quality research studies.

Independently of the used methodology, it is worth noting that the study of sarcoplasmic sub-proteome revealed the overabundance of several heat shock proteins (HSPs) in the high pHu group, which have been widely studied in relation to stress. This interesting finding can be understood considering the apoptosis or programmed cell death process, in which the anti-apoptotic role exerted by HSPs has been described. Triggering of this process (through the action of enzymes called caspases) depends on the nature of the initial stimulus, being stress one of the most relevant factors. Even though many researches have focused on the study of apoptosis and its influence on meat tenderization, these studies have not addressed the possible relationship between *ante mortem* factors influencing animal metabolism and *post mortem* apoptosis. Therefore, in the second part of the present Ph.D. Thesis, the second and third specific objectives were (1) the optimization and use of an enzymatic assay to measure the activity of caspases 3/7 in the skeletal muscle; and (2) the study of animal handling practices on the apoptosis process in bovine muscle at different *post mortem* times.

The optimization of a fluorogenic assay based on the enzymatic release of a fluorophore from a specific substrate was carried out for the measurement of caspase 3/7 activity in muscle extracts characterized by a low concentration of these enzymes. Overall, the optimized instrumental and reaction conditions allowed the measurement of caspase 3/7 activity in a large number of muscle samples with high reproducibility independently of their enzymatic activity level. Furthermore, comparison with a commercial kit showed that better linearity results were achieved for the optimised method compared to the commercial kit, especially in samples with low caspase activity.

Finally, caspase 3/7 and 9 activities were measured using the optimized assay and the influence of different management practices (production system and transport/lairage conditions) on the biochemical muscle characteristics of three Spanish local breeds (Asturiana de los Valles, Retinta and Rubia Gallega) at three *post mortem* times (2, 8 and 24 hours) were studied. It was observed that the activity of both caspases over *post mortem* time greatly depended on breed and the transport/lairage conditions, while the effect of the production system was small. Looking at breed effect, different behaviour of both caspases over time was observed in Rubia Gallega breed compared to Asturiana de los Valles and Retinta breeds. While both caspase activities decreased over time in Rubia Gallega breed, they increased in Asturiana de los Valles and Retinta breeds. This fact could be explained considering the anti-apoptotic role exerted by HSPs, which interact with active caspases under stressful conditions hindering their function and, consequently, slowing down the programmed cell death process. In relation to the effect of transport/lairage conditions, Asturiana de los Valles and

Retinta breeds behaved similarly (increasing pattern over time) and with no differences between mixed and non-mixed animals, however, a very different pattern was observed in Rubia Gallega breed. In this breed, non-mixed animals showed a decreasing activity pattern over time, while mixed animals reached the highest caspase activity at 8 h *post mortem* and then decreased. Taking into account that mixing unfamiliar animals prior to slaughter is one of the most stressful events, this fact could induce the synthesis of HSPs that might delay apoptosis process over *post mortem* time. Overall, caspase activity determinations were able to discriminate among breeds at 2 and 24 h *post mortem* times, especially among those breeds behaving differently (Rubia Gallega *vs* Asturina de los Valles and Retinta). Additionally, the better discriminant capacity of caspase 9 activity compared to caspase 3/7 at 24 h *post mortem* would indicate its preferable use as an indicator to detect factors such as breed and transport/lairage influencing meat quality characteristics.

After studying the effect of management practices (production systems and transport/lairage conditions) on the activity of caspases 3/7 and 9 (especially at 24 hours *post mortem*) in several breeds, the discriminant capacity of enzymatic assay was evaluated to distinguish between normal and high pH samples obtained from the same three breeds (Asturiana de los Valles, Retinta and Rubia Gallega), as well as crossbred animals at 24 h *post mortem*. In general, the activity of both caspases was affected by breed and pHu. Regarding the effect of breed on caspase 9 activity, it was demonstrated that Asturiana de los Valles and Retinta breeds showed higher levels at 24 h *post mortem* compared to Rubia Gallega breed. Looking at caspase 3/7 activity, again, differences were observed between breeds, but these differences depended on the studied pHu group. It is of interest to note that crossbred animals showed the lowest caspase 3/7 and 9 levels, which could be related to the increased resilience of crossbred animals to stress susceptibility. Concerning the effect of pHu, meat samples showing high pHu levels also had higher caspase 3/7 and 9 activities compared to normal pHu samples. This could be associated to the synthesis of HSPs under stress situations, which may delay the apoptosis processes over *post mortem* time. These results confirm that measurement of caspase 3/7 and 9 activities can be good indicators to discriminate between meats with normal and high pHu at 24 hours *post mortem*. However, further research is necessary to understand how the behaviour of each breed can affect the activity of studied caspases.

In conclusion, the results obtained in the present Ph. D. Thesis confirm that proteomic tools and enzymatic assays developed probed to be useful to accurately evaluate proteome changes and apoptosis processes taking place during the conversion of muscle to meat as affected by management practices. These tools have also made possible the discrimination between normal and high pHu meats, thus being able to predict the incidence of this type of defect in cattle. Meanwhile, the results obtained can contribute to the creation of more precise databases about the relationship among biochemical characteristics of meat, handling practices and final meat quality traits with the aim to produce high quality meats under more sustainable management practices.

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PROTEIN NOMENCLATURE

ACTA1	Actin
AK1	Adenylate kinase isoenzyme 1
CKM	Creatine kinase
CRYAB	Alpha-crystallin B chain
DES	Desmin
ENO1	Alpha/beta-enolase-3
ENO3	Beta enolase-1
HSPB1	Heat shock protein beta 1
HSPB6	Heat shock protein beta 6
LGALS1	Galectin 1
MB	Myoglobin
MDH1	Malate deshydrogenase
MYH1	Myosin heavy chain 2x
MYH2	Myosin heavy chain 2a
MYL1	Myosin light chain 1/3, skeletal muscle isoform
PGAM2	Phosphoglycerate mutase 2
PGK1	Phosphoglycerate kinase 1
PGM1	Phosphoglucomutase
PKM	Pyruvate kinase
TPI1	Triosephosphate isomerase

ABBREVIATIONS

1-DE	One-dimensional electrophoresis
2-DE	Two-dimensional electrophoresis
Ac-DEVD-AMC	N-Acetyl-Asp-Glu-Val-Asp-7-Amino-4-methyl-coumarin
Ac-LEHD-AMC	N-Acetyl-Leu-Glu-His-Asp-7-Amino-4-methyl-coumarin
ACN	Acetonitrile
ADP	Adenosine diphosphate
AMC	7-Amino-4-methyl-coumarin
ANOVA	Multifactorial analysis of variance
ATP	Adenosine triphosphate
AV	Asturiana de los Valles
Brij 35®	Polyoxyethylene lauryl ether 30%
BSA	Bovine serum albumin
C18	Octadecylsilane column phase
CAL	Calibration batch
CB	Crossbred
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CV	Coefficient of variation
dd-MS²	Data dependent analysis
DFD	*Dark, firm* and *dry*
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
FA	Formic acid
$FADH_2/FAD^+$	Flavin adenine dinucleotide
FR	Fraction
GO	Gene ontology
h	Hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I	Intensive
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IS	Internal standard
HSPs	Heat shock proteins

LC	Liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LSM	Lest square means
LTL	*Longissimus thoracis et lumborum* muscle
MALDI	Matrix-assisted laser desorption ionization
MALDI-TOF/TOF	Matrix-assisted laser desorption ionisation coupled to time of flight analyser
min	Minutes
mo	Months
Mr	Molecular mass
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m*/*z	mass/charge
NADH/NAD$^+$	Nicotinamide adenine dinucleotide
OFFGEL	Liquid isoelectric focusing
PCA	Principal component analysis
PCr	Phosphocreatine
PCs	Principal component
PGI	Protected Geographical Indication
pHu	Ultimate pH
p*I*	Isoelectric point
PMF	Peptide mass fingerprinting
PMSF	Phenylmethylsulfonyl fluoride
PSE	*Pale, soft* and *exudative*
PSS	Pre-slaughter stress
PVDF	Polyvinylidene difluoride
QC	Quality control
RC	Relative change
RE	Retinta
RFU	Relative fluorescence units
RG	Rubia Gallega
RPL	Replicate batch
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	Semi-extensive
SRM/MRM	Selected reaction monitoring/multiple reaction monitoring
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle
TFA	Trifluoroacetic acid

SECTION I

1 STATE OF THE ART

1.1. GENERAL FRAMEWORK

The increase of worldwide population, which is expected to surpass 9 billion in 2050 [1], makes necessary to meet rising food demands to fulfil the high quality protein needs. In this regard, livestock production has greatly contributed in multiple ways to ending hunger and all forms of malnutrition over the world [2].

In order to meet the human protein demand, in the last 50 years animal production policies of main livestock species have been focused on productivity and intensification practices, moving towards breeding animals that are efficient feed converters and fast growing, producing lean meat with minimum production costs. However, these type of practices have a great impact not only in the maintenance of local breeds and final meat quality attributes, but also in the environment [3]. Therefore, the overall aim of livestock production nowadays is to counteract this situation towards more resilient, efficient and sustainable production practices. Adoption of sustainable farming practices is required for an efficient use of natural resources that can be further optimized with the reduction of food waste and losses along supply chains. This strategy has been clearly described in the European Research Area on Sustainable Animal production as well as in the H2020 – Work Program 2014 -2020: Food security, sustainable agriculture and forestry, marine, maritime and inland water research, and the bioeconomy for which EU Commission has announced the continuity for the next 2021-2027. Linked to the beef cattle sector many actions have been taken in order to increase the efficiency in animal production aiming at reducing the environmental impact that contributes significantly to greenhouse emissions [2,3]. According to this, systems should make use of local, multipurpose breeds and/or productive breeds better suited to their living conditions with the aim to enhance animal ability to overcome endemic and to face emerging diseases and nutritional, reproductive or environmental challenges. Progress here will be the key to improve animal resilience traits that are important for a sustainable livestock sector.

Despite a significant increase in world meat consumption over time, bovine meat consumption has experienced a great decrease over the last decade [4]. The influence of several factors (*i.e.*, changing eating habits, sociological and demographic circumstances) as well as the competition with meat from other livestock species with lower prices have changed the trend of beef consumption. In Spain, beef is the third most consumed meat (4.85 kg per capita, 2019) behind poultry and pork [5], being the national bovine meat trade self-sufficient (110.4 %) to supply the Spanish population. According to the Spanish Agriculture, Fisheries and Food Ministry, the national value of beef cattle sector is increasing gradually over the last decade (32.6 %) reaching up 3,010 million euros in 2019, which means 15.3 % and 5.9 % of the final livestock farming and agriculture production, respectively [6]. In Europe, the Spanish beef production is also important, holding the fourth position in terms of economic value, behind France, UK and Germany.

According to MAPA, the number of living bovine animals was 6,706,000 in 2020, being mostly located in Castile & Leon (21.3 % of the total), followed by Galicia (13.9 %), Extremadura (13.6 %) and Catalonia (10.8 %) [6]. It is of interest to note that in Spain coexist 39 different autochthonous breeds, which most of them are characteristic of each region and endangered breeds. For this reason, public administration takes actions to protect them, not only as part of the Spanish animal genetic and cultural heritage, but also because most of them are reared under extensive or semi-extensive conditions that greatly contribute to the environmental sustainability. However, beef cattle selection aiming to produce carcasses better adapted to market demands (*i.e.*, conformation, fatness, precocity) accelerated the introduction of several foreign breeds, and now more than 50% of beef cattle in Spain are crossbred [7].

Most of the cattle farms (144,510) are classified as meat producers (60.4 %) followed by fattening feedlots (14.6 %), milk producers (9.6 %), those registered as mixed or dual farms (4.0%; specialized in meat and milk production) and other types (11.4 %) [6]. Over the last 10 years, the number of registered Spanish beef cattle producing farms experienced a slight decrease (-1 %) although the census of suckler cows increased 9 %. In general, 71.2 % of beef cattle farms have less than 25 cows and 3.9 % have over 100 cows [8].

In Spain, the climatological and orographic differences across regions make feasible the combination of different livestock farming models (2.3 millions of suckler cows over 24 months (mo) of age dedicated to veal production and 0.8 millions of cows dedicated to milk production). Suckler cows are mostly located in north-western

regions where pastures and 'dehesas' are the predominant grazing areas (26.3 % Castile & Leon, 22.8 % Extremadura, 10.7 % Andalucia, 9.6 % Galicia and 7 % Asturias) (**Figure 1.1.**) [6].

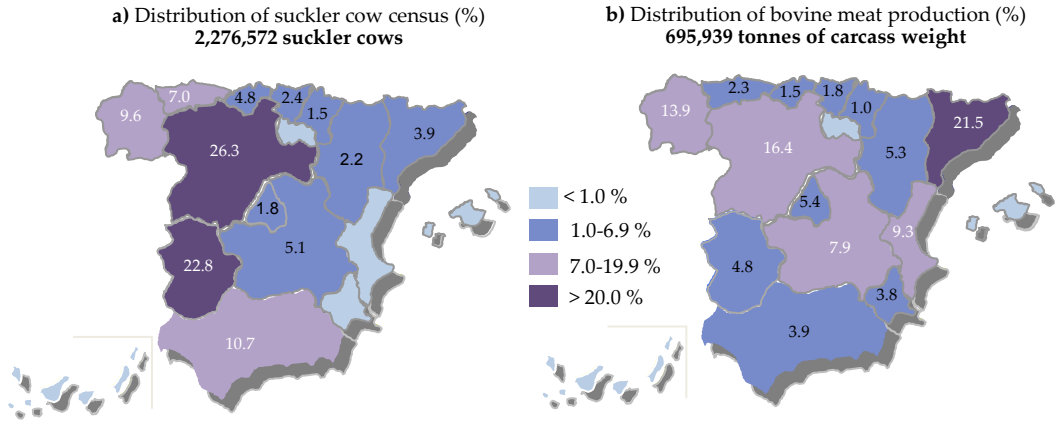

Figure 1.1. National distribution of suckler cows (a) and bovine meat production (b) percentages by regions [6].

The national beef production, in the last decade, has experienced an increase of 16.3 %, reaching a value of 695,939 tonnes in 2019 [6]. As represented in **Figure 1.1.**, the regions where beef suckler cow farms are located do not coincide with the fattening and slaughtering regions. In this sense, Catalonia has become the major bovine meat producer of Spain (21.5 %), followed by Castile & Leon (16.4 %), Galicia (13.9 %), Valencia (9.3 %) and Castile & Mancha (7.9 %).

According to the type of animals slaughtered, most of them are calves between 8 and 12 mo of age (35 %), followed by yearling bulls (30 %). It is of interest to note that the increasing demand for high quality beef resulted in the appearance of several quality labels such as the Protected Geographical Indication (PGI) or Certified Organic Meat. In this regard, Spain has 11 PGIs that represent 5.7 % of the total beef production, being "*Gallega beef*" and "*Asturiana beef*" the most important IGPs in terms of production (53.9 % and 17.3 %, respectively). Regarding Certified Organic Meat (3,521 registered farms), its production has experienced an increase of 13.4 % with respect to 2018, being Andalucia the greatest contributor with 78.2 % of the Organic Meat production [6].

Although these data clearly demonstrate the importance of beef cattle production in Spain, there is a clear drawback related to the lack of uniformity in the quality of the meat reaching the market. Therefore, there is a real need to develop new strategies to study the occurrence and nature of defective bovine meats. This could contribute to implement new policies for reducing losses in the beef production chain, with the

subsequent optimization of the industry. However, due to many factors influencing the muscle to meat conversion, which is a complex process responsible for the development of meat quality traits and the overall acceptance of the meat, the aforementioned goal is very logical but can be complex to achieve.

1.2. BIOCHEMISTRY OF *POST MORTEM* MUSCLE AND ITS RELATION WITH MEAT PRODUCTION

The conversion of muscle to meat is a complex process that involves several physiological, metabolic and structural changes triggered by the animal and its tissues to reinstate an homeostatic control. After slaughter, the blood flow to and from muscles ceases, oxygen supply is cut off and, consequently, energy sources for muscle functioning are depleted. At this point, tissues enter in an ischemic anoxic state, which will affect all biochemical pathways of muscle cells, and it will lead to an adaptation of most metabolic processes (**Figure 1.2.**). The first objective of the cell under such anoxic state is to increase its capacities to produce the energy required for metabolic activities related to cellular functions and muscle contraction. The second step after animal bleeding is to preserve cell functions with an increase in the concentration of several heat shock proteins (HSPs). Due to the inability of cells to maintain reducing conditions, the battle between cell survival and cell death finally turns into cell death processes, (*i.e.*, apoptosis or autophagy) [9-11]. Then, the degradation of myofibrillar proteins by the activation of several proteinase systems, oxidations and other biological processes lead to the well-known meat tenderization process.

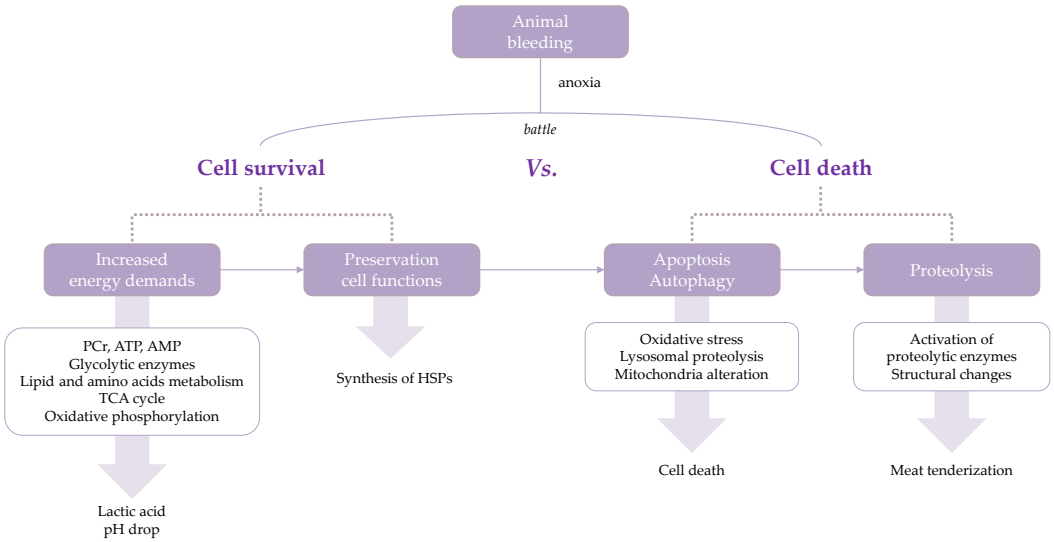

Figure 1.2. Main biochemical pathways involved in the conversion of muscle to meat. (AMP: adenosine monophosphate; ATP: adenosine triphosphate; HSPs: heat shock proteins; PCr: phosphocreatine; TCA cycle: tricarboxylic acid cycle). Addapted from Ouali et al. (2006) [9].

In living organisms, all reactions involved in the oxidation of fuel molecules (amino acids, carbohydrates or fatty acids) enter into tricarboxylic acid (TCA) cycle and oxidative phosphorylation (or electron transport chain) with the main aim of producing energy through the synthesis of mitochondrial ATP [12]. When the blood supply ceases after animal exsanguination there is no source of oxygen and, as a result, mitochondrial ATP synthesis is limited to favour oxidative phosphorylation. At this point, anaerobic metabolism is initiated in order to supply ATP to carry on cellular functions and muscle contraction. Although there are several high-energy compounds that can serve to restore ATP levels (*i.e.*, phosphocreatine (PCr), nucleotide breakdown), anaerobic glycolysis is the predominant pathway for ADP rephosphorylation under anoxic conditions (**Figure 1.3.**).

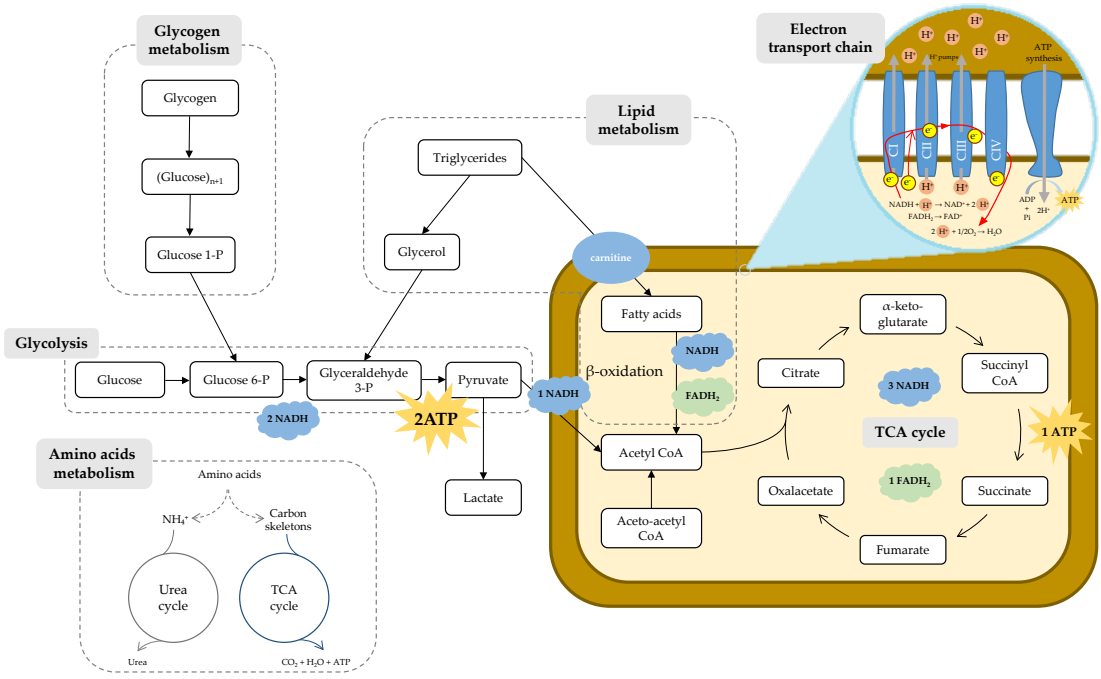

Figure 1.3. Main metabolic pathways involved in energy cell metabolism (ATP: adenosine triphosphate; $FADH_2/FAD^+$: flavin adenine dinucleotide; $NADH/NAD^+$: nicotinamide adenine dinucleotide; TCA cycle: tricarboxylic acid cycle).

Glycolysis is the key metabolic pathway that converts glucose into pyruvate. It can be divided in two phases in which the first one involves five reactions that metabolise six carbon hexoses into glyceraldehyde 3-phosphate. The second set of reactions yield ATP and pyruvate, ending with lactate production. As a primary energy source, glucose is stored as glycogen, essentially in liver and muscles. Glycogenolysis is the sequential liberation of glucose-1-phosphate residues from glycogen to form glucose-6-phosphate that finally enters the glycolytic pathway [12]. (**Figure 1.3.**). Thus,

glycogen stores prior to slaughter and metabolic regulation of glucose/glycogen ratio will have a great influence in *post mortem* muscle metabolism.

When the initial substrate powering glycolysis is limited (*i.e.*, glycogen) by several factors described below (see *section 1.3*), other alternative energy sources (*i.e.*, amino acids, lipids) are needed [12] and other biochemical pathways such as amino acid and lipid metabolism/degradation can be activated even prior to slaughter (**Figure 1.3.**). Fatty acid metabolism, especially fatty acid β-oxidation, is an important pathway for energy production. In this regard, fatty acids are transported to the mitochondria matrix bound to carnitine, where they enter TCA cycle and oxidative phosphorylation for further oxidation. Amino acids are also an important potential source of energy in *post mortem* muscle lacking glucose reserves due to skeletal muscle represents 75 % of the entire free amino acid pool and 25 % of total proteins in the animal [13]. These are degraded to a substrate of TCA cycle or to an intermediate compound that converts into a substrate of this cycle [12].

In essence, TCA cycle, which takes place in the mitochondrial matrix, is the final common pathway for the oxidation of fuel compounds (amino acids, carbohydrates and fatty acids), with the subsequent production of reduction equivalents (NADH and $FADH_2$) that promotes oxidative phosphorylation with the main aim of energy production through the synthesis of mitochondrial ATP. The commented alternative pathways for energy production (amino acid and lipid metabolism/degradation) would be preferably activated before slaughter since mitochondrial ATP production is limited in absence of oxygen in *post mortem* muscle. However, some authors have reported that mitochondria can still consume oxygen in *post mortem* muscle even after 60 days of storage under vacuum packaging [14]. This suggests that oxidative metabolism (*i.e.*, TCA cycle, oxidative phosphorylation) might remain active in *post mortem* muscle in order to maintain cell homeostasis [15,16].

Aforementioned reactions (mainly glycolysis and glycogenolysis) reduce muscle PCr and thus ATP, leading to a concomitant increase of lactate and decrease of muscle pH [17], reaching ultimate pH (pHu) values of 5.5-5.6 at 24 h *post mortem* [18]. The establishment of pHu coincides with the end of glycolysis due to the lack of available substrate (*i.e.*, glycogen) and inactivation of one or more glycolytic enzymes due to the reached acidic conditions [19].

1.3. FACTORS INFLUENCING *POST MORTEM* GLYCOLYSIS RATE

Several factors can influence the *post mortem* glycolysis rate, significantly altering the final pH of meat [20,21] and frequently causing meat quality defects that are associated to an abnormal *post mortem* acidification of the muscle. These factors can be classified in two different groups considering (1) their animal dependence/independence (intrinsic and extrinsic factors) and (2) the timing of their occurrence over the production chain (*ante*, *peri* and *post mortem* factors) (**Figure 1.4.**). Fortunately, most of them can be handled along the production chain; however, those exclusively dependant on the animal (*i.e.*, breed/genotype, muscle fibre type, gender and others) and environmental conditions are beyond control.

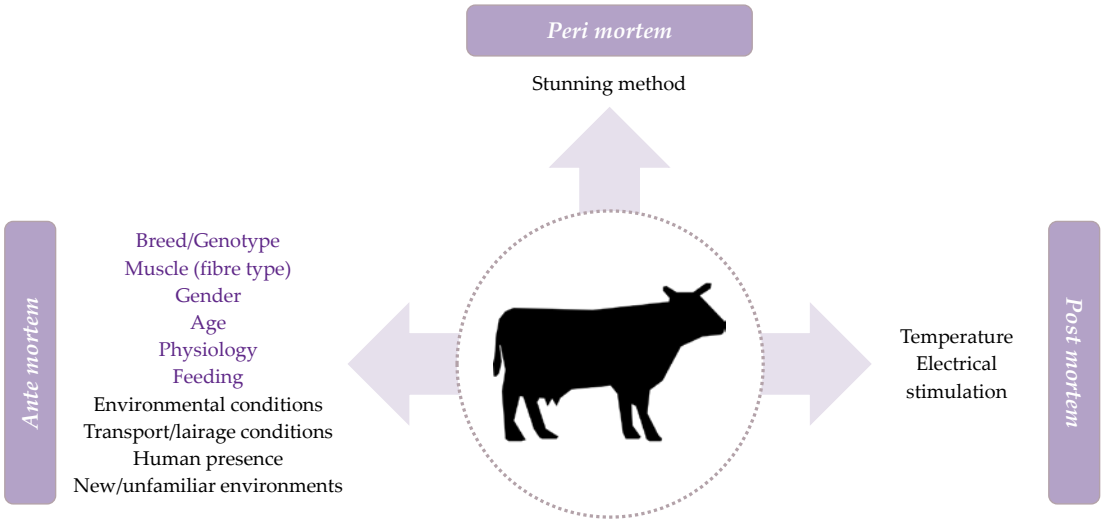

Figure 1.4. Factors influencing glycolysis rate in *post mortem* muscle. Intrinsic factors are represented in purple, while extrinsic factors are in black colour.

Concerning intrinsic factors, breed/genotype, muscle fibre type and gender are the most relevant and they influence majorly before slaughter (*ante mortem*). It is widely described that glycogen concentration varies according to muscle fibre type, influencing the extent of *post mortem* glycolysis and, thus, pHu. Muscles with a high proportion of type I fibres have lower glycogen concentrations and display faster pHu decline rates than those muscles with type II fibres that have higher glycogen concentrations and lower pHu decline rates [22-24]. The genotype also exerts a great influence in muscle fibre type content. In this sense, for example, the presence of myostatin gene mutation in several cattle breeds causes double-muscled or hypertrophied animals [25,26] providing increased type IIB fibre and decreased type IIA fibre proportions, and therefore, influencing the pH decline rate.

Regarding extrinsic factors, these affect all along the production chain (*ante*, *peri* and *post mortem* phases*).* At *post mortem* phase, animal handling procedures such as temperature control and electrical stimulation are key practices to reach a correct meat acidification. Therefore, their use in the meat industry is widespread due to their easy implementation. In this regard, many studies have reported the successful use of temperature control and electrical stimulation to accelerate *post mortem* glycolysis rate and, therefore, minimise the risk of cold-shortening derived from rapid cooling and/or freezing [27,28]. Despite the success of these practices, implementation of such *post mortem* strategies is not enough when pre-slaughter animal handling have not been adequate. Among *ante mortem* factors, the feeding system plays an important role due to the effect of dietary constituents on animal growth and muscle characteristics [29,30]. Beef cattle feeding systems move from completely extensive grazing systems to high-grain intensive production systems, including all the intermediate feeding practices. In general, beef cattle reared under extensive grazing conditions receive imbalanced diets of high-protein content and low-energy pastures, yielding smaller animals than those reared under intensive practices, which receive high-grain diets of high-energy and high-protein contents [29,30]. Moreover, seasonal variations mainly related to temperature fluctuations and undesirable weather events can give low quality pasture and grasslands. As consequence, animal diet has a great impact on live weight, carcass weight, fat thickness, muscle glycogen content and also glycogen depletion rate during transport and slaughter practices. In addition, daily level of physical activity, which can vary between extensive and intensive systems, may also affect *post mortem* glycolysis rate mainly through changes in fibre-type profile [29,31].

During pre-slaughter phase, animals can be exposed to a range of challenging stimuli that may affect the stress status with detrimental effects in animal physiology and thus in the final meat quality characteristics [32]. These stressors perturb animal homeostasis and, as consequence, an adaptive response is activated to restore balance. Although this response is modulated by several animal intrinsic factors (*i.e.*, genetics, gender, age, physiological state), animal housing practices on farm, during transport, and during lairage in the abattoir (*i.e.*, feeding, transport/lairage management, human presence) are highly important to avoid additional stress. Among these factors, transport is one of the most stressful within the meat production chain due to the many handling practices involved before, after and during transport (*i.e.*, noise, vibration, novelty, crowding, climatic factors, loading/unloading, duration of transit, feed/water deprivation) [33]. Moreover, husbandry practices resulting in social mixing or re-grouping of animals may compromise animal welfare, producing

social stress and, thus, increasing aggressiveness and fighting behaviours [34] while feeding system also plays an important role in animal stress taking into account that, directly or indirectly, it influences on animal physiology, social behaviour and responsiveness to stress situations [35]. Despite of aforementioned stressful events, some *peri mortem* practices can reduce animal physical injuries in order to avoid the release of stress hormones that have a direct impact on glycolytic rate. In this line, stunning methods, where animals become unconscious prior to slaughter and bled without causing pain or distress, have been successfully employed to improve animal welfare [36].

The occurrence of pre-slaughter stress (PSS) condition has been widely reported but its study is quite complicated taking into account its multi-factorial origin including both intrinsic and extrinsic factors (**Figure 1.4.**). When animals are handled inadequately before slaughter, they can consume their muscle glycogen reserves as a reaction against acute PSS situations by increasing hormone secretion (*i.e.*, adrenaline, cathecolamines, cortisol). This situation reduces glycogen storages evolving in an abnormal conversion of muscle to meat after slaughter [37]. This phenomenon produces two well-known meat defects; *dark, firm* and *dry* (DFD) meats and *pale, soft* and *exudative* (PSE) meats.

In beef, DFD meats are more common, being also known as dark cutting beef. In these meats, the low *ante mortem* glycogen content in muscle modifies *post mortem* glycolytic metabolism resulting in less lactic acid production and higher pHu (above 6.0) in meats. This results in a poor meat quality characterized by alterations in tenderness and water holding capacity during aging, and fast spoilage by microorganism [38]. On the other hand, PSE meat has been traditionally associated to pig meat production although, recently, this alteration has been also reported in other species such as beef cattle. After slaughter, contrary to DFD meats, the glycolysis rate in PSE meats is very fast, reaching pH values below 5.8 at 45 min *post mortem*. As a consequence of an accelerated *post mortem* glycolysis while carcass temperature is still high, meat turns pale, soft and exudative.

DFD meat is a common problem in many countries, affecting to a wide range of bovine carcasses [39], and provoking important economic losses in the meat industry due to poor quality attributes of the meat. For instance, according to Meat and Livestock Australia, the occurrence DFD meat is around 10 %, representing a loss of about $36 million per year to the Australian beef industry [40]. The USDA Agricultural Marketing Service reported a loss of $165 million due to occurrence of dark-cutting meat in 2000 in USA [41]. Similarly, DFD meats represented a loss of $20

million per year in United Kingdom [42]. In Brazil, 40 % of the total beef carcasses produced from Nellore breed per year are DFD, being an important problem [43]. There is no official information about the incidence of this problem in Spain although available data indicate that 14 % of beef cattle carcasses could be classified as DFD [44].

Australia, one of the countries with the highest incidence of dark cutting beef, considers that meat with a high pHu value (> 5.7) is typically dark in colour and known as DFD [39]. At the same time, FAO reported that pHu values above 6.2 indicate animal stress, injury or sickness prior to slaughter [45]. Therefore, measurement of meat pHu is critical for the industry [41] and, nowadays, it is the most accepted routine practice to control the occurrence of DFD meats. Unfortunately, usefulness of meat pHu assessment is often compromised since high values do not necessarily guarantee the appearance of true DFD meats [46] and, thus, the development of new strategies to unequivocally detect this type of defective meats are demanded.

1.4. DEVELOPMENT OF NEW APPROACHES TO STUDY MEAT BIOCHEMISTRY

Apart from the essential role of energy metabolism during the conversion of muscle to meat, other biochemical pathways can be also involved in this process and, thus, these should be studied to search for other indicators, apart from pHu, that could offer further information of meat biochemical characteristics.

1.4.1 Apoptosis in meat science

The relevance of apoptosis (or cell death process) in meat research was not considered until Ouali et al. [9] proposed cell death as one of the first events triggered during the conversion of muscle to meat. After slaughter, even though the animal tries to preserve cell functions through the increase of HSPs, once the energy is depleted, the battle between cell death and cell survival will turn into the onset of the cell death process [9]. Programmed cell death or apoptosis is a physiological mechanism naturally occurring in living organisms that eliminates excessive, damaged or potentially dangerous cells from an organism without damaging surrounding cells [47]. The main responsible of programmed cell death onset are a group of enzymes named caspases. They are a family of cysteine dependent peptidases that are mainly synthesised as inactive zymogens (pro-caspases). Under internal and/or external

stimuli, they initiate a series of controlled reactions that ultimately lead to cell death. Apoptotic caspases can be further classified according to their point of entry into the cell death pathway as: (1) initiator caspases (caspases 8, 9 10 and 12) and (2) executioner caspases (caspases 3, 6 and 7). The extrinsic pathway (external stimuli) initiates apoptosis through the activation of extracellular receptors that will activate initiator caspase 8. Meanwhile, the intrinsic pathway is activated by internal stimuli (metabolic and/or hypoxic stress) leading to the permeability of the outer mitochondrial membrane that favours cytochrome C release and the formation of the caspase 9 apoptosome complex [48,49], activating executioner caspases 3 and 7 that will proceed with cell dismantling [50] (**Figure 1.5.**).

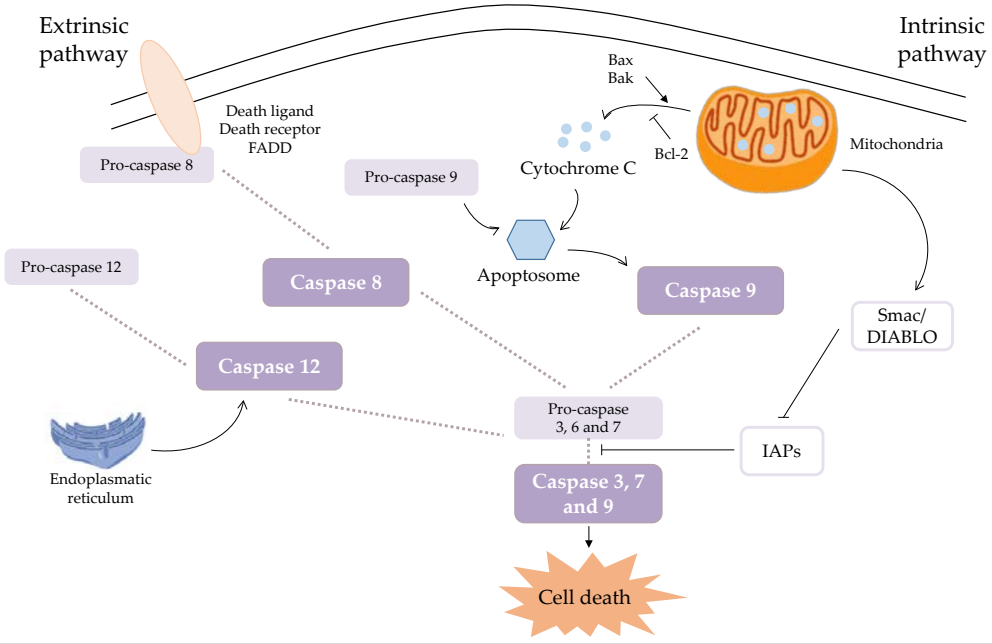

Figure 1.5. Apoptosis pathways triggered by the action of caspases. (FADD: fas receptor-associated death domain; IAPs: inhibitor of apoptosis proteins; Smac/DIABLO: second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low p*I*).

In the last years, major efforts have been conducted to study the metabolic responses associated to intrinsic hypoxia/ischemia that could contribute to *post mortem* proteolysis occurring during meat tenderization. However, these studies did not investigate any possible relationship between *ante mortem* factors influencing animal metabolism and *post mortem* cell death. Concerning the activation of caspases in relation to stress, previous studies have found higher caspase 1 activity in mice when animals were stressed [51] and an activation of caspase 3 in Zebrafish in response to stress induced by heat [52]. In this line, other authors reported higher regulation levels of caspase 3 large subunit in high pHu beef samples compared to normal pHu samples at 24 h *post mortem* [53]. Therefore, the study of apoptosis by measuring

caspase activity stands up as a novel and promising approach to understand the influence of pre-slaughter handling practices on muscle biochemistry and final meat quality.

Most common methods to measure caspase activity are based on assay kits capable to monitor the hydrolysis of specific peptide substrates coupled to cleavable fluorophores, mainly 7-amino-4-methyl-coumarin (AMC) or 7-amino-4-trifluoromethyl-coumarin groups [54]. This methodology has been commonly used in meat research studies for the simultaneous assessment of caspase 3/7 activity [55,56]. However, the low sensitivity of these kits limits their biological application to the characterization of purified enzymes, primary cell cultures or fresh tissue samples with high caspase activity, showing clear limitations in their usefulness for tissues with scarce caspase activity such as skeletal muscle [57]. Alternatively, caspase substrates based on rhodamine dye have been proposed to overcome aforementioned limitations [57], but the high cost of these assays compromises their use when the analysis of high number of samples is required. In this sense, there is a need for an appropriate optimization of current methods in order to accurately determine caspase activity in meat extracts.

1.4.2 Proteomics in meat science

The study of apoptosis applied to meat biochemistry research is a relatively new approach and still deserves further attention even though a remarkable development and implementation of omic technologies (*i.e.*, genomics, transcriptomics, proteomics and metabolomics) have been reported in food-related sciences during the last decade [58].

Skeletal muscle houses a wide variety of proteins that participate in most of the biochemical and structural processes that take place during the conversion of muscle to meat. Most of these proteins are enzymes involved in energy metabolism and structural proteins that play essential roles in the development of meat quality traits and, thus, they are definitely worth studying (**Figure 1.6.**).

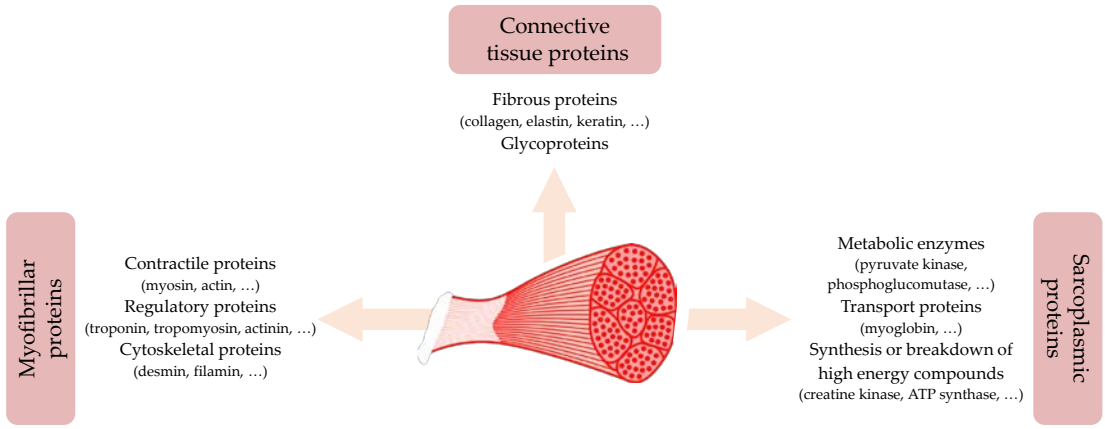

Figure 1.6. Principal groups of proteins integrating the skeletal muscle.

The term "proteome" refers to the total amount of proteins expressed in a cell or tissue at a certain point of time [59], which contains information of gene expression and protein translation [60]. The study of proteome offers many advantages since it is considered the link between the genome and the functional characteristics that take place under different conditions in living organisms. In contrast to genome, proteome is continuously changing with environmental/processing factors, leading to protein interactions or modifications (*i.e.*, degradation, denaturation, oxidation, phosphorylation) [61]. In meat science, proteomics has been used to elucidate the relationship between muscle proteome and several biochemical processes [62,63] involved in meat tenderness [13], color [64,65] and water holding capacity [66,67], with the aim of searching for protein biomarkers related to aforementioned meat quality attributes. However, proteome characterization is complicated. Difficulties arise from its variability over time, sample/sampling complexity and/or low resolution capacity of classical methods. In this sense, over the last 20 years, these problems have been partially solved with the implementation of new methodologies including innovative combinations of different proteomic techniques adapted to meat studies (**Figure 1.7.**) and going from preliminary fractionation to further protein separation, identification and data analysis [68].

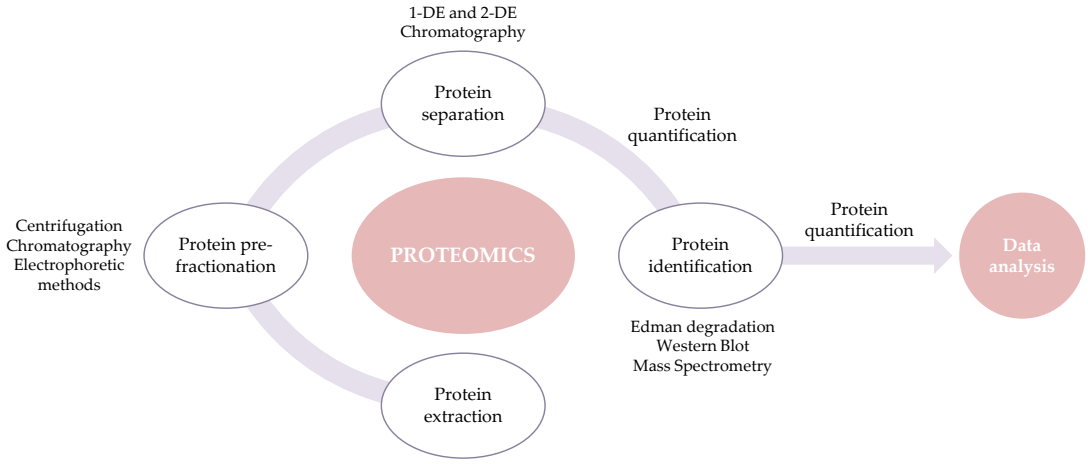

Figure 1.7. Diagram of the main proteomic strategies used in meat proteomic research. (1-DE: one-dimensional electrophoresis; 2-DE: two-dimensional gel electrophoresis).

Two fundamental techniques for protein separation were described in the sixties: (1) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and (2) isoelectric focusing (IEF). These techniques allowed the separation of proteins based on molecular mass (Mr) and surface charge, respectively. The use of one-dimensional electrophoresis (1-DE) under reducing and denaturant conditions of SDS-PAGE had a great impact in meat science since it allowed the study of key enzymatic groups (*i.e.*, calpain, calpastatin) involved in myofibrillar protein degradation [69-71] and, thus, in *post mortem* aging process. However, these studies had important limitations due to their inability to perform large-scale and high throughput protein analysis. Nowadays, most of proteomic analytical strategies are based on at least two dimensional protein separations, using either electrophoretic or chromatographic methods followed by further mass spectrometry (MS) analysis.

One of the most used proteomic separation approach is the two-dimensional gel electrophoresis (2-DE), in which proteins are separated according to their isoelectric point (p*I*) in the first dimension (IEF method) and according to their molecular weight in the second dimension (SDS-PAGE method). In this context, Bouley et al. [72] successfully mapped the bovine skeletal muscle using a combination of 2-DE and MS analysis. They were able to identify 129 protein spots related to metabolism, cell structure/defence and contractile apparatus. Generally, the 2-DE allows the simultaneous separation of 500-2000 individual protein species, and different isoforms or differential post-translational modification can be also distinguished. However, the time and effort required to perform 2-DE separation is considerable and a prior pre-fractionation/enrichment step is usually required in order to simplify sample complexity [61]. In relation to this, a wide range of pre-fractionation methods

have been developed with the aim to separate complex protein mixtures to obtain fractions containing a reduced number of proteins prior to 2-DE and/or MS analysis. One of these emerging technologies is the liquid isoelectric focusing (OFFGEL) that separates proteins according to their p*I*. A multi-compartment chamber integrated by 12 or 24 wells is placed on top of immobilized pH gradient gel (IPG) strips. Solubilised proteins migrate across IPG strips until they respectively reach the pH corresponding to their p*I* [73]. The major advantage of OFFGEL fractionation compared to 2-DE electrophoresis consists in the direct recovery of the fractionated/enriched proteins from the liquid phase and the possibility to further analyse them by either *gel-based* separation steps (SDS-PAGE) or directly by *gel-free* strategies such as liquid chromatography coupled to mass spectrometry (LC-MS). Despite the popularity of *gel-based* strategies, gels have intrinsic disadvantages such as the use of hazardous chemicals (*i.e.*, polyacrylamide), poor representation of less abundant proteins, inaccurate quantitative image analysis and time consumption [74]. In this sense, *gel-free* based approaches like OFFGEL have received more interest as very promising results have been achieved in other areas (*i.e.*, safety, quality and traceability studies of meat and meat products) [75-77]. However, studies proving the advantages of using direct OFFGEL protein fractionation together with LC-MS analysis are still scarce in meat quality research field.

Traditionally, within the framework of protein-oriented investigations, Western Blot and/or automated Edman degradation were the analytical techniques more commonly used for the identification of specific protein species [78]. However, over the past 20 years, significant improvements in MS analysis has opened a new way of characterizing proteins and peptides. Two key innovations have been implemented: (1) the development of different soft ionization methods that convert large, polar and non-volatile compounds into ions in the gas phase, and (2) the subsequent MS analysis capable to separate these ions according to their mass/charge ratio (*m*/*z*). These advances have allowed protein identification following two different strategies: (1) the identification by peptide mass fingerprinting (PMF) for which only the mass list of the intact peptides obtained from protein digestion is required (MS); and (2) the identification by tandem mass spectrometry (MS/MS), in which peptides are fragmented by collision with an inert gas and the generated fragmentation spectrum contains the necessary information to deduce the amino acid sequence of the peptide.

The first strategy, PMF, consists in the comparison of experimental peptide masses obtained by MS analysis and which were previously generated by protein digestion

using a specific enzyme that has a known cleavage pattern (such as trypsin), with theoretical peptide masses of an in-silico-digested protein sequence database. In the case of complex matrices, such as meat, protein identification by PMF is complicated since peptides have been generated from proteolytic processes in which a huge number of peptidases are involved. Therefore, proteins do not present specific proteolytic cleavages of a known enzyme and, thus, the peptide profile is not verifiable with the theoretical masses of protein databases. In addition to this, PMF approach can only be applied to isolated proteins or simple protein mixtures (2-3 proteins). For complex mixtures, peptides derived from digested proteins must be identified by elucidation of their amino acid sequence using MS/MS that is based on coupling two mass analysers separated by a collision chamber (aforementioned second strategy). In this sense, Matrix Assisted Laser-Desorption Ionization (MALDI) coupled to a Time-of-Flight (TOF/TOF) tandem MS has been the most used technique in order to study bovine meat proteome and its relation to energy metabolism [62], pre-slaughter stress [79,80], muscle fibre type [81] and colour stability [64,65]. In contrast, Electro Spray Ionization (ESI) is usually coupled to hybrid instruments such as quadrupole-ionic trap, quadrupole-TOF or triple quadrupole. For instance, when ESI is coupled to MS/MS analysis, this typically yields sequence information of small peptides (3-10 amino acids) from each protein, and these sequence tags allow protein identification through MS/MS by matching experimental data with sequence database information [82].

Another important achievement in proteomics that has been widely used in meat research is the use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (**Figure 1.8.**). Using this approach, an efficient peptide separation and a sensitive identification of a large number of peptides in a single analysis of complex samples can be achieved [15,16,80,83,84].

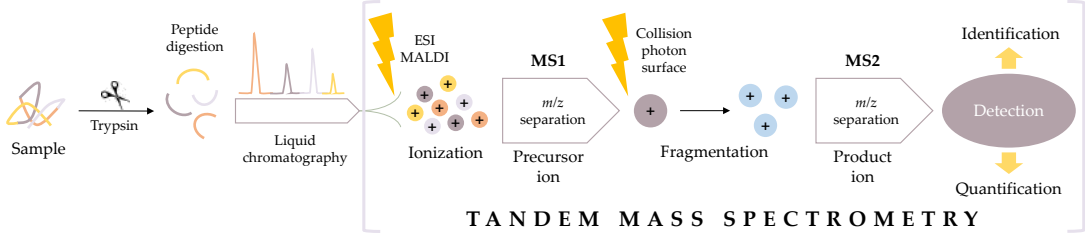

Figure 1.8. General protein analysis workflow using liquid chromatography coupled to tandem mass spectrometry. (ESI: electro spray ionization; MALDI: matrix assisted laser-desorption ionization; MS1/MS2: mass analyser 1 and 2; *m/z*: mass/charge ratio).

In quantitative proteomics, there are different strategies depending on the initial proteomic approach. Protein quantification in *gel-based* approaches using 1-DE or 2-

DE protein separation relies in measuring the intensity of each separated protein band/spot by the use of a densitometric scanner, spectrometric or fluorescence measurements, or computer image analysis. Despite the widespread use of this quantitative method in the study of myofibrillar or sarcoplasmic proteins [16,62,77,79-81], it shows several important limitations such as low specificity, limited dynamic range and band/spot overlapping problems [74].

Considering the shortcomings of *gel-based* separation approaches, there is a need to develop alternative methods based on the extraction of quantitative proteome information from mass spectral data. However, MS is a qualitative rather than a quantitative method, mainly due to unpredictable ionisation abilities of individual peptides [85]. In this line, one of the first methods that allowed a relative quantification of mass spectra were those based on studying differences in protein abundances among different samples. For this purpose, labelling with stable isotopes (*i.e.*, ICAT, SILAC, iTRAQ) is the most precise technique allowing differential chemical tagging of proteins from different samples. However, labelling methods require complex experimental procedures and present additional limitations such as the high cost of the isotopes, the limited number of samples to be analysed and the availability of markers. Concerning meat science research, *label-based* quantification was recently introduced [86] and, due to aforementioned limitations, its utilization in this field is still not popular [87,88].

As an alternative to labelling methods, other *label-free* strategies reported either relative or absolute quantification data [15,84,89]. Even though these are less precise, *label-free* techniques are versatile and affordable since they do not require the use of expensive markers, thus allowing the analysis of a high number of samples [90]. In this sense, targeted LC-MS strategies based on selected reaction monitoring/multiple reaction monitoring (SRM/MRM) full-scan analysis can greatly simplify the routine analysis of known peptides [91] as these can selectively quantify them within complex mixtures. In SRM experiments, normally two mass analysers (MS1 and MS2) are used as static mass filters; the MS1 selects the precursor ions that are fragmented in MS2, providing a particular fragment ion. The specific pair of *m/z* values associated to the selected precursor and fragment ions are referred to as "transition". The main advantages of SRM/MRM approach are affordability of the required MS technology (mainly low-resolution MS, ion-trap and triple-quadrupole devices), and its high sensitivity and specificity while avoiding tedious enrichment/purification steps such as SDS-PAGE gels and OFFGEL fractionations. Even though the implementation of this technology in the food industry for quality evaluations has great potential,

surprisingly, there is a lack of *SRM-based* analytical methodologies applied to the study of *post mortem* meat changes.

Data analysis of MS/MS experiments is essential for both qualitative and quantitative analysis since thousands of mass spectra are generated. As a result, for each detected ion a peak list is obtained containing the observed *m*/*z* values, intensity and charge state. The most common procedure for peptide identification is the use of search engines based on powerful algorithms capable to process huge amounts of spectral data and compare them with existing public protein databases. Some examples of updated search engines are MASCOT, Proteome Discoverer and ProteinPilot. Among protein databases, NCBIprot and UniprotKB/SwissProt are among the most frequently used by scientists. These bioinformatic tools allow the interpretation of tandem mass peptide fragmentation spectra by comparing the experimental results with theoretical peptide fragmentation patterns deducted from the information contained in the databases. Once the peptide sequence is identified, each peptide is assigned to a protein of origin, indicating the confidence threshold of this assignment.

Overall, the complexity of meat biochemistry and its close relation with the final meat quality characteristics makes fundamental the combination of different strategies to advance in the study of the main biochemical pathways underlying the conversion of muscle to meat and to search for reliable meat quality biomarkers linked to these processes. The increase of omics approaches has opened an opportunity to incorporate different strategies that could provide a feasible and integrated solution for these studies. Indeed, the combination of different proteomic approaches, together with the study of cell death processes, may open new ways for the detection and prediction of the different types of meat in relation to quality parameters.

2 HYPOTHESIS AND OBJECTIVES

The conversion of muscle to meat is a complex process that involves several physiological, biochemical and structural changes that have a great influence in the final meat quality traits and overall acceptability of fresh meat. However, its study is hindered by a series of *ante* and *post mortem* factors that can greatly influence this process. Many studies have been conducted searching for biomarkers related to meat quality traits (*i.e.*, tenderness, colour, water holding capacity) but there is still need for further information related to biological changes occurring in bovine muscle. In this sense, the development of new strategies to detect meat quality defects associated to the production factors have become an important challenge within meat quality research.

In this sense, overall objective of the present Ph. D. Thesis was to investigate those biochemical processes taking place in bovine muscle during the first 24 h *post mortem*, such as proteome changes and apoptosis processes, and to study their relationship with defective meat traits depending on management factors influencing the whole production chain. This understanding would help to promote actions contributing to obtain higher quality meats under more sustainable production systems. This will, at the same time, positively influence consumers' purchase decision towards more sustainable and respectful production practices.

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

Objective 1. To develop new proteomic approaches to adequately study relevant changes occurring in skeletal muscle sub-proteome at early *post mortem* times (24 h) and discover new protein biomarkers associated to different ultimate pH meats. This will be addressed by an evolutionary analytical workflow, starting from methods relying on several pre-fractionation steps and standard LC-MS analyses, to more evolved strategies including, simple, high throughput sample preparation steps linked to sophisticated LC-MS analyses. (Publication I to IV).

Objective 2. To optimise an enzymatic assay for the accurate assessment of caspase 3/7 activity in order to study *post mortem* apoptosis processes in skeletal muscle (Publication V).

Objective 3. To apply the developed enzyme assay to study the effect of several management factors (breed, production system and transport/lairage conditions) and meat pHu values during *post mortem* apoptosis processes, and their usefulness to discriminate normal from defective meats (Publication VI and Manuscript I).

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN AND SAMPLE COLLECTION

3.1.1 Experimental Design I: Optimization of *gel-free* and fluorogenic assays

Loin samples (13th rib of the left half carcasses) from three crossbred (CB) beef cattle (n = 3) reared and commercially slaughtered at 18 mo of age in accordance with Directive 2010/63/EU [92] and Council Regulation (EC) Nº 1099/2009 [93], respectively, were collected. At 24 hours (h) *post mortem*, approximately 10 g of *Longissimus thoracis et lumborum* (LTL) muscle samples were taken, epimysium was carefully removed and samples were vacuum packed and stored at -80 °C for further analysis. These samples were destined to (1) the optimization of *gel-free* proteomic approach supported by OFFGEL protein fractionation/enrichment coupled to a LC-MS equipment and (2) the optimization of a fluorogenic assay for caspase activity determinations.

3.1.2 Experimental Design II and III: Study of sarcoplasmic and myofibrillar sub-proteomes

Loin samples (13th rib of the left half carcasses) from eight Asturiana de los Valles (AV) beef cattle (n = 8) and twelve CB animals (n = 12) commercially slaughtered at 14-15 and 14-18 mo of age, respectively, following safety and welfare conditions described above (Experimental Design I) were collected.

From each AV animal, at 2 h *post mortem*, approximately 30 g of LTL muscle sample were taken and a subsample of 10 g was immediately frozen in liquid nitrogen and stored at -80 °C, while the rest of muscle samples (20 g) were transported to the laboratory under refrigeration conditions (4 °C). Later, at 8 and 24 h *post mortem*, subsamples of 10 g were snap-frozen in liquid nitrogen and stored at -80 °C for further analysis. From CB animals, loin samples were collected only at 24 h *post mortem*. Approximately 10 g of LTL muscle sample were frozen in liquid nitrogen and stored at -80 °C for further analysis.

All these meat samples (n = 20) were classified into two different groups according to their pHu values (normal and high pHu). Samples collected from AV breed (n = 8) were normal (pHu < 6.0), while samples collected from CB animals (n = 12), six samples were normal (pHu < 6.0) and the other six were high pHu (> 6.0). All these made a normal set of fourteen samples (n = 14), and a high pHu set of six samples (n = 6) **(Figure 3.1.**). In these sample set (n = 20), the study of sarcoplasmic sub-proteome using a *gel-based* approach was performed (**Figure 3.1., Experimental Design II)**. And the study of myofibrillar (*gel-based*) and sarcoplasmic (*gel-free)* sub-proteomes were performed in the CB sample set (6 normal and 6 high pHu) (**Figure 3.1., Experimental Design III**). The study of sarcoplasmic and myofibrillar sub-proteome was only performed in the sample set collected at 24 h *post mortem*.

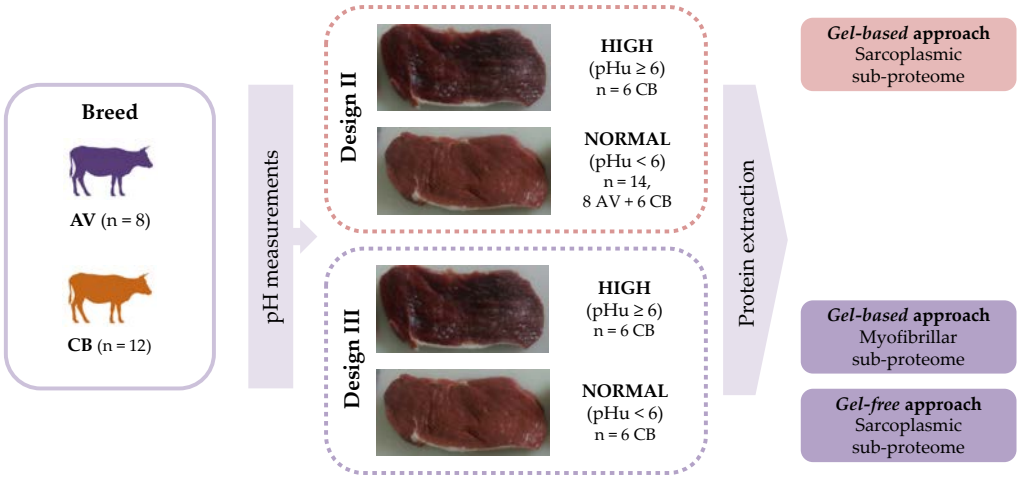

Figure 3.1. Sample description and representation of Experimental Designs II and III. (AV: Asturiana de los Valles; CB: crossbred; pHu: ultimate pH).

3.1.3 Experimental Design IV and V: Study of *post mortem* apoptosis

3.1.3.1 Experimental Design IV

A total of ninety bulls from three Spanish native beef cattle breeds were used: 29 AV, 33 Retinta (RE) and 28 Rubia Gallega (RG) (n = 90). Animals were reared under two different production systems, intensive and semi-extensive, which were typical of each breed and production area (Asturias, Extremadura and Galicia). Within each breed and production system, prior to slaughter, animals were mixed and not mixed with unfamiliar individuals during transport and further lairage at abattoir installations (**Figure 3.2., Experimental Design IV**).

Calves from AV breed suckled their mothers under grazing conditions. They were weaned at 8 mo and supplemented with concentrate *ad libitum* in winter period. At 10-12 mo, calves were finished under one of the two production systems for a period of 100 days until they were slaughtered at 13-15 mo: (a) 18 bulls were managed indoors and fed 8.0 kg/head/day of concentrate and 2.0 kg/head/day of barley straw (intensive), and (b) 11 bulls were managed outdoors, grazing while supplemented with 3.5 kg/head/day of concentrate (semi-extensive). The concentrate was composed of 84 % barley meal, 10 % soya meal, 3 % fat, 3 % minerals, vitamins and oligoelements, and pasture was mainly composed of ryegrass (*Lolium perenne*) and clover (*Trifolium repens*) botanical species.

Calves from RE breed suckled their mothers under grazing conditions until they were weaned at 6 mo. Later, calves were reared under one of the two feeding systems until they were slaughtered at 13-14 mo: (a) 13 bulls were managed indoors and fed 8.0 kg/head/day of concentrate and 2.0 kg/head/day of barley straw (intensive), and (b) 20 bulls were managed outdoors, grazing while supplemented with 4.0 kg/head/day of concentrate (semi-extensive). The concentrate was composed of 51 % corn meal, 34 % barley meal, 8 % soya meal, 3 % fat, 4 % minerals, vitamins and oligoelements, and pasture was mainly composed of ryegrass (*Lolium perenne* and *Lolium rigidum*) and clover (*Trifolium repens*) botanical species.

Management of RG calves was slightly different as they were commercially slaughtered at a younger age (10 mo) compared to other studied breeds. Twelve RG calves were weaned at 1.5 mo and managed indoors with concentrate and barley straw *ad libitum* (intensive). The other 16 RG calves suckled their mothers under grazing conditions of mainly perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) and were supplemented with 3.5 kg/head/day of concentrate until 7 mo. Then, they were finished on concentrates *ad libitum* for 100 days (semi-extensive). The concentrate was composed of 40 % corn meal, 28 % barley meal, 21 % toasted soybean flour, 4 % palmist extraction flour and 2 % palm oil, 2 % calcium carbonate, 2 % fatty acid calcium salt and 1 % sodium chloride.

At the time of slaughter, animals were transported in a lorry with natural ventilation and space allowance of 2.5 m^2 per animal. Prior to slaughter, within each breed and production system, half of the animals were mixed with unfamiliar individuals during transport and further lairage at abattoir installations (intensive: 8 AV, 7 RE and 6 RG; semi-extensive: 5 AV, 12 RE and 6 RG), whereas the other half were not mixed with other animals (intensive: 10 AV, 6 RE and 6 RG; semi-extensive: 6 AV, 8

RE and 10 RG). At arrival to the abattoirs, animals were located in lairage pens and were slaughtered 30 minutes (min) after arrival to avoid additional stress.

Animals were slaughtered in commercial abattoirs of each region following safety and welfare conditions described above (Experimental Design I). The average carcass weights for each breed and production system were 328 ± 51.8 kg for AV, 312 ± 31.0 kg for RE and 237 ± 23.2 kg for RG (intensive) and 247 ± 54.7 kg for AV, 320 ± 17.4 kg for RE and 251 ± 47.5 kg for RG (semi-extensive). Sampling procedure was the same as for AV carcasses described in Experimental Design II. Additionally, the study of *post mortem* apoptosis was performed at different times (2, 8 and 24 h *post mortem*) (**Figure 3.2.**).

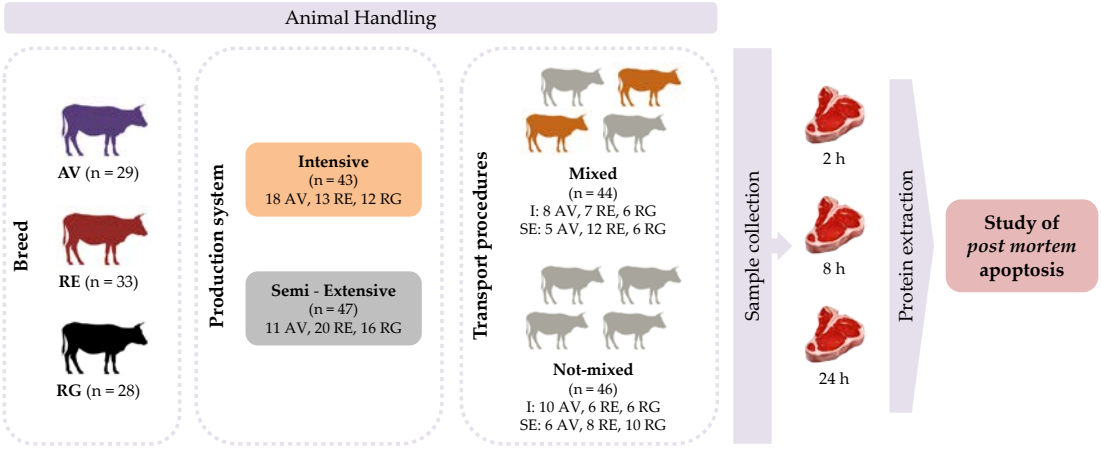

Figure 3.2. Sample description and representation of Experimental Design IV. (AV: Asturiana de los Valles; RE: Retinta; RG: Rubia Gallega; I: intensive; SE: semi-extensive; h: hours).

3.1.3.2 Experimental Design V

As all meat samples obtained under Experimental Design IV were normal, a directed survey towards finding high pHu meat samples was performed in commercial slaughterhouses located near to where animals from Experiment Design IV (AV, RE and RG breeds) were raised. Additionally, the CB sample set (n = 12) collected at 24 h *post mortem* already described in Experiment Design II and III were completed with eight more giving rise a group of twenty samples (n = 20). Thus, a total of sixty three samples were collected from different Spanish local breeds: 18 AV, 20 CB, 14 RE and 11 RG. All these muscle samples (n = 63) were sorted in groups according to their pHu values: (1) high pHu samples over 5.9 (9 AV, 10 CB, 7 RE and 5 RG) and (2) normal samples with pHu values below 5.9 (9 AV, 10 CB, 7 RE and 6 RG) (**Figure 3.3.,**

Experimental Design V). Sampling procedure was the same as for CB carcasses described in Experimental Design II and III.

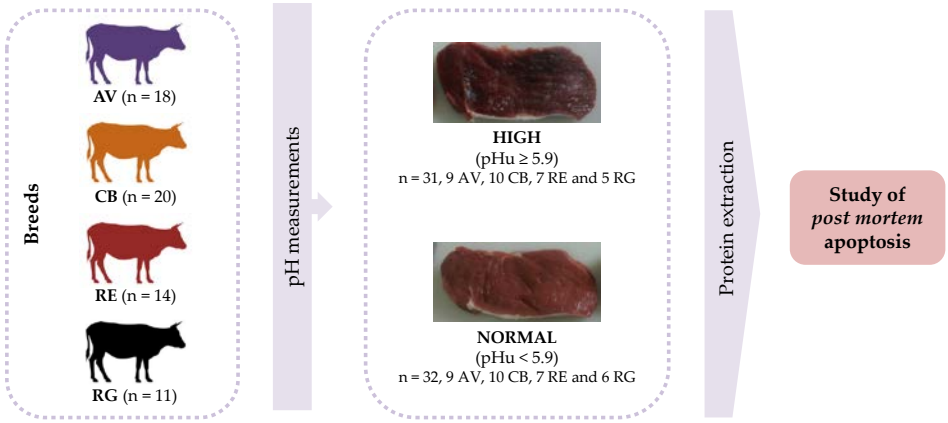

Figure 3.3. Sample description and representation of Experimental Design V. (AV: Asturiana de los Valles; CB: crossbred; RE: Retinta; RG: Rubia Gallega; pHu: ultimate pH).

3.2 METHODOLOGY

3.2.1 Ultimate pH measurements in muscle tissue

At 24 h *post mortem*, measurements of pHu were taken at the 6th rib (LTL muscle) using a penetration electrode (CRISON pH/mV-meter 506, CRISON Instruments SA, Spain). pHu determinations were performed in Experimental Design II, III, IV and V.

3.2.2 Protein extraction from muscle tissue

3.2.2.1 Sarcoplasmic sub-proteome

From each sample group (normal and high pHu meat samples from Experimental Design I, II and III), 0.5 g of meat were homogenised in 4 mL of extraction buffer (10 mM Tris pH 7.6, 1 mM EDTA, 0.25 M sucrose) containing 25 µL of protease inhibitor cocktail (P8340, Sigma-Aldrich Co., St. Louis, MO, USA) using an Ultra-Turrax (Yellow Line Di 25, IKA-Werke GmbH, Staufen, Germany). The homogenate was centrifuged at 20,000 *g* for 20 min at 4 °C using a Beckman Counter centrifuge (Brea, CA, USA) and the obtained supernatant was filtered through a 45 µm PVDF syringe filter (Merck KGaA, Darmstadt, Germany). A subsample of 500 µL was stored at -80

°C for OFFGEL fractionation/enrichment (enriched extracts), while a subsample of 100 µL were cleaned up (OMIX 100 µL C18 pipette tips; Agilent Technologies, Madrid, Spain) and the recovered ACN eluates were completely evaporated (SPD121P Speed Vac concentrator; Thermo Scientific, San Jose, CA, USA equipped with a RVT400 refrigerated vapour trap; Thermo Scientific, San Jose, CA, USA) previous to *in-solution* trypsin digestion for direct protein analysis (un-enriched extracts).

3.2.2.2 Myofibrillar sub-proteome

For the extraction of myofibrillar proteins, 0.5 g of each sample (Experimental Design III) were homogenised in 4 mL of extraction buffer (10 mM Tris pH 7.6, 1 mM EDTA, 0.25 M sucrose) containing 25 µL of protease inhibitor cocktail. The homogenate was centrifuged at 20,000 *g* for 20 min at 4 °C and the retrieved precipitate was washed once in 4 mL of extraction buffer and centrifuged again under the same conditions. The washed precipitate was further dissolved in lysis buffer (10 mM Tris pH 7.6, 7 M urea, 2 M thiourea, 2 % w/v CHAPS, 10 mM DTT) and centrifuged again under the same conditions. The obtained supernatant was filtered through glass wool and stored at -80 °C for further OFFGEL fractionation.

3.2.3 Total protein content of muscle tissue

Muscle sample protein quantification was determined using the Bio-Rad Protein Assay Kit (Hercules, CA, USA) following the Bradford method [94] and using a calibration curve ranging from 0.05 to 0.8 mg/mL of BSA. Samples were 1:20 or 1:10 diluted depending on the final concentration.

3.2.4 Liquid isoelectric focusing of sarcoplasmic and myofibrillar proteins

Sarcoplasmic and myofibrillar protein samples for OFFGEL fractionation were separated according to their p*I* into 12 liquid fractions using 13 cm IPG strips with a specific linear pH gradient (GE healthcare, Sweden), as illustrated in **Figure 3.4**.

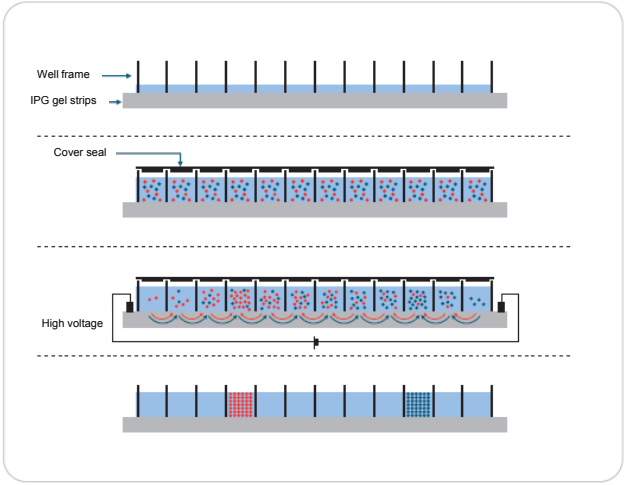

Figure 3.4. Visual diagram of the OFFGEL protein fractionation procedure.

Protein extracts were diluted to a final volume of 2 mL with 1.25X protein OFFGEL stock solution (50 % v/v glycerol, 7 M urea, 2 M thiourea, 65 mM DTT and specific IPG buffer pH, GE healthcare, Sweden) and submitted to protein fractionation/enrichment using an Agilent 3100 OFFGEL fractionator (Agilent Technologies, Waldbronn, Germany) following manufacturer's instructions. IPG strips corresponding to a 12-well frame size covering a specific pH range were fixed onto the tray and rehydrated for 5 min with 1.25X protein OFFGEL stock solution. OFFGEL preparation procedures for specific sarcoplasmic or myofibrillar sub-proteome characteristics are described in **Table 3.1.** A total of 150 µL of each diluted protein extract were loaded into each well. Fractionation was performed at 20 °C with a constant current of 50 µA to reach 20 kVh for about 20 h (depending on sample salt concentration). After focusing, the 12 fractions were separately collected from each well by pipetting. Fractions directed to *gel-based* approach were stored at -80 °C for further analysis by SDS-PAGE, while samples directed to *gel-free* approach were cleaned up and vacuum desiccated and evaporated as described above before *in-solution* trypsin digestion.

Table 3.1. pH gradient values in each of the 12-well fractions obtained during separation of either sarcoplasmic or myofibrillar proteins by OFFGEL fractionation.

	Sarcoplasmic sub-proteome[1]		**Myofibrillar sub-proteome[2]**	
Fraction	*pH in the centre*	*Lower and upper pH*	*pH in the centre*	*Lower and upper pH*
1	3.83	3.59 - 4.08	4.36	4.25 - 4.46
2	4.32	4.08 - 4.56	4.57	4.46 - 4.67
3	4.8	4.56 - 5.05	4.77	4.67 - 4.88
4	5.29	5.05 - 5.53	4.98	4.88 - 5.08
5	5.77	5.53 - 6.02	5.19	5.08 - 5.29
6	6.26	6.02 - 6.50	5.4	5.29 - 5.50
7	6.74	6.50 - 6.98	5.6	5.50 - 5.71
8	7.23	6.98 - 7.47	5.81	5.71 - 5.92
9	7.71	7.47 - 7.95	6.02	5.92 - 6.12
10	8.2	7.95 - 8.44	6.23	6.12 - 6.33
11	8.68	8.44 - 8.92	6.43	6.33 - 6.54
12	9.17	8.92 - 9.41	6.64	6.54 - 6.75

[1] One mg of total protein of each sarcoplasmic extract was diluted to a final volume of 2 mL. IPG buffer and IPG strips corresponding to a specific pH range 3-10 were used.
[2] Two mg of total protein of each myofibrillar extract was diluted to a final volume of 2 mL. IPG buffer and IPG strips corresponding to a specific pH range 4-7 were used.

3.2.5 *Gel-based* approach of sarcoplasmic and myofibrillar proteins

3.2.5.1 SDS-PAGE

The 12 sarcoplasmic/myofibrillar protein fractions obtained from each sample after OFFGEL fractionation were subjected to electrophoretic separation under reducing and denaturant conditions. SDS-PAGE analyses were carried out in duplicate (analytical replica) for each sarcoplasmic/myofibrillar muscle extract (Experimental Design II and III). OFFGEL protein samples were diluted (50:50, v/v) with a buffer solution (0.5 M Tris-HCl pH 6.8, 50 % v/v glycerol, 10 % w/v SDS, 0.2 M DTT and 0.04 % w/v bromophenol blue) and heated at 95 °C for 4 min for protein denaturation. Samples were centrifuged (2,000 *g*, 1 min) before loading onto 1.5 mm x 8 cm x 9 cm 12 % polyacrylamide gels. Each pair of gels (replica) were simultaneously run in a Hoefer Mighty Small II SE260 electrophoresis unit (San Francisco, CA, USA) at a constant current of 50 mA for 2 h. After electrophoresis, gels were fixed into 12 % TCA solution for 1 h and washed twice with bidistilled water for 10 min. Finally, gels were stained overnight with colloidal Blue Coomasie [95], destained with bidistilled

water and stored under refrigeration conditions before image analysis and *in-gel* trypsin digestion.

3.2.5.2 Image analysis of gels

Gel scanning was performed using a LAS-1000 Luminescent Image Analyzer with Intelligent Dark Box II (FUJIFILM, Barcelona, Spain). The intensity of each band was determined using the freeware Gel Analyzer 2010 software (http://www.gelanalyzer.com). Individual band intensities were normalised according to the total band volume per gel. Estimated p*I* and Mr of each band were determined from their position in the OFFGEL fractions and their migration in the polyacrylamide gel, respectively, comparing to commercial standards ranging from 14 to 200 kDa (Cat. No. 161-0317, Bio-Rad, Hercules, CA, USA).

3.2.6 *In-gel* and *in-solution* trypsin digestions

In-gel bands from OFFGEL-SDS-PAGE analysis and *in-solution* samples from enriched OFFGEL protein fractions and un-enriched extracts were digested with trypsin in order to elucidate their protein profiles. In a first step, bands showing quantitatively significant differences in intensity between normal and high pHu sample groups were excised from the gel, cut into pieces and transferred to 0.5 mL Eppendorf tubes. They were then washed twice in 50 mM ammonium bicarbonate for 10 min in continuous agitation and dehydrated twice with 100 % ACN until shrinking and turning opaque. Once the liquid was removed, the remained liquid was evaporated using a Speed Vac concentrator. Both *in-gel* and *in-solution* dried samples were digested overnight at 37 °C with 15 µL of a trypsin solution (Promega, Madison, WI, USA) at a 12.5 µg/mL in 50 mM ammonium bicarbonate (pH 8.5) and with continuous gentle shaking. After incubation, the liquid coming from *in-solution* digested samples was directly evaporated and stored at -80 °C until further resuspension prior to LC-MS analysis. The liquid from *in-gel* digested samples was transferred to clean Eppendorf tubes and the remaining peptides were recovered by washing samples twice with water/ACN (50:50, v/v) acidified with 0.1 % TFA and sonicated for 15 min with Ultrasonic 40000-00301 equipment (Ovan, Barcelona, Spain). This supernatant was combined with the previous one and the resulting liquid was evaporated and stored at -80 °C until further resuspension prior to LC-MS analysis.

3.2.7 Preparation of the internal standard solution and digested samples for LC-MS analysis

3.2.7.1 Preparation of the internal standard solution

A wild almond batch obtained from a local market in Valencia (Spain) was chosen to prepare a protein extract [96]. This extract had a protein content of 10 mg/mL as determined by the Bradford assay kit. The extract was digested with trypsin (3 h, 37 °C and pH 7.8) at an enzyme/substrate ratio of 1.0 % (w/w) and centrifuged at 4,000 *g* for 10 min at 4 °C. Supernatants were 1:46 diluted with aqueous 0.1 % TFA and aliquots stored at -20 °C until they were spiked into the samples and used as internal standard (IS).

3.2.7.2 Resuspension of in-gel digested samples obtained from OFFGEL-SDS-PAGE analysis

In-gel digested samples from the OFFGEL-SDS-PAGE analysis (Experimental Designs II and III) were redissolved in 40 μL of a 0.1 % aqueous TFA solution and transferred into LC-MS vials (**Figure 3.5A.**). This sample set was considered as the NORMAL/HIGH pHu OFFGEL-SDS-PAGE batch.

3.2.7.3 Resuspension of in-solution digested samples from enriched OFFGEL fractions

In-solution digested samples from OFFGEL protein fractions (Experimental Design I) were resuspended in 70 μL of a 0.1 % TFA aqueous solution, and transferred into LC-MS vials (**Figure 3.5B.**). Fifty μL of each OFFGEL fraction were mixed with 15 μL of the IS solution in Eppendorf tubes (total volume of 65 μL), vortexed and centrifuged at 22,000 *g* for 3 min. Then, these were transferred into LC-MS vials and this set was considered as the OFFGEL sarcoplasmic fraction batch. The remaining 20 μL of each fraction were collected together (12 fractions x 20 μL = 240 μL). Seventy μL of this pool were mixed with 20 μL of the IS solution in a separate Eppendorf tube, vortexed, centrifuged (22,000 *g* for 3 min) and transferred into a LC-MS vial, constituting the quality control (QC) batch. The remaining 170 μL of the pooled mix were used to construct the calibration curves of targeted peptides as follows: five different volumes of the mix ranging from 10 to 50 μL (in 10 μL steps) were individually mixed in Eppendorf tubes together with 10 μL of the IS solution and a variable volume of aqueous 0.1 % TFA solution to reach a final volume of 60 μL. Tubes were vortexed,

centrifuged (22,000 *g* for 3 min) and transferred into LC-MS vials constituting the calibration (CAL) batch.

3.2.7.4 Resuspension of in-solution digested samples from un-enriched extracts

In-solution digested samples from un-enriched extracts (Experimental Design III) were resuspended in 80 μL of a 0.1 % TFA aqueous solution and transferred into LC-MS vials (**Figure 3.5C.**). Fifty μL of resuspended un-enriched replicates were spiked with 15 μL of the IS solution, vortexed, centrifuged and transferred into LC-MS vials conforming the normal and high pHu replicate (RPL) batch (NORMAL/HIGH-RPL). The remaining extracts (30 μL) were pooled together but separating normal from high pHu sample sets (6 replicates giving a final volume of 180 μL per set) and the normal and high calibration batch (dNORMAL/dHIGH-CAL) was constructed as previously indicated in section *3.2.7.3* for the CAL batch. All samples were stored at -80 °C until LC-MS analysis.

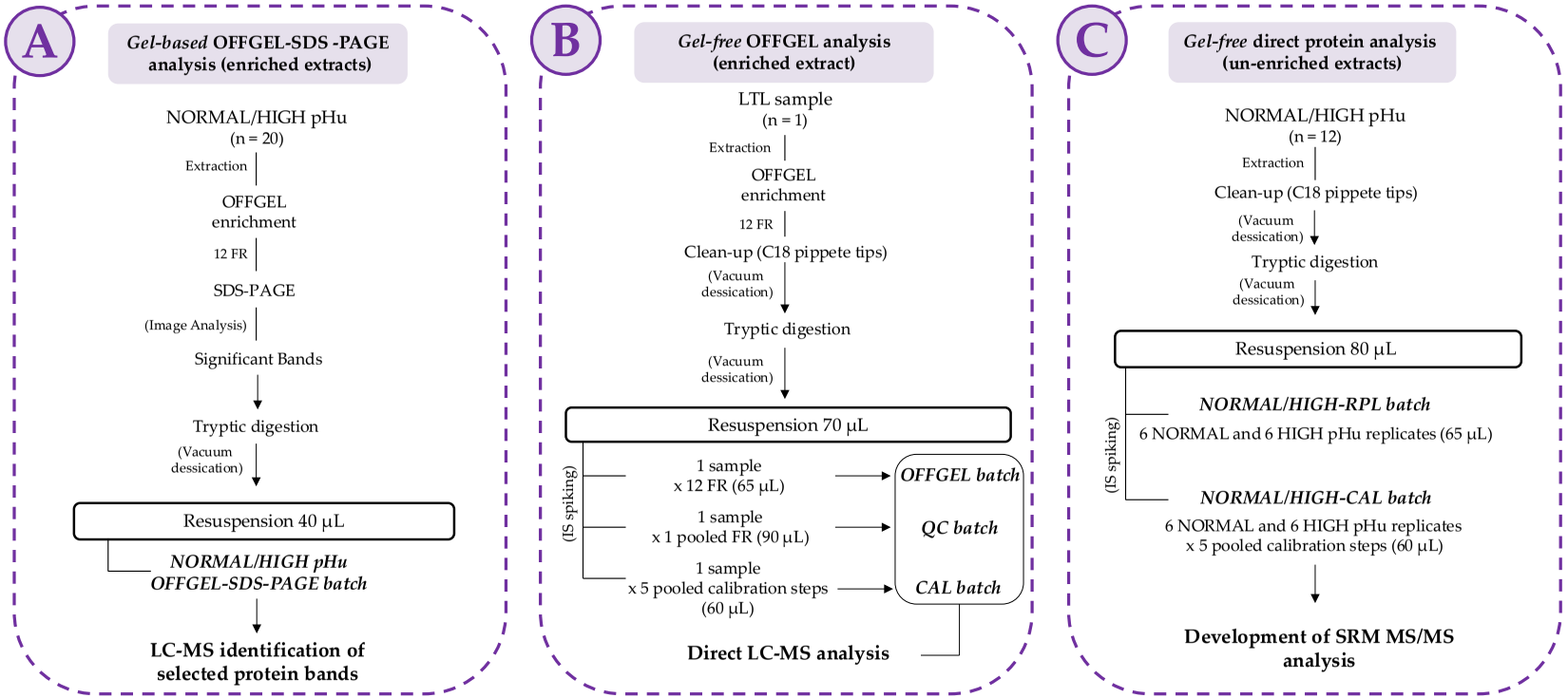

Figure 3.5. Preparation diagrams of (A) *gel-based* analysis of OFFGEL-SDS-PAGE samples; (B) *gel-free* analysis of enriched OFFGEL protein fractions, and (C) *gel-free* direct protein analysis of un-enriched extracts for LC-MS analysis. (FR: Fraction).

3.2.8 Liquid chromatography coupled to mass spectrometry analysis

Chromatographic analyses were carried out in a Thermo Surveyor LC system (equipped with a cooled autosampler, quaternary pump, column oven and vacuum degasser) coupled to a Thermo LCQ Advantage ion-trap MS analyser with an ESI probe operating in positive mode (Thermo Scientific, San Jose, CA, USA). Peptides were separated by a 150 x 0.5 mm, 4 μm particle size reverse-phase C18 Phenomenex Jupiter Proteo column (Phenomenex Inc., Torrance, CA, USA). Mobile phases were solvent A containing 0.1 % FA (v/v) in ultrapure water and solvent B containing 0.1 % FA (v/v) in ACN with the following separation conditions: initially 0 % B, held for 15 min, 0-20 % B in 2 min, held for 4 min, 20-40 % B in 1 min, held for 8 min, 40-100 % B in 1 min, washing with 100 % B for 8 min and column equilibration at initial conditions for 55 min.

Operating parameters of the ion trap detector for untargeted qualitative/quantitative and targeted quantitative peptide analysis merging of full-MS^1 and data dependent MS/MS (dd-MS^2) experiments were the following: ESI operating in positive mode; capillary temperature, 250 °C; spray voltage, 4.0 kV; capillary voltage, 15.0 V. First scan event was full-MS^1 detection in the 400–2000 *m*/*z* range. The second scan event was a dd-MS^2 analysis of the most intense ions with charges from +1 to +4 with the following parameters: repeat count for MS/MS of most intense ion, 3; repeat count duration, 0.5 min; normalised collision induced dissociation energy, 35 %; minimum MS/MS ion intensity threshold, $1x10^5$; exclusion time, 3 min; exclusion mass width, 3 amu; exclusion list, 25 masses including those from spiked almond IS and background noise from a blank injection; number of microscans were 3 and 1 for *in-gel* digested samples and *in-solution* digested samples, respectively; maximum injection time were 200 ms and 300 ms for full-MS^1 and dd-MS^2 experiments, respectively.

Targeted quantitative analysis for hunting potential peptide biomarkers multiplexed full-MS^1 and SRM MS/MS experiments operating under the same general conditions previously described with minimal modifications: number of SRM fragments (transitions) monitored per peptide precursor, 3; number of microscans, 1; maximum injection time, 200 ms.

Control of the platform of MS analysis and manual data processing was performed using Thermo Xcalibur v2.04 software (Thermo Scientific, San Jose, CA, USA).

3.2.9 MS data processing

3.2.9.1 LC-MS identification of selected sarcoplasmic/myofibrillar protein bands from OFFGEL-SDS-PAGE analysis

Identification of significantly different sarcoplasmic/myofibrillar protein bands from *in-gel* digested samples of NORMAL/HIGH pHu OFFGEL-SDS-PAGE batch (Experimental Design II and III) were performed merging full MS^1 and dd-MS^2 analyses. Thus, untargeted qualitative peptide analysis was done by interrogating dd-MS^2 data obtained from tryptic peptides against Uniprot KB v2017_11 (www.uniprot.org) and NCBIprot v20170428 protein databases. To do so, Mascot v2.3 search engine was used applying the following parameters: enzyme, trypsin; no fixed or variable modifications but "Error tolerant" option enabled; mass accuracy was set to 1.2 and 0.8 Da for full MS^1 and dd-MS^2 analyses, respectively; taxonomy restriction parameter, Mammalia. The decoy option was used to estimate the false positive rates by means of False Discovery Rate threshold of 1 %. For identification of protein families obtained from Mascot, only those having a protein score derived from individual ion scores indicating identity or extensive homology ($P \leq 0.05$) were considered as true protein identifications.

3.2.9.2 Direct LC-MS analysis of OFFGEL fractions obtained from sarcoplasmic extracts

Untargeted qualitative and targeted quantitative analysis from *in-solution* digested sample of enriched OFFGEL protein fractions was carried out following the Experimental Design I. In a first approach, untargeted qualitative analysis was performed by interrogating a dd-MS^2 data from OFFGEL batch as described previously in section *3.2.9.1*. Tryptic almond peptides of the IS solution were partially characterized with operating parameters mentioned above but using the 'green plants' option as a taxonomy restriction parameter.

In a second approach, targeted peptide quantification was carried out following the next steps. First of all, full-MS^1 data from OFFGEL batch was manually processed using Xcalibur freeware to study peak symmetry of signals corresponding to peptides that positively covered previously identified proteins by untargeted qualitative analysis. Considered mass tolerance was 0.5 Da and assignments were manually checked by matching experimental dd-MS^2 breakdown patterns with those detailed in Mascot search engine. By this way, an in-house library was built, listing intact

masses and respective retention times of those identified peptides having an acceptable peak shape. The database also listed 16 known/unknown peptides included in the IS used to normalise quantitative results.

It is noteworthy to mention that proteomic research by LC-MS detection is traditionally utilised to perform qualitative analysis, since many signals have poor chromatographic-related features (*i.e.*, peak shape, limited number of scans) compromising quantitative results. Considering the above, a preliminary screening was performed to optimise the scan rate of the MS system to further address the automated quantitative analysis of batches studied (QC, CAL and OFFGEL batches). Freeware MZmine 2 v.2.53 (https://github.com/mzmine/mzmine2/releases) was selected for this purpose since it exhibited important advantages yielding comparable results than those from manual Xcalibur processing. After that, a more detailed validation of automated quantitative analysis of QC data was carried out loading aforementioned in-house library. This validation was performed by comparing manually and automatically processed data obtained by Xcalibur and MZmine freeware, respectively. Then, integrated peak areas of positive assignments (sarcoplasmic peptides and IS) obtained from QC analysis were used to perform the automated quantitative analysis and to test linearity of CAL batch. The final step of the proposed proteomic pipeline was the automated quantification of OFFGEL batch considering these peptides that exhibited acceptable peak areas. Integrated peak areas of positive assignments from QC, CAL and OFFGEL analysis were normalised by the closest IS eluted (analyte/IS ratio) to relatively quantify samples. Differences in the election of IS normalisers among batches can occur since averaged retention times of assignments varied due to different injection volumes assayed.

3.2.9.3 Development of a SRM MS/MS approach for the direct LC-MS analysis of sarcoplasmic extracts (un-enriched samples)

Once automated quantitative analysis was optimised as described above, direct analysis of sample extracts was carried out without any previous enrichment/fractionation step (Experimental Design III). Untargeted qualitative/quantitative peptide analysis from NORMAL/HIGH-RPL batch was carried out processing full-MS^1 and dd-MS^2 data as previously described in sections *3.2.9.1* and *3.2.9.2* with minimal modifications. Briefly, untargeted qualitative analysis was done by interrogating dd-MS^2 data using the Mascot search engine. Untargeted quantitative full-MS^1 analysis was performed using MZmine freeware loading the

aforementioned library. Integrated peak area ratios of signals were normalised by their closest IS eluted to relatively quantify samples.

From the preliminary untargeted full-MS^1 quantitative approach of RPL batch, a list of potential peptide biomarkers was built according to their apparent discrimination features (very high, moderate and none), constituting the SRM library. First, relative quantification of full-MS^1 data using the automated MZmine freeware yielded the initial screening of feasible biomarkers constituting the SRM library. Then, manual inspection of dd-MS^2 results provided by Mascot search engine was also performed to improve the selection of SRM segments in terms of sensitivity and specificity. These databases collected the tentative identification of potential peptide biomarkers (according to their protein of origin) and their intact mass values (*m*/*z* of precursors). Also, these libraries included *m*/*z* of three specific SRM fragments from their breakdown patterns and the windowed SRM scan event segments according to the retention time of peptides provided by the preliminary untargeted full-MS^1 analysis. Since the scan rate of the LC-MS detector used could limit the extension of SRM assay, monitored transitions were windowed across the chromatogram according to the retention time of candidates.

Once the SRM library was built, automated targeted quantitative analysis of SRM data (from NORMAL/HIGH-RPL and -CAL batches) was performed by the freely available Thermo Scientific FreeStyle v.1.6 software (https://thermo.flexnetoperations.com/). Optimization of FreeStyle settings was manually approached by Xcalibur since both freeware share the same algorithm of analysis (ICIS) yielding comparable results. Both, FreeStyle and Xcalibur options, generated one single SRM chromatographic peak corresponding to the automatic summation of signals from all three full-scan MS/MS transitions (integrated peak areas) belonging to the same peptide precursor. Integrated peak area ratios of targeted peptides were normalised by closest IS eluted to relatively quantify SRM data.

3.2.10 Determination of caspase activity

3.2.10.1 Optimization of protein extraction buffer

For caspase extraction, 0.5 g of sample (Experimental Design I) was homogenised with four different extraction buffers (**Table 3.2.**) to optimise the caspase activity

assay. Buffer A was selected since it is a common buffer normally employed to carry out the extraction of sarcoplasmic proteins from muscle tissue [62,97]. The other three buffers are commonly used for caspase extraction from intracellular membranes [98,99]. Once protein extraction buffer was chosen to perform the rest of refinements, homogenates were centrifuged at 20,000 *g* for 20 min at 4 °C. Supernatants were filtered through 0.45 μm PVDF syringe filter and stored at -80 °C until analysed.

Table 3.2. Types of extraction buffers tested for caspase extraction.

	Buffer A	**Buffer B**	**Buffer C**	**Buffer D**
Sucrose (M)	0.25	0.29	0.29	0.29
HEPES, pH 7.5 (mM)	-	10	10	10
Tris, pH 7.6 (mM)	10	-	-	-
EDTA (mM)	1	1	1	1
Brij 35® (%)	-	0.03	0.03	0.03
PMSF (mM)	-	1	1	1
Protease Inhibitor Cocktail (μL)	25	-	-	-
$MgCl_2$ (mM)	-	5	5	-
KCl (mM)	-	10	-	-
Final volume[1] (mL)	4	2	2	2

[1] Final volume to homogenise 0.5 g of muscle.

3.2.10.2 Optimization of experimental conditions for fluorogenic determination of caspase 3/7 activity

The following experimental solutions were used:

Reaction buffer*:* Final composition of selected reaction buffer was 0.29 M sucrose, 0.1 M NaCl, 50 mM HEPES, 0.1 % CHAPS and 1 mM EDTA. Solution was adjusted to pH 7.5 with 50 % HCl.

Substrate and DTT stock solutions: A substrate stock solution was prepared dissolving 5 mg of commercial Ac-DEVD-AMC substrate in 1.48 mL of DMSO with a final concentration of 5 mM and kept at -20 °C until used. Similarly, a DTT stock solution was prepared by dissolving 154 mg of DTT in 20 mL of the reaction buffer and then stored in aliquots at -80 °C until analysed.

Fluorogenic determination of caspase 3/7 activity

The present protocol improved the original fluorescence assay of Stennicke and Salvesen [100] with the aim to enhance sensitivity, flexibility, linearity and affordability of the caspase assay. As aforementioned, different extraction buffers (**Table 3.2.**), together with DTT and enzyme-substrate conditions (**Table 3.3.**) and

main instrumental settings (excitation/emission wavelengths and gain multiplier; **Table 3.4.**) were tested for method optimization.

Table 3.3. Experimental conditions tested to determine caspase 3/7 activity in sarcoplasmic meat extracts.

Volume of sarcoplasmic extract (μL)	Final concentration of DTT[1] in the well (mM)	Final concentration of Ac-DEVD-AMC[2] in the well (mM)	Total volume in each microplate well (μL)	Extract/substrate ratio
30	10	0.025		
30	10	0.05	100	3:5
30	10	0.01		
50	8	0.021		
50	0, 1, 4, 8 and 16	0.042	120	1:1
50	8	0.083		

[1] Volume of DTT in each well was 20 μL.
[2] Volume of Ac-DEVD-AMC in each well was 50 μL.

For the different conditions tested, the caspase enzyme reaction took place in a 96-microtiter-well plate following this procedure: sarcoplasmic extracts (30 or 50 μL) were pre-incubated for 30 min at 37 °C in the presence of DTT as reducing agent (20 μL). Enzyme reaction started with the addition of the substrate solution (50 μL, zero time) and fluorescence intensity was monitored every 2 min over 30 min at 37 °C using a CLARIOstar microplate fluorometer (BMG LABTECH GmbH, Ortenberg, Germany). Analyses were performed in triplicate and results were expressed as mean values of relative fluorescence units (RFU) from the three analytical replicates (fluorescence range from 0 to 260,000 RFU). Additionally, three biological replicates were used and standard deviations and coefficients of variation were calculated in order to validate the assay.

Table 3.4. Instrumental conditions tested to optimise fluorogenic determination of caspase 3/7 activity.

Excitation/emission wavelength (nm)	Gain setting (arbitrary units)
330-20/385-30	600
339-15/439-30	750
350-15/440-20	800
380-15/460-20	1200
380-15/460-20	1500

3.2.10.3 Comparison between the optimised and the commercial fluorogenic determinations of caspase 3/7 activity

Performance of the optimised protocol was compared with a commercial kit previously used by Huang et al. [98] to determine caspase 3/7 activity in meat extracts

based on the same substrate (Ac-DEVD-AMC). Experimental conditions of the commercial kit were the once indicated by the manufacturer. Protocols were compared in terms of sensitivity, linearity and accuracy.

Once the protocol for caspase 3/7 was optimised, samples from Experimental Design IV and V were analysed. In addition to caspase 3/7 activity, the activity of caspase 9 was also determined by using the fluorogenic substrate Ac-LEHD-AMC and following the optimised conditions described for caspase 3/7 activity.

3.3 DATA TREATMENT, STATISTICAL ANALYSIS AND STUDY OF BIOLOGICAL FUNCTION

3.3.1 Data treatment and statistical analysis

Statistical analysis was conducted using SPSS statistical software (version 25.0, New York, USA). Normality and homoscedasticity were checked by Shapiro-Wilk and Leven´s test, respectively.

The univariate non-parametric Mann-Whitney test and parametric *t*-student test were applied to compare the protein band intensities and normalised SRM results, respectively, from NORMAL/HIGH pHu OFFGEL-SDS-PAGE and NORMAL/HIGH-RPL batches. Significant level was declared at $P \leq 0.05$.

Multifactorial analysis of variance (ANOVA) was applied to study the differences on caspase 3/7 and 9 activity depending on the factors studied (breed, production system, transport/lairage conditions, *post mortem* time and pHu). To do so, caspase activity data from Experimental Design IV were subjected to Linear Mixed Model procedures of ANOVA considering the individual animal as a subject and carcass weight as a covariate in the model. Caspase activity (3/7 and 9) data were analysed using breed, production system and transport as fixed factors and *post mortem* time as a repeated measure factor. Production system was nested within breed and transport was nested within production system and breed. For muscle pHu, the effect of *post mortem* time was excluded from the model. The parameters of the model were estimated using the restricted maximum likelihood method and the Huynh-Feldt matrix was selected for the repeated measures covariance structure following the Akaike information criterion. In contrast, caspase activity (3/7 and 9) data from Experimental Design V were subjected to the General Linear Model of ANOVA considering breed and pHu as fixed factors. Additionally, for both models used, least

square means of dependent variables for the levels of fixed factors were compared using the LSD test. Significance level was declared at $P \leq 0.05$.

For multivariate analysis of data, principal component analysis (PCA) as an extraction method, was also performed on a correlation matrix of selected variables using SPSS and XLSTAT 2010.5.02 software (Addinsoft, Paris, France). The two dimensional coordinate systems defined by the first two principal components (PCs) was used to study the distribution of each sample according to the intensity of selected protein bands from NORMAL/HIGH pHu OFFGEL-SDS-PAGE batch. Same PCA strategy was also applied to full-MS^1 data and normalised SRM results of NORMAL/HIGH-RPL batch to create the SRM library and to study sample distribution according to SRM results, respectively. Variables with communality values over 0.6 were included and rotated PCs (extracted by Varimax analysis) explaining over 5 % of the variance were selected.

Relative change (RC) coefficient was estimated in normalised SRM results of NORMAL/HIGH-RPL batch to assess the peptide quantitative change between normal and high pHu groups. This coefficient was calculated using Least Square Means (LSM) values of peptides as follows:

$$RC = dLSM \,/\, |LSM_{High}|$$

in where *dLSM* is a measure of the differential normalised peak areas between the normal and high pHu groups. *RC* coefficient ranges between -1.0 and +1.0 and takes zero value when intensity change is absent.

Comprehensive analysis of proteomics data was performed by Perseus software v.1.6.5.0 (http://www.perseus-framework.org) that allowed hierarchical clustering [101]. Matrix data from normalised SRM results of NORMAL/HIGH-RPL batch was previously transformed (2x log) and missing values were replaced by random numbers that were drawn from a normal distribution. Hierarchical clustering of peptides (rows) and biological replicates (columns) from each sample set (NORMAL/HIGH-RPL) were used to design a heat map representation of the clustered matrix using Euclidean distance for both row and column trees.

3.3.2 Study of biological function

Each of the identified protein showing statistical differences between normal and high pHu sample sets (from either OFFGEL-SDS-PAGE or RPL batch) was classified according to its biological function using Gene Ontology (GO) term from AmiGO

website (http://amigo.geneontology.org/amigo/). Moreover, protein-protein interactions were assessed to elucidate protein networks using STRING v.10.5 freeware software (ELIXIR, Wellcome Genome Campus, Hinxton, Cambridgeshire, UK, https://string-db.org) and open source platform Cytoscape [102]. *Bos taurus* species was used to perform this analysis.

4 RESULTS AND DISCUSSION

Muscle conversion to meat is a complex process and the responsible for the development of meat quality attributes. Despite numerous studies carried out over decades addressing different factors affecting beef quality, the lack of uniformity in quality of meat products reaching the market is still a major problem for the industry. In relation to this, several biochemical processes (*i.e.*, energy metabolism, proteome changes or cell death processes) still remain unclear and deserve further attention, especially in ruminant species. The reason for this is undoubtedly the complexity and multi-factorial factors influencing meat quality characteristics. Although many factors are responsible for the occurrence of defective meats, such as high pHu meats, animals suffering from PSS conditions are the most relevant contributors. It is worldwide recognized that pH values over 6.0 at 24 h *post mortem* are intimately associated to animals that suffered from PSS [45] providing, most of the times, DFD meats [39], and therefore, pHu is a highly important quality control measurement for the meat industry [41] which is routinely employed as a method to detect defective meats. However, meats with high pHu values do not always relate to true DFD defective meats [46] and, therefore, alternative and more reliable approaches are nowadays highly demanded in order to face this problem. In this regard, several approaches have been tested in the present thesis in order to elucidate protein biomarkers that could relate to aforementioned defective meats.

4.1 PROTEOMIC APPROACH

Proteomics has become a powerful tool to unveil reliable protein biomarkers related to defective meats [103,104] and, therefore, capable to predict these type of meats. Most proteomic approaches addressing this type of meats (with high pHu) from beef cattle are based on the study of total protein extracts [79,80]. In this sense, it is known that sarcoplasmic proteins (*i.e.*, stress/defence proteins, metabolic enzymes) are the ones mainly related to stress and metabolic proccesses, although studies reporting changes in myofibrillar proteome are scarce and deserve further investigation [16]. Moreover, the idea to deepen on the study of myofibrillar sub-proteome is supported

by the fact that texture properties are notably altered in high pHu meats in comparison to normal meats.

4.1.1 *Gel-based* approach: OFFGEL-SDS-PAGE-LC-MS analysis

As a starting point, in the present work, changes occurring in both, sarcoplasmic (**Publication I**, see *Appendix I*) and myofibrillar (**Publication II**, see *Appendix II*) sub-proteomes, of normal (< 6.0) and high (> 6.0) pHu bovine meats were studied in order to elucidate main biochemical pathways responsible of pHu that could produce DFD type of meats. Thus, normal and high pHu samples of bovine LTL muscle sampled at early *post mortem* times (24 h) were analysed by *gel-based* proteomic approach consisting on OFFGEL fractionation/enrichment followed by SDS-PAGE and LC-MS analysis. The proposed OFFGEL-SDS-PAGE-LC-MS methodology was previously implemented in our laboratory [77], which has demonstrated to be suitable for the study of muscle sub-proteome. Therefore, it was expected that this proteomic strategy would facilitate the characterization of different proteins as reliable biomarkers that could contribute to the quality assessment of commercialized meat and meat products.

Overall, protein distribution profiles achieved by OFFGEL-SDS-PAGE enrichment were highly reproducible in all retrieved fractions, enabling the study and comparison of sarcoplasmic and myofibrillar proteome between normal and high pHu meat samples. While sarcoplasmic proteins were mostly focused along neutral and basic fractions (fractions 6 to 12, **Figure 4.1A.**), myofibrillar proteins were distributed in all fractions (fractions 1 to 12, **Figure 4.2A.**).

4.1.1.1 Study of the sarcoplasmic sub-proteome

The study of sarcoplasmic sub-proteome revealed that the abundances of five proteins (A to E bands), which were further identified by LC-MS, were significantly different between normal and high pHu meats ($P \leq 0.05$). Abundance of PGM1 (see *Protein Nomenclature*) was significantly higher, whereas ACTA1 was significantly less abundant in normal samples compared to high pHu samples. Interestingly, it was observed that CRYAB, HSPB6 and HSPB1 were only present in high pHu sample group (**Figure 4.1B.**). Additionally, the PCA representation of factor scores of each sample (animal) according to the intensity of each selected band (A to E) clearly discriminated between normal and high pHu sample groups, indicating that several

of these proteins could be considered as potential indicators of high pHu meats (**Figure 4.1C.**). These proteins were, moreover, classified according to their biological role as chaperones/HSPs (CRYAB, HSPB6 and HSPB1), metabolic (PGM1) and structural (ACTA1) proteins. Among those proteins and under normal conditions (pHu < 6.0), ACTA1 was the only one that exhibited a poor aqueous solubility although high pHu values (over 6.0) enhanced its solubility making this protein more easily detectable in the soluble extracts of high pHu samples [81,105,106]. This is in good agreement with the results obtained by Mahmood et al. [16] since they also found actin up-regulation in high pHu meat samples. Keeping in mind that animals that suffered PSS condition have depleted glycogen stores before slaughter, the regulation levels of glycolytic enzymes will be different between normal and high pHu meats as in the case of PGM1. In this sense, other studies found a down-regulation of PGM1 in high pHu samples [84]. However, its activity might also depend on its phosphorylated form, which is also influenced by differences in meat pHu [79]. It is well-known that HSPs play a major role as essential molecular chaperones involved in several mechanisms (*i.e.*, stress response, actin stability, apoptotic signalling pathway) interacting with damaged proteins to preserve their function under stressful conditions [107,108]. Taking into account their protective role, animals suffering from PSS condition may have a great abundance of HSPs to avoid the activation of apoptosis signalling pathways and hindering the activity of caspases. Moreover, the overabundance of HSPs and their relation with actin stability could explain the up-regulation of ACTA1 in high pHu meat samples. In this regard, other authors also observed an increased regulation levels of several HSPs in high pHu meat samples [15].

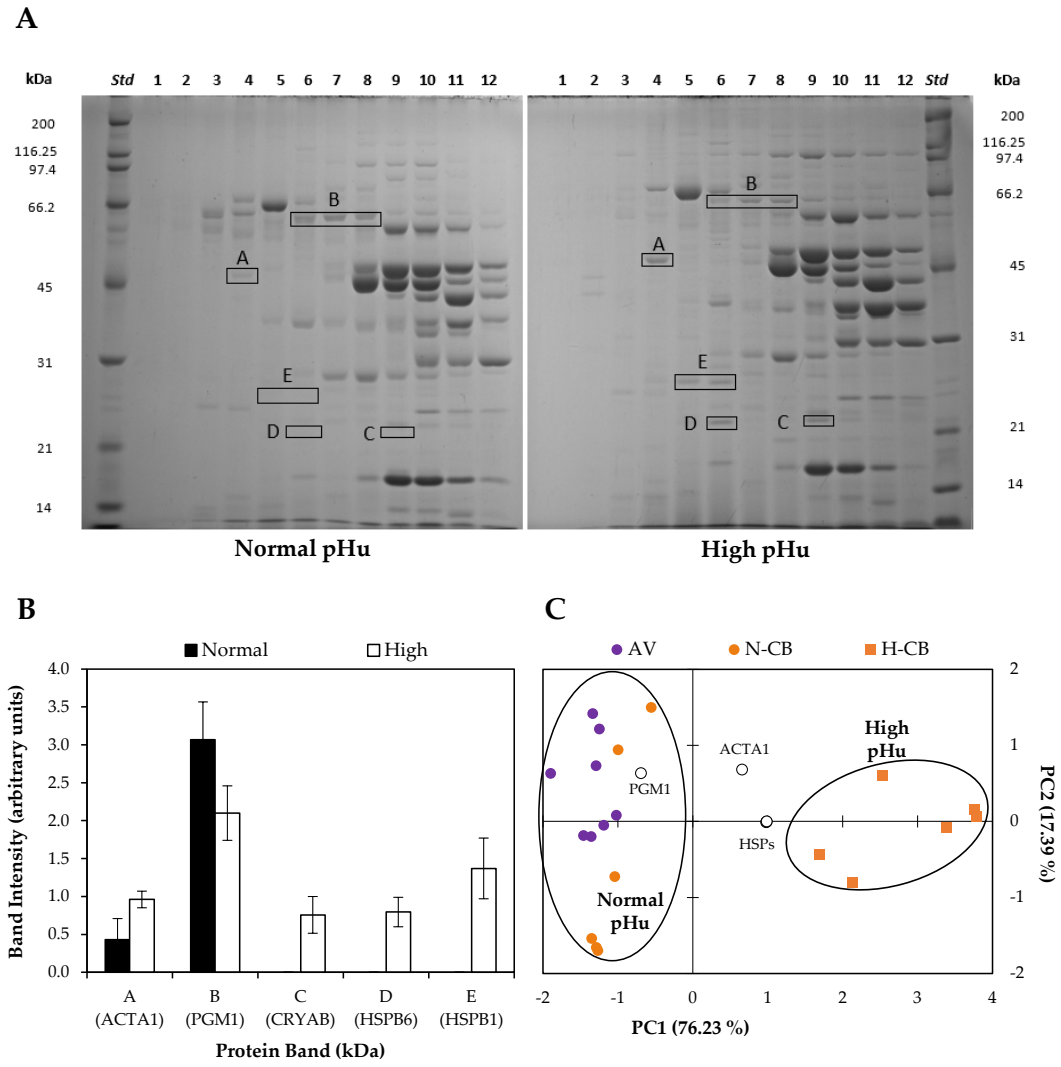

Figure 4.1. (**A**) 12 % SDS-PAGE of sarcoplasmic fractions obtained after OFFGEL enrichment/fractionation (3-10 pH range) from normal and high pHu meat samples. *Std*: Molecular standard. (**B**) Mean values of abundances of the five selected sarcoplasmic protein bands obtained by image analysis in normal (■, n = 14) and high pHu (□, n = 6) meat sample groups. Error bars indicate the standard deviation for each group. (**C**) Animal sample distribution on the two-dimensional coordinate system defined by principal component (PC) 1 and 2. Each point represents an individual sample obtained from a specific breed (● AV: Asturiana de los Valles animals; ● N-CB: normal crossbred animals; ■ H-CB: high pHu crossbred animals) or the proposed PSS protein biomarkers (○).

4.1.1.2 Study of the myofibrillar sub-proteome

Regarding the study of myofibrillar sub-proteome, the abundances of four proteins (A to D bands) were significantly different ($P \leq 0.05$) between normal and high pHu samples (**Figure 4.2B.**). Further LC-MS analysis confirmed the identification of DES, PKM, MYL1 and the coexistence of both, MYH1 and MYH2 isoforms, in band D.

While DES, PKM and MYL1 were more abundant in normal compared to high pHu meat samples, both MYH1 and MYH2 isoforms were up-regulated in high pHu samples. Again, PCA analysis showed that normal and high pHu meat samples were clearly separated through the PC1 according to proposed pHu proteins biomarkers (DES, PKM, MYL1, MYH1 and MYH2) (**Figure 4.2C.**). As previously indicated, myofibrillar fraction is mainly integrated by structural (insoluble) rather than metabolism (soluble) proteins but the presence of soluble proteins in the myofibrillar fraction has been described [106]. This can be explained considering protein solubility changes induced by pH reduction and the high temperature at early *post mortem* stage [109], which can cause protein denaturation and aggregations that precipitate onto myofibrils [110]. Changes in protein solubility could be the reason for the higher abundance of PKM in normal pHu samples, however, it also interesting to note that PKM plays a key role in glycolysis pathway. Considering the glycogen depletion occurring in cattle suffering PSS conditions might be related to reduced concentration and/or activity of muscle glycolytic enzymes during *post mortem* phase [16], this could also explain different abundance of PKM between normal and high pHu meat samples. Due to high meat pHu allows proteolytic enzymes to increase the hydrolysis of myofibrillar proteins such DES or MYL1 [111-113], the abundance of these proteins should be lower in meat samples with high pHu compared to normal meat samples as observed in the present study. In addition, greater oxidative stress and ROS production in animals that suffered PSS condition [15,84] have been associated to higher myofibrillar proteolysis due to calpain and caspase systems, resulting in a greater myofibrillar degradation [114]. Despite these explanations, more research is needed as up and down-regulated myofibrillar proteins have been reported in both types of meat samples, normal and high pHu [79,80]. On the other hand, MYH1 and MYH2 isoforms showed higher abundance in high pHu compared to normal meat samples. The myosin isoforms present in a muscle are related to its fibre type. Taking into account that both isoforms (2a and 2x) are predominant in fast-twitch oxidative-glycolytic fibres (fibres type IIA), obtained results were logical considering that fibre type II have been described to display lower rates of pH decline compared to type I fibres [22]. Moreover, fibre type IIA (rich in 2a isoforms) seems to be positively related to the production of dark cutting (high pHu) meat caused by PSS as previously reported [26,115,116]. Anyway, further investigations are required in order to better understand the role that these isoforms play in animals suffering PSS.

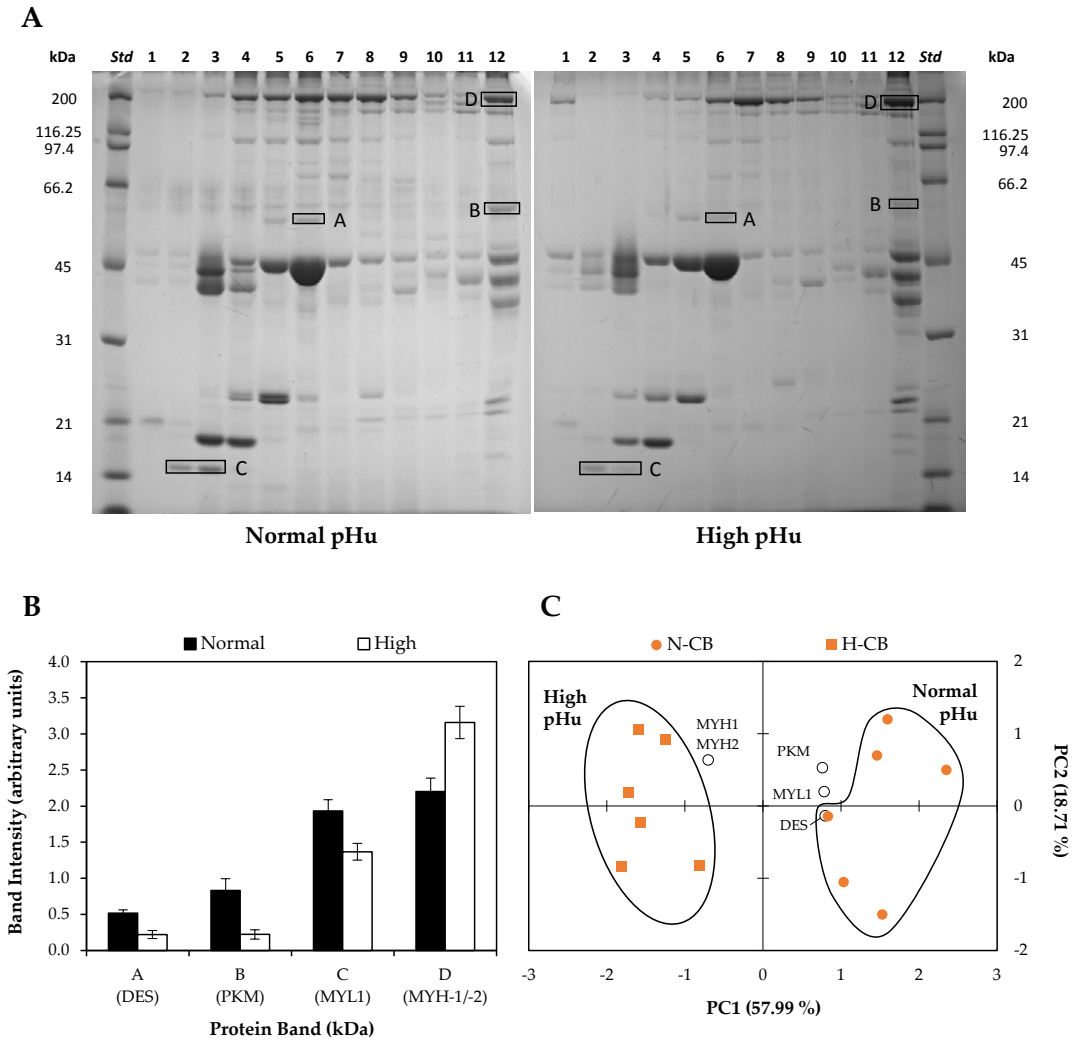

Figure 4.2. (**A**) 12 % SDS-PAGE of myofibrillar fractions obtained after OFFGEL enrichment/fractionation (4-7 pH range) from normal and high pHu meat samples. *Std*: Molecular standard. (**B**) Mean values of abundances of the four selected myofibrillar protein bands obtained by image analysis in normal (■, n = 6) and high pHu (□, n = 6) meat sample groups. Error bars indicate the standard error of the mean for each group. (**C**) Animal sample and protein distribution on the two-dimensional coordinate system defined by principal component (PC) 1 and 2. Each point represents either an individual sample obtained from a crossbred animal (● N-CB: normal pHu samples; ■ H-CB: high pHu samples) or the proposed PSS protein biomarkers (○).

4.1.2 *Gel-free* approach: OFFGEL-LC-MS analysis

As a second step of the present work, several different proteomic approaches were studied in order to evaluate their usefulness to analyse bovine meat sub-proteome. In this regard, it was clearly demonstrated that the use of OFFGEL as a preliminary enrichment/pre-fractionation step followed by SDS-PAGE and LC-MS approach

allowed protein discrimination between normal and high pHu meat samples (see *4.1.1 section*). In general, OFFGEL enrichment has received positive feedbacks as promising results have been achieved when directly coupled to a LC-MS, but the implementation of an automated processing of MS data in quantitative proteomics is still a limitation. Thus, there is a need for a robust, easy, affordable and reliable approach supported by LC-MS for an automated qualitative/quantitative protein research. In this sense, in the present work, the potential use of OFFGEL enrichment coupled to LC-MS as a *gel-free* proteomic approach was considered (**Publication III,** see *Appendix III*) where complementary automated qualitative/quantitative strategies combining full-MS^1 and $ddMS^2$ experiments were evaluated to analyse MS data (**Figure 4.3.**).

Regarding untargeted protein qualitative analysis, dd-MS^2 data from OFFGEL samples were interrogated using Mascot search engine and yielding an unambiguous identification of 47 sarcoplasmic proteins. The majority of identifications belonged to *Bos taurus* species with only six exceptions since used protein databases (UniprotKB/SwissProt and NCBIprot) did not include the sequence information of several proteins from bovine species. On the other hand, the implementation of targeted peptide quantitative approach was more complex due to the involvement of different validation steps (QC, CAL and OFFGEL batches; see *3.2.7.3* and *3.2.9.2 sections*).

First of all, full-MS^1 data from OFFGEL batch corresponding to peptides that positively covered previously identified proteins (untargeted qualitative analysis) was used to build an in-house library. A first validation step demonstrated that peak area integrations of peptides reached by MZmine and Xcalibur were comparable throughout QC samples. Then, a confidence threshold was established to automatically integrate peak areas above $7x10^6$ which was similar to manual processing and yielding reproducible results with acceptable deviations. However, under the present conditions, this methodology was limited to medium- and highly-abundant peptides (> $7x10^6$ peak areas). Next step was to test linearity of those sarcoplasmic peptides that exhibited acceptable results (CV ≤ 25 %) in the QC experiment. In this line, the automated quantitative analysis of CAL batch yielded 18 calibration curves of bovine peptides, finding that achieved linearity was rather good in all cases (r^2 ranged between 0.95 and 0.99). These results from pooled samples (QC and CAL batches) may suggest feasibility of direct quantitative peptide analysis of raw (un-enriched/un-fractionated) protein extracts after the preliminary qualitative approach.

The final step of the proposed study was to perform an automated quantitative analysis of peptides with peak areas above $7x10^6$ from OFFGEL batch. The sum of relative abundances of those peptides belonging to the same parent protein enabled visualization of 20 muscle sarcoplasmic proteins over OFFGEL fractionation. Results were comparable to those regarding the same proteins previously characterized by the untargeted qualitative study. Interestingly, normalized protein abundances generally followed the same trend as Mascot scores, suggesting that higher protein abundances were correlated with higher scores. It is worth noting that the number of proteins found in the OFFGEL batch is lower since many of the peptides identified by Mascot analysis had a poor chromatographic response, thus hindering further quantitative analysis. Moreover, the automated quantitative processing of OFFGEL sample data was also constrained by confidence threshold of integrations, which evidently compromised the determination of peptides with low abundances. On the other hand, the identification of peptides in OFFGEL experiment, previously not observed in the QC study, demonstrated the effectiveness of OFFGEL fractionation as a protein enrichment strategy.

Independently of the different experimental conditions assayed to analyse sample batches, ratios among closest IS must remain constant among studied batches. In general, averaged ratios from different batches were comparable, confirming the reliability of the proposed methodology (in general, average CV ≤ 20% considering 20 replicates; data not shown).

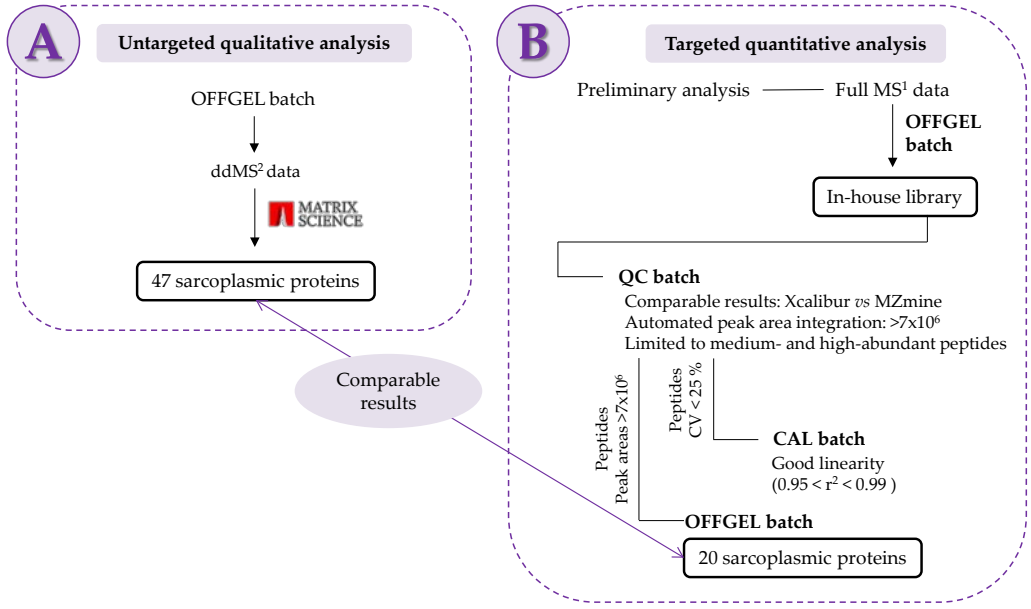

Figure 4.3. Diagram of the methodology proposed in the present study: *gel-free* pipeline combining untargeted qualitative and targeted quantitative analysis of QC, CAL and OFFGEL samples through ddMS² and full-MS¹ approaches. (CV: coefficient of variation; CAL: calibration; QC: quality control).

4.1.3 *Gel-free* approach: direct LC-MS analysis

As a third step of the present work, the bovine sarcoplasmic sub-proteome was studied using an innovative *gel-free* LC-MS proteomic approach with the aim to identify biomarkers capable to differentiate meat samples groups, normal and high pHu. It was demonstrated that OFFGEL fractionation followed by LC-MS analysis allowed simultaneous qualitative/quantitative analysis of bovine proteome (see *section 4.1.2*). However, inclusion of the OFFGEL enrichment step increases the sample processing time while limitations associated to LC-MS methodology introduce uncertainties in peptide quantification, complicating the analysis. In this respect, targeted LC-MS strategies based on SRM analysis can greatly simplify routine analysis of previously characterized peptides because of its high sensitivity and specificity avoiding tedious enrichment/purification steps. Furthermore, the accuracy of SRM determinations overcome uncertainties of low resolution MS. This was tested with un-enriched extracts (NORMAL/HIGH-RPL sample batch) that were analysed by a conventional ion-trap featuring a preliminary full-MS^1 and $ddMS^2$ untargeted approach to search for potential biomarker candidates, followed by a targeted SRM assay to accurately test their discrimination efficiency (**Publication IV,** see *Appendix IV*).

A preliminary untargeted qualitative/quantitative peptide approach merging full-MS^1 and $ddMS^2$ data from NORMAL/HIGH-RPL sample batch was carried out to create a SRM library and tentatively identifying 24 potential peptide biomarkers. It must be highlighted that from the 24 biomarker candidates proposed by preliminary full-MS^1 analysis, only one had an erratic behaviour and was discarded for further SRM research. From this, targeted quantitative analyses of NORMAL/HIGH-RPL and NORMAL/HIGH-CAL batches were performed through the automated analysis of SRM data. Seventeen peptides showed significant differences ($P \leq 0.05$, **Table 4.1.**) between the two groups assayed (NORMAL/HIGH-RPL) with generally higher values in high pHu compared to normal samples, and with positive RC coefficients (**Figure 4.4.**). Only those peptides with statistically non-significant differences in the analysis of replicates had noticeable RC deviations (**Figure 4.4.**).

Table 4.1. Normalised SRM 1 and 2 results (LSM ± SEM, arbitrary units) from peptides obtained from normal (n = 6) and high (n = 6) pHu meat samples (NORMAL/HIGH-RPL batch).

Peptide ID[a]	m/z	RT	Normal	High	SEM	*P*-value[b]	Acronym[c]
Myoglobin-2	629.4	2.2	0.175	0.273	0.025	0.043	**MB**-2
Galectin 1-1	968.5	2.4	0.00626	0.0168	0.00266	0.016	**LGALS1**-1
Actin-1	978.6	2.5	0.0178	0.0665	0.0093	0.006	**ACTA1**-1
Beta-enolase-1	941.7	2.7	0.0992	0.235	0.0287	0.007	**ENO3**-1
Phosphoglycerate kinase 1-1	734.3	2.7	nd	0.0357	0.0047	0.001	**PGK1**-1
Beta-enolase-2	1296.3	3.0	0.00694	0.00815	0.00150	ns	**ENO3**-2
Phosphoglucomutase 1	907.5	3.2	0.0276	0.0469	0.0064	ns	**PGM1**
Triosephosphate isomerase-2	770.5	4.8	nd	0.0285	0.0126	0.001	**TPI1**-2
Creatine kinase M-1	998.3	5.1	0.110	0.238	0.033	0.009	**CKM**-1
alpha/beta-enolase-3	903.2	63.3	0.488	0.673	0.068	ns	**ENO1**
Triosephosphate isomerase-4	802.1	66.1	0.245	0.473	0.051	0.012	**TPI1**-4
Creatine kinase M-2	754.8	66.2	0.0556	0.142	0.0257	0.016	**CKM**-2
Creatine kinase M-3	893.5	66.3	0.0647	0.194	0.0270	0.005	**CKM**-3
Actin-2	1123.5	66.8	0.00603	0.0211	0.00398	0.010	**ACTA1**-2
Phosphoglycerate kinase 1-2	1056.4	67.6	0.351	0.470	0.033	ns	**PGK1**-2
Phosphoglycerate mutase 2	821.6	67.8	0.282	0.377	0.040	ns	**PGAM2**
Phosphoglycerate kinase 1-4	1012.2	68.4	0.0604	0.102	0.0097	0.010	**PGK1**-4
Adenylate kinase isoenzyme 1	1031.5	69.0	0.0333	0.0406	0.0023	ns	**AK1**
Heat shock prot b6-1	850.7	71.5	nq	0.187	0.071	0.001	**HSPB6**-1
Heat shock prot b6-2	1275.5	71.9	nd	0.0688	0.0240	0.001	**HSPB6**-2
Galectin 1-2	863.2	72.1	nd	0.00608	0.00224	0.001	**LGALS1**-2
Malate deshydrogenase-2	893.0	72.3	nd	0.00259	0.00045	0.001	**MDH1**
Heat shock prot b1	1597.5	73.2	nd	0.0140	0.0041	0.001	**HSPB1**

LSM, least square mean, SEM, standard error of the mean; nd, not detected; nq, not quantified; ns, not significant.
[a] Peptide identification.
[b] Statistical differences were declared at $P \leq 0.05$.
[c] Entire acronym corresponds to the peptide name and the part highlighted **in bold** refers to the corresponding parent protein.

Interestingly, 6 peptides were only found in studied high pHu replicates and, from them, only HSPB1 was previously reported to be exclusive of high pHu meat samples (**Publication I**, see *section 4.1.1.1*). In contrast, peptide HSPB6-1 (*m/z* at 850.7), found to be exclusive of high pHu meats in the previous approach (**Publication I**, see *section 4.1.1.1*), it was observed in low abundance and only in 3 out of 6 replicates of normal meat samples, evidencing the usefulness of SRM analysis compared to less specific LC-MS untargeted alternatives previously used (**Publication I** and **II**, see *sections 4.1.1.1* and *4.1.1.2*). Moreover, calibration curves obtained from the analysis of NORMAL/HIGH-CAL batch revealed that better results were achieved in terms of number of signals and linearity in high pHu meat samples (data not shown). The analysis of CAL batch also confirmed that these 6 peptides were only found in HIGH-CAL sample group as previously shown by HIGH-RPL samples. In any case, NORMAL/HIGH-CAL assay demonstrated robustness of SRM determinations and confirmed reliability of results from the replicates batch study.

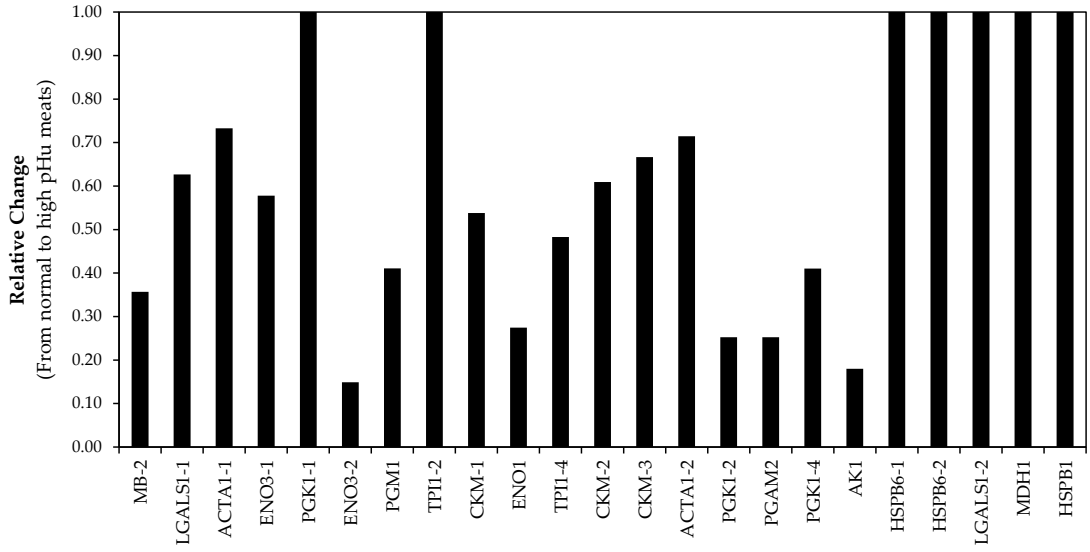

Figure 4.4. Quantitative changes between normal and high pHu meat samples of RPL batch as assessed by the RC coefficient.

In order to visually explain individual sample and peptide distribution from SRM assay between the two groups (NORMAL/HIGH-RPL), a PCA analysis and a heat map were performed. PCA depicting meat samples distribution according to the normalised SRM results of the 23 peptides showed that normal and high pHu replicates were clearly separated by PC1. Whereas high pHu samples had positive factor scores, normal pHu samples showed negative factor scores (**Figure 4.5A.**). The representation of individual regulation level of peptides in a heat map with hierarchical clustering allowed the classification of samples. Peptides (rows) were grouped in two main clusters depending on their regulation levels, being peptides of cluster 1 (top) down-regulated compared to those of cluster 2 (bottom). Similarly, biological sample replicates (columns) were grouped in two clusters (normal and high pHu) according to differences on peptide abundance. Overall, peptides showing statistically significant differences were up-regulated in high pHu samples (**Figure 4.5B.**).

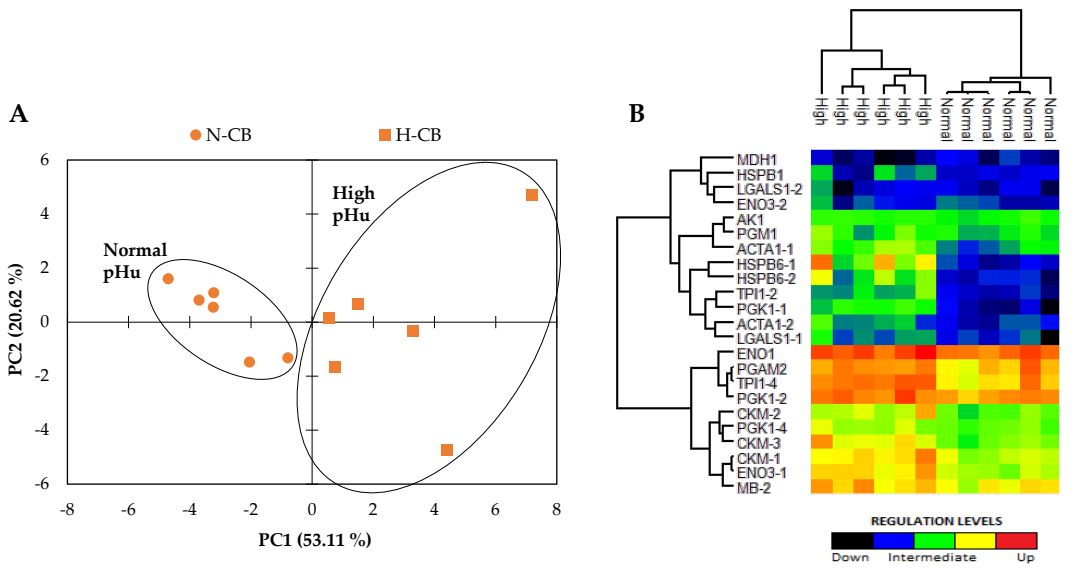

Figure 4.5. (**A**) Animal sample distribution on the two-dimensional coordinate system defined by the principal component (PC) 1 and 2. Each point represents an individual sample obtained from a crossbred animal (● N-CB: normal pHu samples; ■ H-CB: high pHu samples. (**B**) Heat map showing the normalised SRM results of peptides from each biological replicate.

After demonstrating the efficiency of 17 peptides as potential biomarkers of high pHu meat samples, the next step was to get the biological explanation of the proteome changes found between normal and high pHu meat sample groups. Only those peptides exhibiting significant quantitative differences ($P \leq 0.05$) between normal and high pHu meat samples were considered for functional classification using GO terms and were referred to their proteins of origin. A total of 10 proteins were classified according to their biological processes: carbohydrate metabolism (ENO3, MDH1, PGK1 and TPI1), abiotic stimulus response (CKM, MB and PGK1) and biological regulation (ACTA1, HSPB1, LGALS1, MB and PGK1). Taking into account that animals suffering PSS conditions have limited glycogen reserves prior to slaughter, the regulation levels of glycolytic enzymes is expected to be different between normal and high pHu meats as in the case of those proteins involved in carbohydrate metabolism (ENO3, MDH1, PGK1 and TPI1). Additionally, muscle cells would need to use, apart from glycogen, other alternative energy sources for restoring ATP levels and maintaining cell homeostasis (*i.e.*, amino acids, lipids). This fact could explain the overabundance in high pHu meats of those proteins involved in energy production (CKM and MB) and TCA cycle (MDH1), which is the final common pathway for the oxidation of fuel molecules to produce energy, as stated in the scientific literature [15,84,117]. In the present work was also found high regulation levels of ACTA1 in meat samples with high pHu (**Publication I,** see *section 4.1.1.1*) due to the fact that high pHu values can enhance its solubility [16,81,105,106]. Meanwhile, HSPB1 and

HSPB6 proteins were up-regulated in high pHu samples as previously stated (**Publication I**, see *section 4.1.1.1*). As reported in the scientific literature, taking into account the protective role of these proteins under stressful conditions [107,108], overabundance of these proteins in high pHu meat samples was expected [15]. Finally, LGALS1 has been described to participate in a wide range of biological processes [118]. However, the relevance of this protein in *post mortem* muscle is highly controversial and contradictory findings have been reported in the literature as reviewed by Ouali et al. [13]. The role of LGALS1 in high pHu meat samples still remains unclear and deserves further investigation.

Overall, the *gel-free* proteomic strategy presented in the third step of the present work demonstrated its usefulness to study quantitative differences in muscle proteome of beef cattle. Appropriate optimization of a conventional ion-trap allowed untargeted qualitative/quantitative and targeted quantitative analysis of samples through full-MS^1, $ddMS^2$ and SRM approaches. Higher efficiency in terms of little sample preparation, affordability and quality of results exhibited by the straightforward analysis of soluble protein extracts has demonstrated the possibility of implementing this methodology in meat quality research studies.

4.2 CASPASE ACTIVITY APPROACH

As stated at the beginning of Results and Discussion chapter, different proteomic approaches have been developed to thoroughly search for protein biomarkers related to high pHu meat samples. In this regard, it had been demonstrated that HSPs, widely studied proteins in relation to stress [107,108], were only found in high pHu meat samples, as others did [15]. This interesting finding can be understood considering the apoptosis process. Triggering of this process depends on the nature of initial stimulus, being animal stress one of the relevant factors where the involvement of HSPs has been described as anti-apoptotic players [119]. Even though many researchers have been focused on the study of apoptosis as one of the first events triggered in *post mortem* muscle and having a great influence in meat tenderisation [9], these studies have not propounded in any possible relationship among *ante mortem* factors influencing animal metabolism and *post mortem* cell death. Therefore, in the second block of the present work and for the first time, the study of apoptosis as a way to understand the influence of animal handling practices on beef quality was proposed. To achieve this objective, first of all, the limitations of commercial kits to

accurately measure caspase activity in samples with scarce enzymatic activity such as skeletal muscle [57] had to be overcome.

4.2.1 Optimization of the fluorogenic assay

Optimization of the fluorogenic assay in terms of affordability, sustainability and sensitivity was considered critical to determine caspase 3/7 activity in tissues with low enzymatic activity level such as skeletal muscle as overcome in **Publication V** (see *Appendix V*).

To do this, a preliminary enzyme activity approach of samples obtained using 4 different extraction buffers was performed (see *Table 3.2.*). As explained in Materials and Methods chapter, buffer A was selected since it is a commonly used buffer for the sarcoplasmic protein extraction in muscle tissues [62,97]. The other 3 were commonly used for caspase extraction from intracellular membranes [98,99]. In this regard, no enzyme activity was found in samples extracted with buffer A because the presence of protease inhibitor cocktail might have inhibited any residual protease action (**Figure 4.6A.**). On the contrary, the presence of 1 mM of PMSF in buffers B, C and D did not seem to affect caspase activity because it inhibits serine peptidases but not cysteine peptidases such as caspases [120]. The main difference among buffers B to D was the presence or absence of $MgCl_2$ and/or KCl. In the literature, authors have reported either the use or not of these compounds for caspase extraction [98,121,122]. In this case, the maximum caspase activity was reached with buffer D (**Figure 4.6A.**), and thus, this buffer, which contains few chemical compounds, was selected to test under final optimised assay conditions.

Figure 4.6B. displays caspase 3/7 activity of meat samples differing in final substrate concentrations (mM) and sample to substrate ratio (R-) (see *Table 3.3.*). The highest caspase 3/7 activity was achieved at 0.083 mM and R-1:1 after 46 min of reaction at 37 °C. However, such experimental conditions were discarded to avoid saturation of the fluorescence response in the case of samples showing higher enzymatic activity, thus limiting the dynamic range of the proposed methodology. Since R- had a great influence on sensitivity (see R-1:1 *vs* R-3:5) and considering the cost of processing high number of samples, an intermediate substrate concentration of 0.042 mM, R-1:1 and 30 min of reaction time were considered as the final optimised assay conditions. Previous studies have reported that 0.01 mM of substrate would be enough to determine activity of purified caspases 3, 6, 7 and 8 expressed in *E. coli* [100], but as

previously mentioned, determination of low activity levels in meat extracts required higher substrate concentrations for reliable determinations.

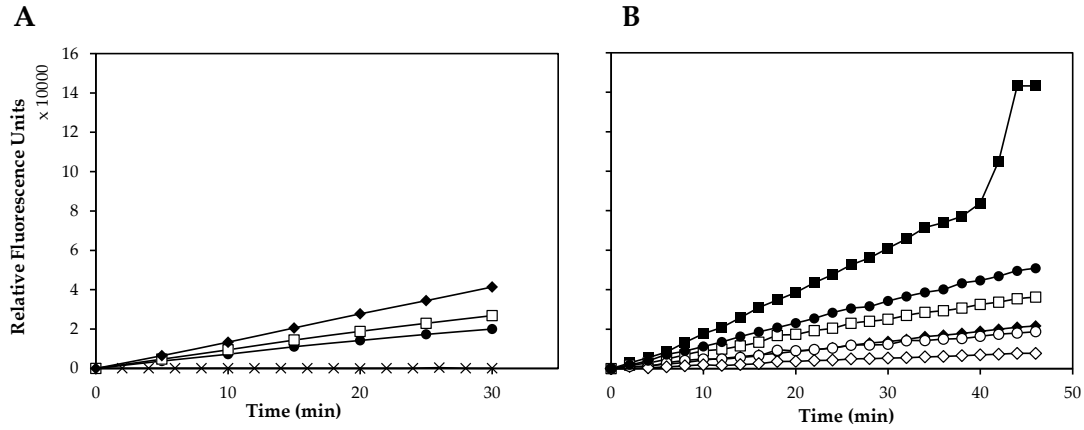

Figure 4.6. (**A**) Effect of extraction buffer on caspase 3/7 activity: (x) buffer A; (●) buffer B (□) buffer C; and (♦) buffer D (final optimised buffer). (**B**) Effect of substrate concentration (mM) and extract to substrate ratio (R-) on caspase 3/7 activity: (♦) 0.021 mM (R-1:1); (■) 0.083 mM (R-1:1); (◊) 0.025 mM (R-3:5); (○) 0.05 mM (R-3:5); (□) 0.1 mM (R-3:5) and (●) 0.042 mM and R-1:1 (final optimised conditions).

Sensitivity limitations of the fluorogenic caspase assay could be improved by appropriate optimization of the fluorescence detection settings (see *Table 3.4.*). Enzyme activity was only detected at excitation and emission wavelengths of 380-15 and 460-20 nm, respectively, in accordance to previous results in meat extracts using the same fluorogenic peptide substrate (**Figure 4.7A.**) [98,99]. Furthermore, maximal fluorogenic response was achieved at the maximum amplification gain assayed (1500).

The optimised assay clearly demonstrated that reproducibility was good either within or between-runs, obtaining CV below 5 % and 14 %, respectively (data not shown). Higher CVs were found between-runs due to the fact that caspase 3/7 activity can slightly change over long storage times. Comparison with a commercial kit showed that better linearity results were achieved for the optimised methodology yielding higher correlation values (**Figure 4.7B.**), in samples with either high or low caspase activity compared to commercial kit.

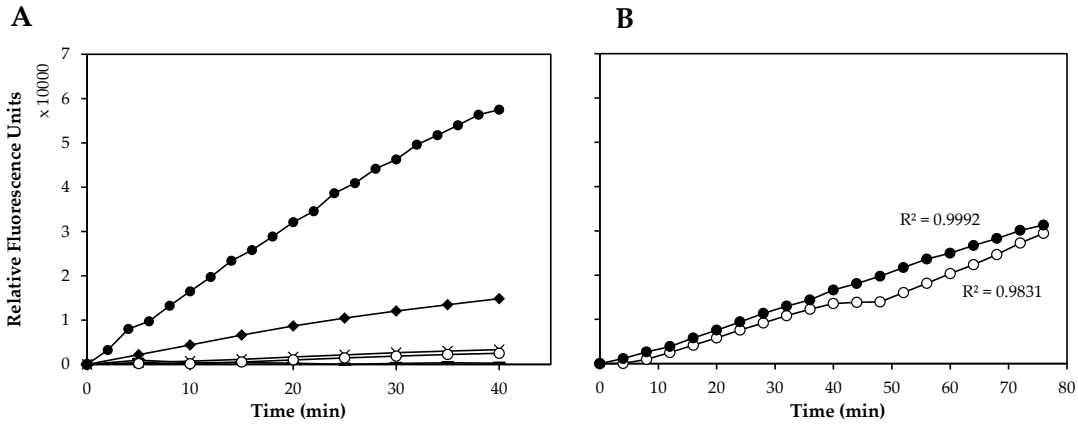

Figure 4.7. (**A**) Effect of instrumental conditions on caspase 3/7 activity: (**—**) 330-20 and 385-30 nm; Gain: 600; (○) 339-15 and 439-30 nm; Gain: 750; (x) 350-15 and 440-20 nm; Gain: 800; (♦) 380-15 and 460-20 nm; Gain: 1200 and (●) final optimised conditions (380-15 and 460-20 nm; Gain: 1500). (**B**) Comparison between the optimised method of the present work (●) and a commercial caspase 3/7 assay kit (○) in terms of linearity, sensitivity and reliability in the measurement of a meat sample representative of high caspase activity levels.

Overall, the linearity of the fluorogenic assay to measure low caspase 3/7 activities in meat/muscle extracts was greatly improved in the optimised protocol as compared to the commercial kit. Reaction and instrumental conditions proposed in the present work made possible the study of a wide range of tissue samples independently of their caspase 3/7 activity. Furthermore, the use of a 96-microtiter-well format loading a customized substrate concentration enabled an affordable analysis of a high number of meat extracts with a good reproducibility.

4.2.2 Study of apoptosis process using caspase activity measurements

Using the optimized fluorogenic assay, the activities of executioner caspases 3/7 and initiator caspase 9 were investigated over time (2, 8 and 24 h *post mortem*) in loin samples of three Spanish beef cattle breeds managed under different feeding systems (intensive *vs* semi-extensive) and transport/lairage conditions (mixed *vs* non-mixed with unfamiliar animals) (**Publication VI,** see *Appendix VI*).

All animals from the several breeds, feeding systems and transport/lairage conditions studied, presented meat with normal pHu (< 6.0). However, these values were significantly affected by breed ($P \leq 0.001$) while feeding and transport/lairage effects were not significant. Animals from RE breed showed significantly higher pH values (5.64 ± 0.12) compared to animals from the other two breeds (5.49 ± 0.06 for AV and 5.55 ± 0.14 for RG), which could be related to a more excitable temperament of RE

breed [123]. As the behaviour of both caspase 3/7 and caspase 9 activities was similar, both enzymes were discussed together.

Overall, results obtained in the present work showed that caspase activity over *post mortem* time greatly depends on breed and transport/lairage conditions ($P \leq 0.05$), while the effect of production system was not significant. Looking at breed effect, different behaviour of caspases over time was observed in RG breed, showing significantly higher activities at 2 h and lower activities at 24 h *post mortem* compared to AV and RE breeds (**Figure 4.8.**). In general, intermediate and statistically not different values were observed at 8 h *post mortem* among breeds, except for RG breed and caspase 3/7 activity. It is assumed that caspases are activated immediately after animal exsanguination, and therefore, highest activities would be expected at early *post mortem* times while subsequently decreasing over time [98,124-127]. In this sense, as previously reported in the literature [128-130], this pattern was only observed in RG breed, while low caspase activities were observed in AV and RE breeds at early *post mortem* time which could be indicative of higher stress levels reached before slaughter. It is known that HSPs interact with active caspases under stressful conditions hindering their function and, consequently, slowing down the cellular death process. In this regard, breed effect and its relation to excitable temperament and stress susceptibility have been previously reported for RE breed [123], but never for AV and RG breeds [131,132]. The low caspase activity observed in AV breed could be also related to the myostatin-related muscle hypertrophy (double-muscled) of these animals [133] and, therefore, are considered more susceptible to stressful conditions [134]. Another important factor that could also explain the differences observed in *post mortem* muscle metabolism is the age of the animals associated to specific management practices. For instance, at slaughter, animals from RG were younger (10 mo) than other animals (13 to 15 mo), and in this sense, some authors have reported that several apoptotic mechanisms, including caspase 3 activation, were more pronounced in young rat compared to mature rat brains after inducing cerebral hypoxia-ischemia [135]. Although both, initiator caspase 9 and executioner caspase 3/7 activities, showed a similar breed-dependant pattern, caspases 3/7 seemed to be slightly less sensitive, with not significant differences between AV and RE breeds at 24 h *post mortem* (**Figure 4.8B.**). On the contrary, caspase 9 activity was significantly different among studied breeds (**Figure 4.8A.**).

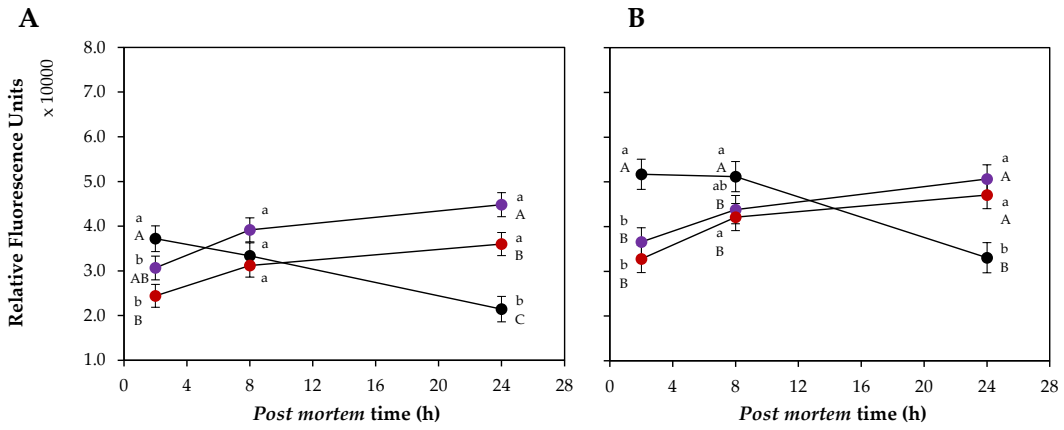

Figure 4.8. Interaction between breed and *post mortem* time factors for caspase 9 (**A**) and 3/7 (**B**) activity. Least square means and standard error of the means have been represented. Different capital letters indicate significant differences among breeds and different lower case letters indicate significant differences among *post mortem* times ($P \leq 0.05$).
●, Asturiana de los Valles; ●, Retinta; ●, Rubia Gallega.

In relation to the effect of transport/lairage conditions, **Figure 4.9.** represents the evolution of caspase 9 and 3/7 activities over *post mortem* time, in mixed and non-mixed animal groups. While AV and RE breeds behaved similarly (increasing activities over time) and with no differences between mixed and non-mixed animals (**Figure 4.9A., 9B., 9D.** and **9E.**), a very different pattern was observed in RG breed (**Figure 4.9C.** and **9F.**). At 2 h *post mortem*, both caspase activity values for RG breed were higher compared to the other breeds, but with no differences between mixed and non-mixed animals. At later *post mortem* times, differences were significant between mixed and non-mixed animal groups. Non-mixed animals showed a decreasing caspase pattern while mixed animals reached the highest caspase activity at 8 h *post mortem* and then decreased. Taking into account that mixing unfamiliar animals prior to slaughter is one of the most stressful events that influence bovine species [32,136,137], this event could have triggered the activation of HSPs delaying the apoptosis process over *post mortem* time.

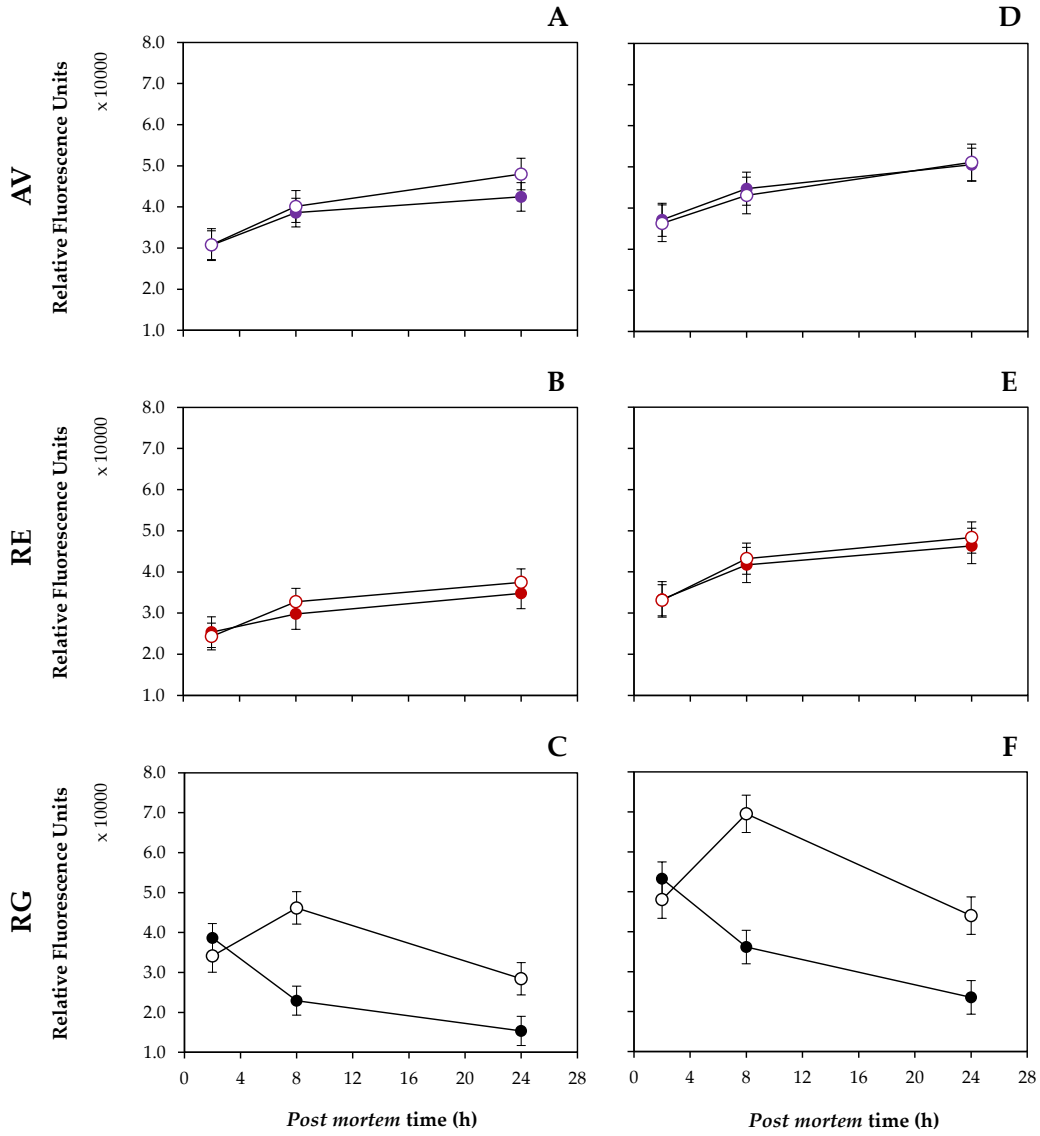

Figure 4.9. Evolution of caspase 9 (**A**, **B** and **C**) and 3/7 (**D**, **E** and **F**) activity over *post mortem* time as related to transport/lairage for mixed (○) and non-mixed (●) animal groups in each studied breed: **A**/**D**) Asturiana de los Valles; **B**/**E**) Retinta, and **C**/**F**) Rubia Gallega breed. Least square means and standard error of the means have been represented. (AV: Asturiana de los Valles; RE: Retinta; RG: Rubia Gallega).

Overall, caspase activity pattern was different among breeds at 2 and 24 h *post mortem* times, especially among those breeds behaving most differently (RG *vs* AV and RE). Additionally, the better discriminant capacity of caspase 9 activity compared to caspase 3/7 at 24 h *post mortem* would indicate its usefulness as an indicator of factors such as breed and transport/lairage influencing meat quality characteristics.

After demonstrating the different activity pattern over time of caspase 9 and 3/7 activity, especially the differences observed at 24 h *post mortem* among samples collected from several breeds managed under different feeding systems and

transport/lairage conditions, the activity of these enzymes was studied in normal and high pHu muscle samples obtained from several Spanish breeds and crossbred animals at 24 h *post mortem* (**Manuscript VII,** see *Appendix VII*).

In general, the activity of both caspases was affected by breed ($P \leq 0.001$) and pHu ($P \leq 0.001$), and the behaviour of both caspases was quite similar as observed in **Figures 4.10A.** and **4.11**. However, there was an interaction between breed and pHu for caspase 3/7 activity ($P \leq 0.001$).

Regarding the effect of breed on caspase 9 activity at 24 h *post mortem* (**Figure 4.10B.**), AV showed a significantly higher caspase activity compared to CB and RG ($P \leq 0.05$), while intermediate activities where observed for RE breed ($P > 0.05$). Observed activity data variability was also higher in AV breed, with highest maximum and lowest minimum activities, compared to other breeds. These results are partially in accordance to those reported in the previous experiment (**Publication VI**, see *Appendix VI*), where AV and RE breed showed significantly higher caspase 9 activities at 24 h *post mortem* compared to RG breed, which would be indicative of high stress levels reached before slaughter by these breeds. Concerning the effect of pHu, significantly higher caspase activity at 24 h *post mortem* was observed in high compared to normal pHu meat samples (**Figure 4.10C.**). Although there are not studies that analyse caspase 9 activity in relation to meat pHu, results would be comparable to those reported for caspase 3/7 activity. In this regard, some authors have revealed differences in caspase 3 regulation levels between normal and high pHu samples, reporting high expression of caspase 3 large subunit in high compared to normal pHu meat samples at 24 h *post mortem* [53]. This finding agrees with the fact that stress can induce the synthesis of HSPs, event that may delay the *post mortem* apoptosis. Taking into account that caspase activation occurs immediately after animal exsanguinations and decreases over time, the idea of anti-apoptotic role exhorted by HSPs is becoming stronger.

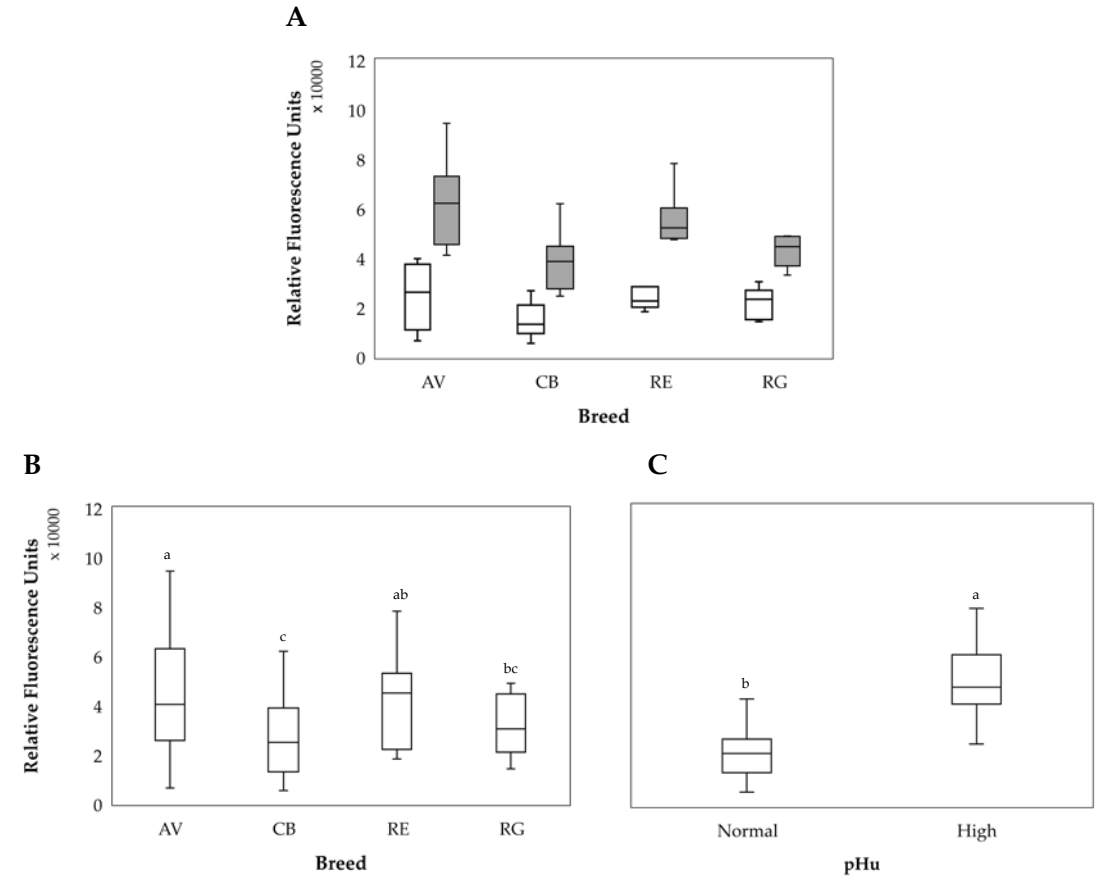

Figure 4.10. Box-plot with upper and lower whiskers representing the effect of (**A**) breed and meat pHu interaction (□ normal pHu meat samples; ■ high pHu meat samples) (**B**) breed and (**C**) meat pHu on caspase 9 activity at 24 h *post mortem*. Different letters indicate significant differences among breeds ($P \leq 0.05$).

As for caspase 9 activity, the activity of caspase 3/7 was significantly higher in meat samples with high compared to meat samples with normal pHu in all studied breeds ($P \leq 0.05$) (**Figure 4.11.**). However, this pattern was quite different in RE breed with strong differences in caspase 3/7 activity between normal and high meat samples, reinforcing again the idea of the major role played by bovine breed in caspase activity. In this sense, as previously indicated, it has been described the aggressive behaviour and stress susceptibility of RE breed [123] compared to AV and RG breed that are similar to each other [131,132]. On the other hand, overall, crossbred animals showed the lowest caspase levels, in normal and high pHu meat samples, which could be related to the increased resilience to stress susceptibility of these animals [138,139]. In terms of variability in activity, in general this was similar in both types of meat samples (normal and high pHu) for all studied breeds except for RG breed where activity variability was greater in high pHu meat samples (comparable to AV breed) compared to normal samples.

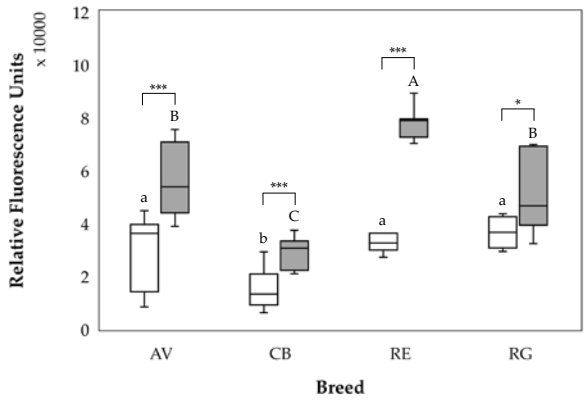

Figure 4.11. Box-plot with upper and lower whiskers representing the effect of breed and meat pHu on caspase 3/7 activity. Different lower case letters indicate significant differences among breeds in meat samples with normal pHu, and different capital letters indicate significant differences among breeds in meat samples with high pHu. Asterisks represent differences between the normal (□) and high (■) pHu meat samples for each studied breed. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Overall, these results confirm that both caspase 9 and 3/7 activities were significantly higher in meat samples with high compared to normal pHu, independently of the studied breed and demonstrating that caspase activity measured at 24 h *post mortem* can be a good indicator of high pHu meat samples. However, differences among breeds are also important that need to be taken into account. In this regard, further research is necessary to understand how the behaviour of each breed can affect the activity of studied caspases.

5 REFERENCES

[1] United Nations. World Population Prospects: the 2019 Revision. Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat 2019.

[2] FAO. World Livestock: Transforming the livestock sector through the Sustainable Development Goals. Food and Agriculture Organization of the United Nations 2018:222.

[3] FAO. The future of food and agriculture. Trends and challenges. Food and Agriculture Organization of the United Nations 2017.

[4] Henchion M, McCarthy M, Resconi VC, Troy D. Meat consumption: Trends and quality matters. Meat Science 2014;98:561-8.

[5] MAPA. Informe del Consumo de Alimentación en España. Ministerio de Agricultura, Pesca y Alimentación 2019.

[6] MAPA. Indicadores Económicos del Sector de la Carne de Vacuno. Ministerio de Agricultura, Pesca y Alimentación 2020.

[7] Jiménez-Montero JA, González-Recio O, Alenda R. Consideraciones sobre el nivel productivo del ganado vacuno de carne en España. Tierras de Castilla y León. Ganadería 2011;183:46-9.

[8] MAPA. Estudio del sector español de vacas nodrizas. Datos SITRAN. Ministerio de Agricultura, Pesca y Alimentación 2018.

[9] Ouali A, Herrera-Mendez CH, Coulis G, Becila S, Boudjellal A, Aubry L, Sentandreu MA. Revisiting the conversion of muscle into meat and the underlying mechanisms. Meat Science 2006;74:44-58.

[10] García-Macia M, Sierra V, Palanca A, Vega-Naredo I, de Gonzalo-Calvo D, Rodríguez-González S, Oliván M, Coto-Montes A. Autophagy during beef aging. Autophagy 2014;10:137-43.

[11] Lana A, Zolla L. Apoptosis or autophagy, that is the question: Two ways for muscle sacrifice towards meat. Trends in Food Science & Technology 2015;46:231-41.

[12] Lehninger AL. Princípios de bioquímica. Barcelona: Ediciones Omega; 2001.

[13] Ouali A, Gagaoua M, Boudida Y, Becila S, Boudjellal A, Herrera-Mendez CH, Sentandreu MA. Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. Meat Science 2013;95:854-70.

[14] Tang J, Faustman C, Hoagland TA, Mancini RA, Seyfert M, Hunt MC. Postmortem oxygen consumption by mitochondria and its effects on myoglobin form and stability. Journal of Agricultural and Food Chemistry 2005;53:1223-30.

[15] Wu S, Luo X, Yang X, Hopkins DL, Mao Y, Zhang Y. Understanding the development of color and color stability of dark cutting beef based on mitochondrial proteomics. Meat Science 2020;163:108046.

[16] Mahmood S, Turchinsky N, Paradis F, Dixon WT, Bruce HL. Proteomics of dark cutting longissimus thoracis muscle from heifer and steer carcasses. Meat Science 2018;137:47-57.

[17] Bendall JR, Bourne GH. The structure and function of muscle. Structure 1973:243-309.

[18] Pearson AM. Muscle function and postmortem changes. In: Price JF, Schweigert BS, editors. The science of the meat and meat products. Westport, Connecticut: Food & Nutrition Press Inc; 1986, p. 155-191.

[19] Lawrie RA, Ledward DA. The structure and growth of muscle. In: Lawrie RA, editor. Meat science. Cambridge: Woodhead Publishing Limited,1998, p. 31-57.

[20] Tarrant PV, Mothersill C. Glycolysis and associated changes in beef carcasses. Journal of the Science of Food and Agriculture 1977;28:739-49.

[21] Ferguson DM, Gerrard DE. Regulation of post-mortem glycolysis in ruminant muscle. Animal Production Science 2014;54:464-81.

[22] Devine CE, Ellery S, Averill S. Responses of different types of ox muscle to electrical stimulation. Meat Science 1984;10:35-51.

[23] Talmant A, Monin G, Briand M, Dadet M, Briand Y. Activities of metabolic and contractile enzymes in 18 bovine muscles. Meat Science 1986;18:23-40.

[24] Pethick DW, Fergusson DM, Gardner GE, Hocquette JF, Thompson JM, Warner R. Muscle metabolism in relation to genotypic and environmental influences on consumer defined quality of red meat. In: Hocquette JF, Gigli S, editors. Indicators of milk and beef quality, vol 112: EAAP Publication. Netherlands: Wageningen Academic Publishers; 2005, p. 95-110.

[25] Holmes J, Ashmore CR. A histochemical study of development of muscle fiber type and size in normal and double muscled cattle. Growth 1972;36:351-372.

[26] Ashmore CR. Phenotypic expression of muscle fiber types and some implications to meat quality. Journal of Animal Science 1974;38:1158-64.

[27] Marsh BB, Lochner JV, Takahashi G, Kragness DD. Effects of early post-mortem pH and temperature on beef tenderness. Meat Science 1981;5:479-83.

[28] Bendall JR. Electrical stimulation of carcasses of meat animals. In Lawrie RA, editor. Developments in meat science. London: Applied Science Publishers; 1980, p. 37.

[29] Vestergaard M, Oksbjerg N, Henckel P. Influence of feeding intensity, grazing and finishing feeding on muscle fibre characteristics and meat colour of semitendinosus, longissimus dorsi and supraspinatus muscles of young bulls. Meat Science 2000;54:177-85.

[30] Muir PD, Deaker JM, Bown MD. Effects of forage-and grain-based feeding systems on beef quality: A review. New Zealand Journal of Agricultural Research 1998;41:623-35.

[31] Aalhus JL, Price MA. Endurance-exercised growing sheep: I. Post-mortem and histological changes in skeletal muscles. Meat Science 1991;29:43-56.

[32] Ferguson DM, Warner RD. Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants?. Meat Science 2008;80:12-9.

[33] Tarrant PV. Transportation of cattle by road. Applied Animal Behaviour Science 1990;28:153-70.

[34] Bøe KE, Færevik G. Grouping and social preferences in calves, heifers and cows. Applied Animal Behaviour Science 2003;80:175-90.

[35] Marco-Ramell A, Arroyo L, Saco Y, García-Heredia A, Camps J, Fina M, Piedrafita J, Bassols A. Proteomic analysis reveals oxidative stress response as the main adaptative physiological mechanism in cows under different production systems. Journal of Proteomics 2012;75:4399-411.

[36] Gregory NG, Grandin T. Animal welfare and meat science. Wallingford: CABI Publising; 1998.

[37] McVeigh JM, Tarrant PV, Harrington MG. Behavioral stress and skeletal muscle glycogen metabolism in young bulls. Journal of Animal Science 1982;54:790-5.

[38] Newton KG, Gill CO. The microbiology of DFD fresh meats: A review. Meat Science 1981;5:223-32.

[39] Loudon KMW, Lean IJ, Pethick DW, Gardner GE, Grubb LJ, Evans AC, McGilchrist P. On farm factors increasing dark cutting in pasture finished beef cattle. Meat Science 2018;144:110-7.

[40] Meat and Livestock Australia. Dark cutting in livestock. 2014.

[41] Poleti MD, Moncau CT, Silva-Vignato B, Rosa AF, Lobo AR, Cataldi TR, Negrão JA, Silva SL, Eler JP, de Carvalho Balieiro JC. Label-free quantitative proteomic analysis reveals muscle contraction and metabolism proteins linked to ultimate pH in bovine skeletal muscle. Meat Science 2018;145:209-19.

[42] Adzitey F, Nurul H. Pale soft exudative (PSE) and dark firm dry (DFD) meats: Causes and measures to reduce these incidences - a mini review. International Food Research Journal 2011;18:11-20.

[43] Rosa AF, Ventura R, Oliveira G, Perez BC, Mattos EC, Carvalho ME, Bonin M, Rezende FM, Eler JP, Ferraz J. Can genetics be associated with the high frequency of dark, firm and

dry meat in brazilian nellore beef cattle? In: Troy D, Mc Donnel C, Hinds L, Kerry J, editors. 63rd International Congress of Meat Science and Technology: Nurturing locally, growing globally. Netherlands: Wageningen Academic Publishers; 2017, p. 71.

[44] Mach N, Bach A, Velarde A, Devant M. Association between animal, transportation, slaughterhouse practices, and meat pH in beef. Meat Science 2008;78:232-8.

[45] Chambers PG, Grandin T, Heinz G, Srisuvan T. Guidelines for humane handling, transport and slaughter of livestock. Bangkok: FAO; 2001.

[46] Ponnampalam EN, Hopkins DL, Bruce H, Li D, Baldi G, Bekhit AE. Causes and contributing factors to "dark cutting" meat: Current trends and future directions: A review. Comprehensive Reviews in Food Science and Food Safety 2017;16:400-30.

[47] Fidzianska A, Kaminska A, Glinka Z. Muscle cell death. Ultrastructural differences between muscle cell necrosis and apoptosis. Neuropatologia Polska 1991;29:19-28.

[48] Grilo AL, Mantalaris A. Apoptosis: A mammalian cell bioprocessing perspective. Biotechnology Advances 2019;37:459-75.

[49] Boatright KM, Salvesen GS. Mechanisms of caspase activation. Current Opinion in Cell Biology 2003;15:725-31.

[50] Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nature reviews Molecular Cell Biology 2008;9:231.

[51] Towers AE, Oelschlager ML, Lorenz M, Gainey SJ, McCusker RH, Krauklis SA, Freund GG. Handling stress impairs learning through a mechanism involving caspase-1 activation and adenosine signaling. Brain, Behavior, and Immunity 2019;80:763-76.

[52] Alderman SL, Leishman EM, Fuzzen MLM, Bernier NJ. Corticotropin-releasing factor regulates caspase-3 and may protect developing zebrafish from stress-induced apoptosis. General and Comparative Endocrinology 2018;265:207-13.

[53] Díaz-Luis A, Díaz F, Diñeiro Y, González-Blanco L, Arias E, Coto-Montes A, Oliván M, Sierra V. Nuevos indicadores de carnes (DFD): estrés oxidativo, autofagia y apoptosis. ITEA, Información Técnica Económica Agraria: revista de la Asociación Interprofesional para el Desarrollo Agrario (AIDA) 2021;117:3-18.

[54] Gurtu V, Kain SR, Zhang G. Fluorometric and Colorimetric Detection of Caspase Activity Associated with Apoptosis. Analytical Biochemistry 1997;251:98-102.

[55] Cao JX, Ou CR, Zou YF, Ye KP, Zhang QQ, Khan MA, Pan DD, Zhou G. Activation of caspase-3 and its correlation with shear force in bovine skeletal muscles during postmortem conditioning. Journal of Animal Science 2013;91:4547-52.

[56] Zhang J, Yu Q, Han L, Chen C, Li H, Han G. Study on the apoptosis mediated by cytochrome c and factors that affect the activation of bovine longissimus muscle during postmortem aging. Apoptosis 2017;22:777-85.

[57] Wang Z, Liao J, Diwu Z. N-DEVD-N′-morpholinecarbonyl-rhodamine 110: novel caspase-3 fluorogenic substrates for cell-based apoptosis assay. Bioorganic & Medicinal Chemistry Letters 2005;15:2335-8.

[58] Zamaratskaia G, Li S. Proteomics in meat science—current status and future perspective. Theory and practice of meat processing 2017;2:18-26.

[59] Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez J, Yan JX, Gooley AA, Hughes G, Humphery-Smith I. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and arnino acid analysis. Bio/technology 1996;14:61-5.

[60] Anderson NG, Anderson NL. Twenty years of two-dimensional electrophoresis: past, present and future. Electrophoresis 1996;17:443-53.

[61] Hollung K, Veiseth E, Jia X, Færgestad EM, Hildrum KI. Application of proteomics to understand the molecular mechanisms behind meat quality. Meat Science 2007;77:97-104.

[62] Jia X, Hildrum KI, Westad F, Kummen E, Aass L, Hollung K. Changes in enzymes associated with energy metabolism during the early post mortem period in longissimus thoracis bovine muscle analyzed by proteomics. Journal of Proteome Research 2006;5:1763-9.

[63] Jia X, Ekman M, Grove H, Færgestad EM, Aass L, Hildrum KI, Hollung K. Proteome changes in bovine longissimus thoracis muscle during the early postmortem storage period. Journal of Proteome Research 2007;6:2720-31.

[64] Canto AC, Suman SP, Nair MN, Li S, Rentfrow G, Beach CM, Silva TJ, Wheeler TL, Shackelford SD, Grayson A. Differential abundance of sarcoplasmic proteome explains animal effect on beef Longissimus lumborum color stability. Meat Science 2015;102:90-8.

[65] Joseph P, Suman SP, Rentfrow G, Li S, Beach CM. Proteomics of muscle-specific beef color stability. Journal of Agricultural and Food Chemistry 2012;60:3196-203.

[66] Liu J, Arner A, Puolanne E, Ertbjerg P. On the water-holding of myofibrils: Effect of sarcoplasmic protein denaturation. Meat Science 2016;119:32-40.

[67] Di Luca A, Elia G, Hamill R, Mullen AM. 2D DIGE proteomic analysis of early post mortem muscle exudate highlights the importance of the stress response for improved water-holding capacity of fresh pork meat. Proteomics 2013;13:1528-44.

[68] D'Alessandro A, Zolla L. Meat science: From proteomics to integrated omics towards system biology. Journal of Proteomics 2013;78:558-77.

[69] Koohmaraie M. Muscle proteinases and meat aging. Meat Science 1994;36:93-104.

[70] Koohmaraie M, Kennick WH, Anglemier AF, Elgasim EA, Jones TK. Effect of postmortem storage on cold-shortened bovine muscle: Analysis by SDS-polyacrylamide gel electrophoresis. Journal of Food Science 1984;49:290-1.

[71] Bandman E, Zdanis D. An immunological method to assess protein degradation in post-mortem muscle. Meat Science 1988;22:1-19.

[72] Bouley J, Chambon C, Picard B. Mapping of bovine skeletal muscle proteins using two-dimensional gel electrophoresis and mass spectrometry. Proteomics 2004;4:1811-24.

[73] Vincent D, Solomon PS. Development of an in-house protocol for the OFFGEL fractionation of plant proteins. Journal of Integrated OMICS 2011;1:216-25.

[74] Abdallah C, Dumas-Gaudot E, Renaut J, Sergeant K. Gel-based and gel-free quantitative proteomics approaches at a glance. International Journal of Plant Genomics 2012;2012:494572.

[75] Sentandreu MA, Fraser PD, Halket J, Patel R, Bramley PM. A proteomic-based approach for detection of chicken in meat mixes. Journal of Proteome Research 2010;9:3374-83.

[76] Surowiec I, Koistinen KM, Fraser PD, Bramley PM. Proteomic approach for the detection of chicken mechanically recovered meat. Meat Science 2011;89:233-7.

[77] Beldarrain LR, Aldai N, Picard B, Sentandreu E, Navarro JL, Sentandreu MA. Use of liquid isoelectric focusing (OFFGEL) on the discovery of meat tenderness biomarkers. Journal of Proteomics 2018;183:25-33.

[78] Talamo F, D'Ambrosio C, Arena S, Del Vecchio P, Ledda L, Zehender G, Ferrara L, Scaloni A. Proteins from bovine tissues and biological fluids: defining a reference electrophoresis map for liver, kidney, muscle, plasma and red blood cells. Proteomics 2003;3:440-60.

[79] Mato A, Rodríguez-Vázquez R, López-Pedrouso M, Bravo S, Franco D, Zapata C. The first evidence of global meat phosphoproteome changes in response to pre-slaughter stress. BMC Genomics 2019;20:590.

[80] Franco D, Mato A, Salgado FJ, López-Pedrouso M, Carrera M, Bravo S, Parrado M, Gallardo JM, Zapata C. Tackling proteome changes in the longissimus thoracis bovine muscle in response to pre-slaughter stress. Journal of Proteomics 2015;122:73-85.

[81] Jia X, Hollung K, Therkildsen M, Hildrum KI, Bendixen E. Proteome analysis of early post-mortem changes in two bovine muscle types: M. longissimus dorsi and M. semitendinosis. Proteomics 2006;6:936-44.

[82] Bendixen E. The use of proteomics in meat science. Meat Science 2005;71:138-49.

[83] Yu Q, Wu W, Tian X, Hou M, Dai R, Li X. Unraveling proteome changes of Holstein beef M. semitendinosus and its relationship to meat discoloration during post-mortem storage analyzed by label-free mass spectrometry. Journal of Proteomics 2017;154:85-93.

[84] Kiyimba F, Hartson SD, Rogers J, VanOverbeke DL, Mafi GG, Ramanathan R. Changes in glycolytic and mitochondrial protein profiles regulates postmortem muscle acidification and oxygen consumption in dark-cutting beef. Journal of Proteomics 2021; 232:104016.

[85] Lim H, Eng J, Yates JR, Tollaksen SL, Giometti CS, Holden JF, Adams MW, Reich CI, Olsen GJ, Hays LG. Identification of 2D-gel proteins: a comparison of MALDI/TOF peptide mass mapping to μ LC-ESI tandem mass spectrometry. Journal of the American Society for Mass Spectrometry 2003;14:957-70.

[86] Bjarnadottir SG, Hollung K, Høy M, Bendixen E, Codrea MC, Veiseth-Kent E. Changes in protein abundance between tender and tough meat from bovine Longissimus thoracis muscle assessed by isobaric Tag for Relative and Absolute Quantitation (iTRAQ) and 2-dimensional gel electrophoresis analysis. Journal of Animal Science 2012;90:2035-43.

[87] Mao Y, Hopkins DL, Zhang Y, Li P, Zhu L, Dong P, Liang R, Dai J, Wang X, Luo X. Beef quality with different intramuscular fat content and proteomic analysis using isobaric tag for relative and absolute quantitation of differentially expressed proteins. Meat Science 2016;118:96-102.

[88] Wei Y, Li X, Zhang D, Liu Y. Comparison of protein differences between high- and low-quality goat and bovine parts based on iTRAQ technology. Food Chemistry 2019;289:240-9.

[89] Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature 2003;422:198-207.

[90] Zhu W, Smith JW, Huang C. Mass spectrometry-based label-free quantitative proteomics. Journal of Biomedicine and Biotechnology 2009;2010:840518.

[91] Wu W, Dai R, Bendixen E. Comparing SRM and SWATH methods for quantitation of bovine muscle proteomes. Journal of Agricultural and Food Chemistry 2019;67:1608-18.

[92] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

[93] Regulation (EC) Nº 1099/2009 of the European Parliament and of the Council of 24 September 2009 on the protection of animals at the time of killing.

[94] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976;72:248-54.

[95] Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 2004;25:1327-33.

[96] Mirzapour M, Rezaei K, Sentandreu MA, Moosavi-Movahedi AA. In vitro antioxidant activities of hydrolysates obtained from Iranian wild almond (A mygdalus scoparia) protein by several enzymes. International Journal of Food Science and Technology 2016;51:609-16.

[97] Melody JL, Lonergan SM, Rowe LJ, Huiatt TW, Mayes MS, Huff-Lonergan E. Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. Journal of Animal Science 2004;82:1195-205.

[98] Huang F, Huang M, Zhang H, Guo B, Zhang D, Zhou G. Cleavage of the calpain inhibitor, calpastatin, during postmortem ageing of beef skeletal muscle. Food Chemistry 2014;148:1-6.

[99] Cao J, Ou C, Zou Y, Ye K, Zhang Q, Khan MA, Pan D, Zhou G. Activation of caspase-3 and its correlation with shear force in bovine skeletal muscles during postmortem conditioning. Journal of Animal Science 2013;91:4547-52.

[100] Stennicke HR, Salvesen GS. Caspases: preparation and characterization. Methods 1999;17:313-9.

[101] Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, Mann M, Cox J. The Perseus computational platform for comprehensive analysis of (prote) omics data. Nature methods 2016;13:731.

[102] Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Research 2003;13:2498-504.

[103] Marco-Ramell A, Gutiérrez AM, Velarde A, Cerón JJ, Bassols A. The Use of Proteomics to Study Biomarkers of Stress and Welfare in Farm Animals. In: de Almeida AM, Eckersall D, Miller I, editors. Proteomics in Domestic Animals: from Farm to Systems Biology. Switzerland: Springer Cham; 2018, p. 339-360.

[104] Schilling MW, Suman SP, Zhang X, Nair MN, Desai MA, Cai K, Ciaramella MA, Allen PJ. Proteomic approach to characterize biochemistry of meat quality defects. Meat Science 2017;132:131-8.

[105] Bjarnadóttir SG, Hollung K, Færgestad EM, Veiseth-Kent E. Proteome changes in bovine longissimus thoracis muscle during the first 48 h postmortem: shifts in energy status and myofibrillar stability. Journal of Agricultural and Food Chemistry 2010;58:7408-14.

[106] Laville E, Sayd T, Morzel M, Blinet S, Chambon C, Lepetit J, Renand G, Hocquette JF. Proteome changes during meat aging in tough and tender beef suggest the importance of apoptosis and protein solubility for beef aging and tenderization. Journal of Agricultural and Food Chemistry 2009;57:10755-64.

[107] Larkins NT, Murphy RM, Lamb GD. Influences of temperature, oxidative stress, and phosphorylation on binding of heat shock proteins in skeletal muscle fibers. American Journal of Physiology-Cell Physiology 2012;303:C654-65.

[108] Xing T, Wang MF, Han MY, Zhu XS, Xu XL, Zhou GH. Expression of heat shock protein 70 in transport-stressed broiler pectoralis major muscle and its relationship with meat quality. Animal 2017;11:1599-607.

[109] Zapata I, Zerby HN, Wick M. Functional proteomic analysis predicts beef tenderness and the tenderness differential. Journal of Agricultural and Food Chemistry 2009;57:4956-63.

[110] Stryer L. Metabolism: basic concepts and design. Biochemistry.4th ed. New York: WH Freeman & Company; 1995.

[111] Lametsch R, Roepstorff P, Møller HS, Bendixen E. Identification of myofibrillar substrates for μ-calpain. Meat Science 2004;68:515-21.

[112] Beltrán JA, Jaime I, Santolaria P, Sañudo C, Albertí P, Roncalés P. Effect of stress-induced high post-mortem pH on protease activity and tenderness of beef. Meat Science 1997;45:201-7.

[113] Camou JP, Marchello JA, Thompson VF, Mares SW, Goll DE. Effect of postmortem storage on activity of μ-and m-calpain in five bovine muscles. Journal of Animal Science 2007;85:2670-81.

[114] Smuder AJ, Kavazis AN, Hudson MB, Nelson WB, Powers SK. Oxidation enhances myofibrillar protein degradation via calpain and caspase-3. Free Radical Biology and Medicine 2010;49:1152-60.

[115] Ozawa S, Mitsuhashi T, Mitsumoto M, Matsumoto S, Itoh N, Itagaki K, Kohno Y, Dohgo T. The characteristics of muscle fiber types of longissimus thoracis muscle and their influences on the quantity and quality of meat from Japanese Black steers. Meat Science 2000;54:65-70.

[116] Young OA, Foote DM. Further studies on bovine muscle composition. Meat Science 1984;11:159-70.

[117] Cônsolo NRB, Rosa AF, Barbosa, Garibaldi Simon LC, Maclean PH, Higuera-Padilla A, Colnago LA, Titto EAL. Preliminary study on the characterization of Longissimus lumborum dark cutting meat in Angus× Nellore crossbreed cattle using NMR-based metabolomics. Meat Science 2020;172:108350.

[118] Zhou B, Long Y, Song G, Li Q, Cui Z. Molecular characterization of the lgals1 gene in large scale loach Paramisgurnus dabryanus. Gene 2016;577:65-74.

[119] Ray PS, Martin JL, Swanson EA, Otani H, Dillmann WH, Das DK. Transgene overexpression of αB crystallin confers simultaneous protection against cardiomyocyte apoptosis and necrosis during myocardial ischemia and reperfusion. The FASEB Journal 2001;15:393-402.

[120] Ishisaka R, Utsumi T, Yabuki M, Kanno T, Furuno T, Inoue M, Utsumi K. Activation of caspase-3-like protease by digitonin-treated lysosomes. FEBS Letters 1998;435:233-6.

[121] Chen L, Feng X, Zhang Y, Liu X, Zhang W, Li C, Ullah N, Xu X, Zhou G. Effects of ultrasonic processing on caspase-3, calpain expression and myofibrillar structure of chicken during post-mortem ageing. Food Chemistry 2015;177:280-7.

[122] Chen L, Feng XC, Lu F, Xu XL, Zhou GH, Li QY, Guo XY. Effects of camptothecin, etoposide and Ca2+ on caspase-3 activity and myofibrillar disruption of chicken during postmortem ageing. Meat Science 2011;87:165-74.

[123] García FIH, Cruz AMR. Nuevo tratamiento de induccion y sincronizacion ovulatoria que posibilita la concentracion de parideras y la utilizacion practica de la inseminacion artificial en vacuno retinto. ITEA, Información Técnica Económica Agraria: revista de la Asociación Interprofesional para el Desarrollo Agrario (AIDA) 2005;26:416-8.

[124] Huang F, Huang M, Zhang H, Zhang C, Zhang D, Zhou G. Changes in apoptotic factors and caspase activation pathways during the postmortem aging of beef muscle. Food Chemistry 2016;190:110-4.

[125] Kemp CM, Bardsley RG, Parr T. Changes in caspase activity during the postmortem conditioning period and its relationship to shear force in porcine longissimus muscle. Journal of Animal Science 2006;84:2841-6.

[126] Kemp CM, King DA, Shackelford SD, Wheeler TL, Koohmaraie M. The caspase proteolytic system in callipyge and normal lambs in longissimus, semimembranosus, and infraspinatus muscles during postmortem storage. Journal of Animal Science 2009;87:2943-51.

[127] Herrera-Mendez CH, Becila S, Boudjellal A, Ouali A. Meat ageing: Reconsideration of the current concept. Trends in Food Science and Technology 2006;17:394-405.

[128] Wang L, Han L, Ma X, Yu Q, Zhao S. Effect of mitochondrial apoptotic activation through the mitochondrial membrane permeability transition pore on yak meat tenderness during postmortem aging. Food Chemistry 2017;234:323-31.

[129] Wang L, Ma G, Zhang Y, Shi X, Han L, Yu Q, Zhao S, Ma J. Effect of mitochondrial cytochrome c release and its redox state on the mitochondrial-dependent apoptotic cascade reaction and tenderization of yak meat during postmortem aging. Food Research International 2018;111:488-97.

[130] Wang L, Yu Q, Han L, Ma X, Song R, Zhao S, Zhang W. Study on the effect of reactive oxygen species-mediated oxidative stress on the activation of mitochondrial apoptosis and the tenderness of yak meat. Food Chemistry 2018;244:394-402.

[131] Eusebi PG, Sevane N, Cortés O, Contreras E, Cañon J, Dunner S. Aggressive behavior in cattle is associated with a polymorphism in the MAOA gene promoter. Animal Genetics 2020;51:14-21.

[132] Ruiz Tena JL. Fomento, conservación y ordenación de las razas autóctonas españolas. In: El Arca: Boletín de la Sociedad Española para los Recursos Genéticos Animales, editor. Córdoba: Universidad de Córdoba, Servicio de Publicaciones; 1999, p. 18-27.

[133] Oliván M, Martínez A, Osoro K, Sañudo C, Panea B, Olleta JL, Campo MM, Oliver MÀ, Serra X, Gil M, Piedrafita J. Effect of muscular hypertrophy on physico-chemical, biochemical and texture traits of meat from yearling bulls. Meat Science 2004;68:567-75.

[134] Fiems LO. Double muscling in cattle: genes, husbandry, carcasses and meat. Animals 2012;2:472-506.

[135] Zhu C, Wang X, Xu F, Bahr BA, Shibata M, Uchiyama Y, Hagberg H, Blomgren K. The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. Cell Death and Differentiation 2005;12:162-76.

[136] Swanson JC, Morrow-Tesch J. Cattle transport: Historical, research, and future perspectives. Journal of Animal Science 2001;79:E102-9.

[137] Warriss PD, Kestin SC, Brown SN, Wilkins LJ. The time required for recovery from mixing stress in young bulls and the prevention of dark cutting beef. Meat Science 1984;10:53-68.

[138] Mäki-Tanila A. An overview on quantitative and genomic tools for utilising dominance genetic variation in improving animal production. Agricultural and Food Science 2007;16:188-98.

[139] Peric T, Comin A, Corazzin M, Montillo M, Cappa A, Campanile G, Prandi A. Short communication: Hair cortisol concentrations in Holstein-Friesian and crossbreed F1 heifers. Journal of Dairy Science 2013;96:3023-7.

SECTION II

CONCLUSIONS

Based on the experimental results obtained in the present Ph.D. Thesis, the following conclusions were drawn:

1. The use of liquid isoelectric focusing pre-fractionation/enrichment method combined with sodium dodecyl sulphate polyacrylamide gel electrophoresis and coupled to liquid chromatography – mass spectrometry analysis facilitated the study of sarcoplasmic and myofibrillar sub-proteomes from bovine meat through the identification of several marker proteins. In addition, the high reproducibility of this methodology allowed the discrimination of normal and high pHu meat samples through the quantitative analysis of key marker proteins found in both, sarcoplasmic and myofibrillar muscle extracts.
2. The development and optimization of a *gel-free* methodology based on liquid isoelectric focusing enrichment directly coupled to liquid chromatography – mass spectrometry analysis proved to be successful to perform untargeted qualitative and automated targeted quantitative analysis of muscle sarcoplasmic proteome. Moreover, this fractionation greatly facilitated protein mapping and peptide quantification of bovine sarcoplasmic extracts despite of using a conventional three dimensional ion-trap.
3. The development and implementation of an innovative *gel-free* strategy based on direct analysis of sarcoplasmic protein extracts coupled to liquid chromatography – mass spectrometry analysis allowed the discrimination of normal and high pHu meat samples using untargeted qualitative/quantitative and targeted quantitative approaches based on selected reaction monitoring strategies.
4. The study of sarcoplasmic sub-proteome revealed overabundance of several heat shock proteins in high pHu meat samples independently of the methodology used. This could be associated to animals that have presumably suffered pre-slaughter stress condition and the activation of the apoptosis process.

5. The optimization of a fluorogenic assay based on the enzymatic release of a fluorophore from a synthetic and specific peptide substrate allowed the measurement of caspase 3/7 activity in skeletal muscle extracts characterized by low caspase activity level and proved to be successful to investigate the apoptosis process through the measurement of caspase 3/7 and 9 activities at different *post mortem* times.
6. Different management practices evidenced differences in caspase 3/7 and 9 activities over *post mortem* time. The highest caspase 3/7 and 9 activities were observed in meat samples from Asturiana de los Valles and Retinta breeds and in meat samples from mixed Rubia Gallega breed that could be associated to animals suffered pre-slaughter stress.
7. The optimised fluorogenic assays of caspase 3/7 and 9 activities allowed a clear discrimination between normal and high pH meat samples at 24 h *post mortem*. This confirms the usefulness of caspase 3/7 and 9 activities as new indicators to detect the occurrence of high pHu meats.
8. Proteomic tools and enzymatic assays proved their potential to accurately evaluate proteome changes and the apoptotic processes occurring during the conversion of muscle to meat.
9. These results provide more accurate and valuable information about the relationship between biochemical characteristics of meat, animal handling practises and final meat quality traits. This will help stakeholders to establish a link between sustainable animal handling practises and production of high quality meats.

SECTION III

1 APPENDIX I PUBLICATION I

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Search for proteomic biomarkers related to bovine pre-slaughter stress using liquid isoelectric focusing (OFFGEL) and mass spectrometry[1]

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Abstract

Proteome changes derived from animals that have suffered pre-slaughter stress are a fact. In this study, Proteomic analysis was carried out on 20 bovine loin samples from Asturiana de los Valles and crossbreds cattle previously classified as normal and DFD meat at 24 h post-mortem using pH measurements. Sarcoplasmic sub-proteome of *Longissimus thoracis* at 24 hours post-mortem was fractionated by the use of liquid isoelectric focusing (OFFGEL) in the pH range 3-10, followed by SDS-PAGE analysis of each retrieved fraction. The protein fractionation profile showed high reproducibility along the different sample groups. Five protein bands showed significant differences ($p<0.05$) between the two groups, allowing discrimination between them. Proteins present in these bands, which were identified by LC-MS, were actin, phosphoglucomutase-1, alpha-crystallin B, heat shock protein beta-6 and heat shock protein beta-1.

Significance: The significance of this study relies on the optimization of OFFGEL fractionation as a promising technology to search for reliable biomarkers of pre-slaughter stress. This method separates proteins along different liquid fractions according to their isoelectric point; the obtained fractions can be further characterized by SDS-PAGE or directly identified by LC-MS. This achievement stands out as an alternative to the use of 2-DE electrophoresis in protein separation and analysis.

Keywords

DFD Meat ● Pre-Slaughter Stress ● OFFGEL● Protein Biomarkers ● Mass Spectrometry

1.1 INTRODUCTION

Proteomics has thoroughly sought a deep understanding of gene expression and protein translation. This discipline concerns protein interactions or modifications (phosphorylation, denaturation, methylation, etc) but also the biochemical pathways that take place under different conditions in living organisms [1]. It has been widely used to search for protein biomarkers related to meat tenderness, colour and water holding capacity (WHC) [2]. However, few research efforts have been put in the discovery of pre-slaughter stress biomarkers leading to discriminate among meat from either normal or stressed animals as stated by Franco et al. [3], reporting for the first time the usefulness of Proteomics to study pre-slaughter stress condition (PSS) in Rubia Gallega cattle breed.

PSS arises from both intrinsic and extrinsic factors of slaughtered animals. Among the intrinsic factors, physiology, age, sex and genetics are among the most relevant. Regarding extrinsic stressors such as temperature, handling activities, human presence, lairage time or feed/water deprivation in the abattoir [4] they are not only prevalent but also manageable. Before slaughter, animals exposed to PSS consume their glycogen reservoirs to produce ATP through glycolysis. This perturbs glycolysis after slaughter, lowering its rate due to the prior glycogen reduction and generating not enough lactic acid to enable a normal pH reduction, thus giving rise to higher pH values like 6.0 and above, which evolves in an abnormal conversion of muscle into meat [5]. This condition produces dark, firm and dry (DFD) meat, which causes economical losses in the meat industry because of its lower quality attributes such as alterations in tenderness during aging, higher WHC or spoilage by microorganism contamination [6]. To reduce the incidence of DFD meat, current practices performed by meat producers, food industry and governments consider the improvement of animal welfare regarding their transportation, lairage, handling at abattoirs and slaughter methods.

Although there are many biochemical indicators (glucose, lactate, pH, etc.) that can be quickly measured to assess animal stress [7], they do not provide further information about biological changes occurring due to PSS. In this respect, Proteomics has become a powerful tool to unveil reliable protein biomarkers to detect meat quality defects [8].

Skeletal muscle houses a huge variety of proteins with different roles such as structural, defensive/stress and metabolic functions [9]. Some of them have been suggested as stress biomarkers, as the case of creatine kinase and lactate dehydrogenase in animals that have suffered pre-slaughter stress [10]. Increased levels of these enzymes in plasma and blood would be indicative of muscle damage due to stressor factors [11]. Moreover, Heat Shock Proteins (HSPs) normally involved in defence mechanisms, have been also studied in relation to stress. It should be emphasized that HSPs play a major role as essential molecular chaperones under

environmental and physiological stress conditions [12,13].

Regarding protein fractionation techniques, the most common approach was sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by two-dimensional polyacrylamide gel electrophoresis (2-DE) [14]. Despite the popularity of gel-based strategies, novel gel-free alternatives have recently been proposed to overcome drawbacks of traditional fractionation concerning costs in routine analysis (mainly 2-DE) and limitations for covering the entire proteome (small biomolecules) of samples. One of these emerging technologies is the liquid isoelectric focusing (OFFGEL) that separates proteins according to their isoelectric point (p*I*) [15]. Thus, a multi-compartment chamber with 12 or 24 wells is placed on top of an immobilized pH gradient gel (IPG) strips that provides the pH gradient. Solubilised proteins migrate across IPG strips until meeting the pH corresponding to their respective p*I* and stop. Time required for fractionation depends on sample complexity, reaching up to 24 h. Retrieved liquid fractions containing fractionated/enriched proteins can be submitted to further gel-based separation steps or directly digested and analysed by liquid chromatography coupled to mass spectrometry (LC-MS).

Advantages in fractionation/enrichment of protein samples using OFFGEL were recently demonstrated [16,17] and promising results were also achieved when it was implemented in studies assessing safety, quality and origin of meat and meat products [18,19,20]. However, its use in the fractionation/enrichment of muscle proteome is still limited.

This work aims to study changes in the sarcoplasmic proteome of meat to elucidate which proteins and biochemical mechanisms are involved in PSS. Then, normal and DFD samples of bovine *Longissimus thoracis* (LT) muscle were analysed by OFFGEL fractionation/ enrichment followed by SDS-PAGE and LC-MS analysis. The proposed methodology would facilitate the elucidation of different proteins as reliable PSS biomarkers that could be useful to assess quality of commercialized meat and meat products.

1.2 MATERIALS AND METHODS

1.2.1 Animal samples

In this research, a total of twenty (n=20) beef samples were analysed. Eight 'normal' meat samples with pH values lower than 5.5 at 24 h post-mortem were collected from Asturiana de los Valles (AV) breed. Another twelve samples were collected from crossbred animals (AV x Friesian) from which six samples were considered as 'DFD' meat (PSS-CB: pre-slaughter stressed crossbred animals), with pH values over 6.0, and the other six were considered as 'normal' meat (N-CB: normal crossbred animals), with pH values below 6.0. These made a 'normal' meat group of 14 samples, and a 'DFD' meat group of 6 samples. The pH measurements were taken at the

Longissimus thoracis (LT) muscle of the 6th rib at 24 h post-mortem.

Animals were slaughtered and dressed in commercial abattoirs at the age of 12 to 18 months (yearling bulls) following the safety and welfare conditions established by the European Union (Council Regulation (EC) No 853/2004 and No 1099/2009). At 2 h post-mortem, a LT muscle sample from the 13th rib was excised from the left half of each carcass. After muscle sampling, the epimysium was carefully dissected, and about 10 g of each muscle sample were taken. Samples were then transported to the laboratory in refrigerated conditions and, at 24 h post-mortem, they were frozen in liquid nitrogen and stored at -80 °C until analysed.

1.2.2 Extraction and quantification of sarcoplasmic proteins

Half a gram of each type of meat, normal and DFD, was homogenized in 4 mL of extraction buffer (10 mM Tris pH 7.6, 1 mM EDTA, 0.25 M Sucrose) containing 25 μL of inhibitors cocktail (P8340, Sigma-Aldrich Co., St. Louis, MO, USA) using an Ultra-Turrax Yellow Line Di 25 (IKA-Werke GmbH, Staufen, Germany). The homogenate was centrifuged at 20,000 *g* for 20 min at 4 °C using a Beckman Coulter centrifuge (Brea, CA, USA), being the obtained supernatant filtered through a 45 μm PVDF syringe filter and stored at -80 °C until analysed. Protein quantification of samples was assessed using the Bio-Rad Protein Assay Kit (Hercules, CA, USA) following the Bradford method and using a calibration curve ranging from 0.05 to 0.8 mg/mL of BSA.

1.2.3 Liquid isoelectric focusing (OFFGEL)

Sarcoplasmic proteins were fractionated according to their p*I* into 12 liquid fractions using 13 cm IPG strips with a linear gradient in the pH range 3-10 (GE healthcare, Uppsala, Sweden). One mg of each sarcoplasmic extract was diluted to a final volume of 2 mL with 1.25X protein OFFGEL stock solution (6% v/v glycerol, 7 M urea, 2 M thiourea, 65 mM DTT and IPG buffer pH 3-10, GE healthcare, Uppsala, Sweden) and used for protein fractionation/enrichment using an Agilent 3100 OFFGEL fractionator (Agilent Technologies, Waldbronn, Germany) following the manufacturer's instructions. IPG strips were fixed onto the tray and rehydrated with 1.25X protein OFFGEL stock solution. Fractionation was performed at 20 °C with a constant current of 50 μA to reach 20 kVh for about 20 h (depending on salt concentration of samples). After focusing, the 12 fractions were separately collected and stored at -80 °C.

1.2.4 SDS PAGE and image analysis

SDS-PAGE analyses were carried out in duplicate (analytical replica) for each of the twenty fractionated muscle extracts. OFFGEL protein samples were diluted (50:50, v/v) with a sample buffer solution (0.088 M Tris-HCl pH 6.8, 10% v/v glycerol, 2% w/v SDS, 0.2 M DTT and 0.04 w/v bromophenol blue) and heated at 95 °C for 4 min to denaturalize proteins. Samples

were centrifuged (2,000 *g*, 1 min) before loading onto 1.5 mm x 8 cm x 9 cm 12% polyacrylamide gels. Each pair of gels was simultaneously run in a Hoefer Mighty Small II SE260 electrophoresis unit (San Francisco, CA, USA) at a constant current of 50 mA for two hours. After electrophoresis, gels were fixed into 12% trichloroacetic acid solution for 1 h, then washed two times with bidistilled water for 10 min. Finally, gels were stained with colloidal Blue Coomasie [21] overnight and finally distained with bidistilled water. For scanning gels, LAS-1000 Luminescent Image Analyzer with Intelligent Dark Box II (FUJIFILM, Barcelona, Spain) was used. The intensity of each band was determined using the freeware Gel Analyzer 2010 software (http://www.gelanalyzer.com). Individual band intensities were normalized according to the total band volume per gel. Estimated p*I* and molecular mass (Mr) of each band was determined from their position in the OFFGEL fractions and their migration in the polyacrylamide gel, respectively, comparing to commercial standards ranging from 14 to 200 kDa (Cat. No. 161-0317, Bio-Rad, Hercules, CA, USA).

1.2.5 *In gel* digestion of selected protein bands

Those bands showing statistically significant intensity differences between normal and DFD samples were digested with trypsin to elucidate their protein profiles. Thus, bands were excised from the gel and placed into 0.5 mL Eppendorf tubes. Bands were washed twice in 50 mM ammonium bicarbonate for 10 min in continuous agitation and solvent discarded by pippeting. Bands were dried until turning shrink and opaque by washing twice with 100% ACN. Once the liquid was removed by pippeting, the remaining liquid was evaporated using a Savant SPD121P Speed Vac concentrator equipped with a RVT400 refrigerated vapour trap (Thermo Scientific, San Jose, CA, USA). Dried samples were digested with 15 μL of 12.5 μg/mL sequencing grade modified trypsin (Promega, Madison, WI, USA) solution and mixed with 50 μM ammonium bicarbonate (pH 8.5) overnight at 37 °C with continuous gently shaking. Peptides were recovered by washing samples twice with water/ACN (50:50, v/v) acidified with 0.1 % TFA, sonicated for 15 min, the supernatants poured into clean Eppendorf tubes and evaporated as previously mentioned. Finally, samples were redissolved in 40 μL of a 0.1 % aqueous TFA solution and poured into LC-MS vials.

1.2.6 Mass spectrometry analysis

Twenty-five μL of each sample prepared as described in section 2.5 were injected in a Thermo Surveyor LC system equipped with a refrigerated autosampler and coupled to a Thermo LCQ Advantage (Thermo Scientific, San Jose, CA, USA) ion trap MS instrument loading an electrospray (ESI) probe. Separation of peptides was carried out on a Jupiter Proteo reversed-phase C18 column (15.0 cm × 0.5 mm; Phenomenex, Torrance, CA,

USA). Mobile phases consisted of solvent A containing 0.1% formic acid (v/v) in bidistilled water and solvent B containing 0.1 % formic acid (v/v) in ACN. Chromatographic analysis was at 23 °C and separation conditions consisted of stepwise isocratic gradients including 15 min of 0% B, 5 min of 20% B, 10 min of 40 %B and finally 10 min of 100% B at a flow rate of 30 μL/min, then returning to initial conditions having 0% of solvent B.

Operating parameters of the ion trap detector were the following: electrospray ionization in positive mode; capillary temperature, 250 °C; spray voltage, 4.0 kV; capillary voltage, 15.0 V. First scan event was full MS detection in the 400–2000 *m/z* range. The second scan event was a data dependent MS/MS analysis of the most intense ions with charges from +2 to +4 with the following parameters: repeat count for most intense ion, 3; repeat count duration, 0.5 min; exclusion duration, 10 min; normalized collision energy, 35%; minimum MS signal for MS/MS analysis, $1x10^5$. Both scan events shared the same number of microscans and maximum injection time that were 3 and 300 ms, respectively. Control of the LC-MS system and data acquisition was done through a PC loading the Thermo Xcalibur v2.0 (Thermo Scientific, San Jose, CA, USA) software. Peptide identification was achieved by the interpretation of generated MS/MS data against Uniprot KB v2017_11 (www.uniprot.org) and NCBIprot v20170428 protein databases using Mascot v2.3 as search engine with the following parameters: enzyme, trypsin; no fixed or variable modifications but "Error tolerant" option enabled; mass accuracy was set to 1.2 and 0.8 Da for MS and MS/MS analyses, respectively; The option "Mammalia" was selected as taxonomy restriction parameter; decoy option was used to estimate the false positive rates by means of False Discovery Rate threshold from 1%. For selection of protein family identifications obtained from Mascot, only those having a protein score derived from individual ion scores indicating identity or extensive homology ($p<0.05$) were considered as true protein identifications.

1.2.7 Statistical and protein functional analysis

Obtained results were analysed using univariate and multivariate statistics. The univariate Mann-Whitney non-parametric test was applied to find significant differences between normal and DFD sample groups ($p<0.05$) using SPSS 23.0.0 software (SPSS Inc., Chicago, IL, USA). The intensity of each of the selected protein bands of the fourteen (normal) and six (DFD) individuals were compared. For multivariate analysis of data, principal component analysis (PCA) was utilised using the XLSTAT 2010.5.02 software (Addinsoft, Paris, France). The two-dimensional coordinate system defined by the first two principal components was used to study the distribution of each animal according to the intensity of selected protein bands.

Each identified protein was classified according to its biological function by means of Gene Ontology (GO) term from AmiGO website: (http://amigo.geneontology.org/amigo/).

Protein-protein interactions from bands that showed significantly different abundances between normal and DFD samples were assessed using STRING v.10.5 freeware software (ELIXIR, Wellcome Genome Campus, Hinxton, Cambridgeshire, UK, https://string-db.org). *Bos taurus* species was used to perform the analysis.

1.3 RESULTS AND DISCUSSION

The twenty bovine meat samples analysed in this work were classified as normal and DFD according to pH values measured at 24 h post-mortem (data not shown). The main aim of this study was to compare normal and DFD animal's proteome using liquid isoelectric focusing in combination with SDS-PAGE and LC-MS.

After OFFGEL separation, 12 protein fractions were obtained for each sample along the assessed pH range (3 to 10). These were further analysed by SDS-PAGE in order to reveal the protein distribution along these fractions.

Fig. 1 shows representative examples of SDS-PAGE gels of the fractionated sarcoplasmic sub-proteome from normal (1A) and DFD (1B) meats. Protein distribution profiles achieved by OFFGEL separation/ enrichment were highly reproducible in all retrieved fractions, enabling the study of differences between normal and DFD groups. In both cases, acidic fractions (1 to 5) housed only few proteins in contrast to the most populated neutral and basic fractions (6 to 12).

The most noticeable differences in protein abundances between normal and DFD gels were observed in bands A to E (squared in **Fig. 1A and 1B**). This finding was confirmed by densitometry analysis (**Fig. 2**) that revealed significant differences (p<0.01) among both meat groups. So, abundance of band B was notoriously higher in normal samples, whereas band A was significantly less abundant in the normal group in comparison to DFD meat. Interestingly, it was observed that bands C, D and E were exclusive of the DFD group.

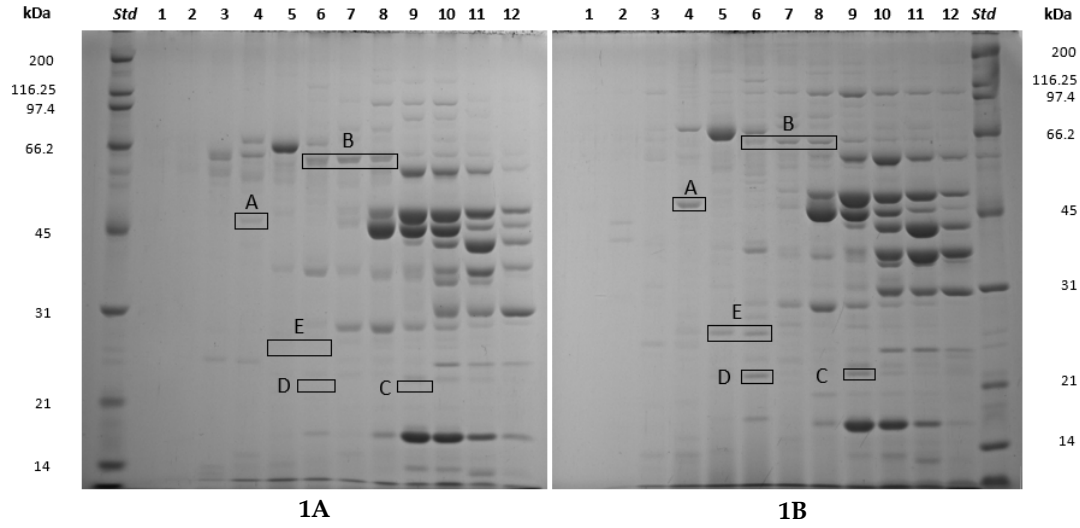

Fig. 1. 12 % SDS-PAGE of fractions obtained after OFFGEL isoelectric focusing in the pH range 3-10 from two representative samples: 1A) normal and 1B) DFD meat. *Std*: Molecular standard.

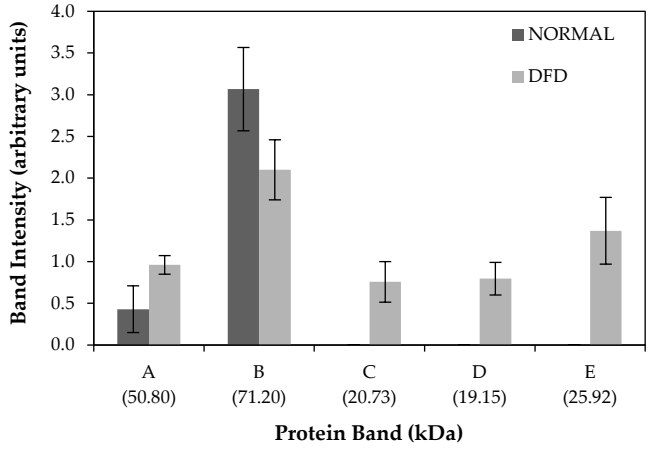

Fig. 2. Mean values of abundances (and standard deviations) of the five selected protein bands obtained by densitometry analysis (described in **Table 1**) in normal (dark, n=14) and DFD (light, n=6) meat sample groups. Error bars indicate the standard deviation for each group.

Further LC-MS analysis of bands with the same molecular mass clustered in neighboring fractions (bands B and E in **Fig. 1**) was carried out to confirm same protein assignment for each particular band (**Table 1**). This outcome was previously reported [16] and seemed to be related to protein phosphorylation, which induces changes in the p*I* value of the protein. Both phosphoglucomutase-1 (PGM1; band B in **Fig. 1**) and heat shock protein beta-1 (HSPB1; band E in **Fig. 1**) have a phosphorylation site. Thus, their degree of phosphorylation could distribute them over different OFFGEL fractions according to their final p*I* value. Furthermore, PGM1 participates in the transference of the phosphate group from

glucose-1-P to glucose-6-P as well as HSPs can suffer a post-translational phosphorylation that may alter their p*I* value [22].

Table 1. Proteins from bovine *Longissimus thoracis* muscle showing significant differences between normal and DFD meat that were identified by LC-ESI-MS/MS using the MASCOT search engine and NCBInr and Uniprot KB protein databases. All identified proteins belonged to *Bos taurus* species.

Band	Protein Identification/ Gene[a]	Theoretical[a]/ apparent Mr (kDa)[b]	Theoretical[a]/ apparent p*I* (pH value in the OFFGEL fraction)	Accession number[c] (UNIPROT)	Mascot Score[a]	Protein coverage[a] (%)	*p*-value[d]	Biological function
A	Actin ACTA1	42.02/50.80	5.23	5.05-5.53	P68138	388	25	<0.01	Structural maintenance
B	Phosphoglucomutase-1 PGM1	61.55/71.20	6.36	6.02-7.47	Q08DP0	366	19	<0.01	Regulation of glycogen metabolism
C	Alpha-crystallin B CRYAB	20.02/20.73	6.76	7.47-7.95	P02510	388	43	<0.01	Stress resistance
D	Heat shock protein beta-6 HSPB6	17.46/19.15	5.95	6.02-6.50	Q148F8	418	61	<0.01	Stress resistance
E	Heat shock protein beta-1 HSPB1	22.38/25.92	5.98	5.53-6.50	Q3T149	613	43	<0.01	Stress resistance and actin organization

[a] Protein and gel identification, theoretical Mr and p*I*, Mascot Score, Coverage and Nº. of peptides indentified were remove from Swiss-Prot/UniProt KB database.
[b] Apparent Mr was calculated through band position of the gel.
[c] Accession number was derived from UNIPROT database.
[d] *p*-value was estimated using Mann-Whitney test.

Fig. 3 illustrates PCA representation of factor scores of each sample (animal) studied according to the intensity of selected protein bands (A to E). The first component (PC1) explained 76.23% of the total variability found among beef samples and clearly discriminated between normal and DFD groups in which AV and N-CB had negative factor scores, while PSS-CB had positive factor scores. Among the normal sample group, all individuals (AV and N-CB) were mixed in the left side of the PC1 and it was observed there was no separation between AV and N-CB groups neither through PC1 nor PC2. Then, we can conclude that breed effect does not have remarkable influence on the normal sample group, being this factor not relevant in our study of searching for biomarkers related to PSS.

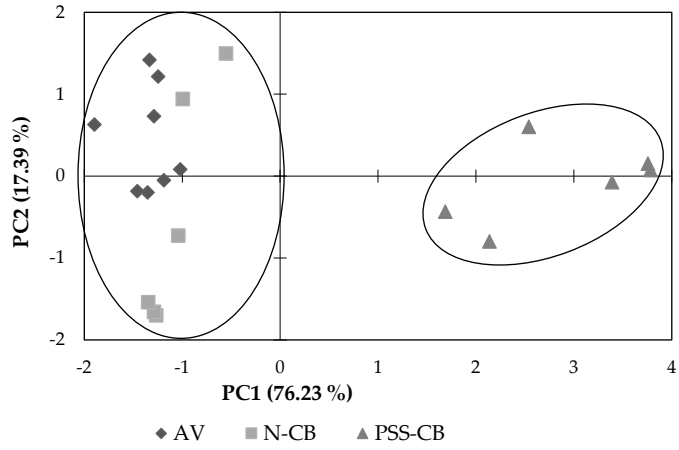

Fig. 3. Animal sample distribution on the two-dimensional coordinate system defined by principal component (PC) 1 and 2. Each point represents an individual sample (♦ AV: Asturiana de los Valles animals; ■ N-CB: normal crossbred animals; ▲ PSS-CB: pre-slaughter stressed crossbred animals).

1.3.1 Functional analysis of identified proteins

As mentioned, LC-MS study characterized proteins from bands A to E, being classified (**Table 1**) according to their biological role as stress (alpha-crystallin B, heat shock protein beta-6 and heat shock protein beta-1), metabolic (phospho-glucomutase-1) and structural (actin) proteins. Among those proteins and under normal conditions (pH below 6.0 after slaughter), actin is the only one that exhibits a poor aqueous solubility even if a small part of it can be extracted together with sarcoplasmic proteins [9,23,24]. In contrast, pH values of DFD meat (above 6.0) are far enough from the p*I* of actin (5.23), enhancing its solubility and turning more noticeable the presence of this protein in the soluble extracts of stressed animals (see **Fig. 1** and **2**).

Protein-protein interaction network of the five characterized bovine proteins is depicted in **Fig. 4**. Phosphoglucomutase-1 (PGM1) and heat shock protein beta-6 (HSPB6) remain out of the protein network, with no interaction between these proteins and the others. PGM1 plays an important role in the regulation of

glycogen metabolism through the inter-conversion of glucose-1-phosphate to glucose-6-phosphate. This latter enters the glycolytic pathway to produce ATP in order to maintain cell homeostasis [25]. The catalytic site of phosphoglucomutase-1 molecule contains a phosphorylated serine residue at position 117 of its polypeptide chain. The phosphoryl group is transferred to the C-6 hydroxyl group of glucose 1-phosphate to form glucose 1,6-biphosphate. The C-1 phosphoryl group of this intermediate is then shifted to the same serine residue, resulting in the formation of glucose 6-phosphate and the regeneration of the phosphoenzyme.

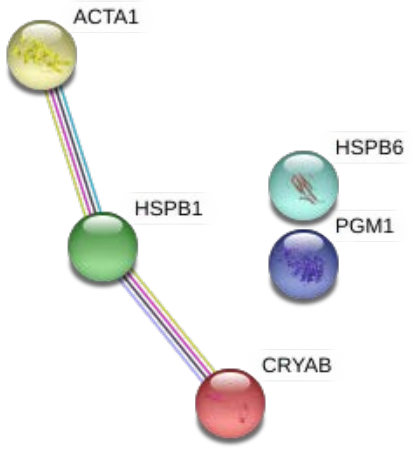

Fig. 4. Protein-protein interaction network of five selected protein bands in normal and DFD meats using String 10.5. The network nodes (circles) represent proteins, the edges represent protein-protein functional associations, line colour indicates the type of interaction and line thickness denotes the association strength (threshold: 0.4, medium confidence level). ACTA1: Actin; HSPB1: Heat shock protein beta-1; CRYAB: Alpha-crystallin B; HSPB6: Heat shock protein beta-6; PGM1: Phosphoglucomutase-1.

Heat shock protein beta-1 (HSPB1) is in the centre of the network node and is related to alpha-crystallin B (CRYAB) and actin (ACTA1). Although HSPB6 is not linked to HSPB1 and CRYAB, this protein is well known in terms of its function, so we could conclude that the database lacks of information to relate this protein to the other two. All of them belong to the heat shock protein family and have been widely described as molecular chaperones involved in several mechanisms (protein assembly and disassembly, protein folding and unfolding, translocation, ...) and can interact with damaged proteins under stressed conditions to preserve their function [26]. Some authors have associated HSPB6 to meat tenderness [27] and apoptosis pathway [28], although the mechanism involved remains unclear.

Interaction between actin and heat shock protein beta-1 comes from the protective action exerted by HSPs under stress conditions as reported by Jia et al. [29], detailing their patronizing effect on actin filaments against aggregation promoted by oxidative stress. However, it has been stated that increased phosphorylation of mammalian HSPs can appropriately modify the oligomeric conformation of proteins, turning them a more stable resultant forms and protecting against stress [30].

1.3.2 Relationship between protein abundance and meat quality derived from PSS

Tenderness is one of the most relevant traits of meat quality together with juiciness and flavour, and many efforts have been carried out to understand the mechanisms involved [27]. This attribute is

influenced by a wide variety of both pre-slaughter (animal species, breed, age, muscle type and handling activities) and post-mortem (pH, WHC, sarcomere shrinkage, temperature and proteolysis of myofibrillar proteins) factors [31]. Some studies reported that DFD meat from animals that suffered PSS was significantly more tender than normal meat at 24 h post-mortem [3,32]. Abnormally higher pHs (>6.0) reached in DFD meat may contribute to an increased tenderness in different ways. Firstly, raising the electrostatic repulsion between myofibrillar proteins confers less lateral shrinkage of muscle fibres [33]. Secondly, overall protein denaturation is lower and stimulates the water-binding capacity of myosin. We can conclude that both mechanisms characterize DFD meat with higher WHC.

There was reported the importance of phosphoglucomutase-1 on meat and fish quality regarding tenderness [8,34]. This enzyme seems to be downregulated in tender meats as demonstrated by Ciaramella et al. [35] in stressed catfish and by Picard et al. [36] in tender compared to tough beef steaks. In this line, other studies stated that DFD meat is more tender than normal meat at 24 h post-mortem [3,32]. All those results are in agreement with findings illustrated by **Fig. 1** and **2** regarding higher abundance of PGM1 in normal compared to DFD samples. This can be understood considering the potential nine phosphorylation sites of PGM1, one of them involved in the glycogen pathway that seems to play an important role in meat tenderization [34]. In this study, this enzyme spread along three different OFFGEL fractions (lanes 6, 7 and 8 in **Fig. 1** A and B) in both sample groups due to phosphorylation. More concretely, one peptide of PGM1 was observed to be phosphorylated at Ser-117 of the protein chain (**Table 2**) in these fractions for both normal and DFD meat. This suggested the activation of the catalytic site of PGM1 that enabled its participation into the aforementioned glucose interconversion via glycolysis to produce ATP. The lower abundance of this protein in DFD samples (**Fig. 2**) could be related to glycogen depletion before slaughter due to PSS, notably reducing the metabolism of the enzyme.

Table 2. Phosphorylated peptides identified from PGM1 in both normal and DFD meat from the analysis of MS/MS data using MASCOT search engine and NCBInr and Uniprot KB protein databases. Underlined aminoacids correspond to those that have suffered phosphorylation.

Fraction	Protein identified	Specific sequence showing a phosphorylated position[a]	Peptide Score[1]	Nº of peptides identified[b]	Precursor ion m/z	Charge	Probability[d] (%)
6	Phosphoglucomutase-1 PGM1	^{108}K.AIGGIILTA<u>S</u>HNPGGPNGDFGIK.F^{130} + phosphorylation (S117)	57	2	1144.15	2+	81.78
7	Phosphoglucomutase-1 PGM1	^{108}K.AIGGIILTA<u>S</u>HNPGGPNGDFGIK.F^{130} + phosphorylation (S117)	56	4	1143.27	2+	59.50
8	Phosphoglucomutase-1 PGM1	^{108}K.AIGGIILTA<u>S</u>HNPGGPNGDFGIK.F^{130} + phosphorylation (S117)	44	2	1143.71	2+	100

[a] Superscripts indicate the position of the peptide into the PGM1 protein sequence.
[b] Peptide score was derived from the higher score value among all phosphorylated peptides identified.
[c] Nº of peptides identified is referring to phosphorylated peptides in their serine 117.
[d] It is referring to the probability that the modification occurs in that specific amino acid of the sequence that it has been studied.

Although the action of HSPs has been extensively studied in living muscle, their role in post-mortem muscle is still unclear. In this study, the three stress-related proteins indentified (bands C, D and E in **Table 1**) were only found in DFD meat samples (**Fig. 1**). This finding seems to agree with evidence that stress can induce the synthesis of protective proteins such as HSPs to preserve cellular proteins against denaturation and possible loss of function [37] in living animals. Furthermore, there is an existing controversy regarding the pro- or anti-apoptotic role of HSPs, suggesting that their function in apoptosis may depend on its phosphorylation state. Ouali et al. [27] proposed apoptosis as a novel way to explain meat tenderisation and suggested that HSPs might delay meat aging, negatively affecting meat tenderness. Several mechanisms were also reported in which stress-generated HSPs could play diverse roles as anti-apoptotic factor by complexation with active caspases, protection of target proteins and restoration of the active structure of proteins [38]. This is in accordance to results obtained by Rosa et al. [39] for HSPB6 since they reported a negative correlation between the expression of this protein and tenderness. In contrast, these same authors found that HSPB1 was positively correlated with beef tenderness in LT muscle, in accordance to results obtained by Morzel et al. [40] and Carvalho et al. [41]. As observed, despite many studies addressing HSPs, stress and meat tenderness, relevance of these proteins in post-mortem muscle and meat quality still remains unclear and deserves further investigations.

1.4 CONCLUSIONS

The comparison of sarcoplasmic sub-proteomes of normal and DFD meat samples was successfully achieved using liquid isoelectric focusing (OFFGEL) hyphenated to SDS-PAGE and LC-MS analyses. Five bands associated to proteins actin, phosphoglucomutase-1, alpha-crystallin B, heat shock protein beta-6 and heat shock protein beta-1, with different abundances and biological functions, were found for normal and DFD meat groups and firstly proposed as biomarkers of the PSS condition.

The innovative proteome fractionation approach presented in this work can constitute an interesting alternative to traditional 2-DE electrophoresis. Highly reproducible protein profiles achieved by implementing OFFGEL fractionation enabled the accurate comparison of the proteome from different meat quality groups.

This research may facilitate future studies addressing the biological role and post-translational modification of these biomarkers under specific stress conditions, as well as the direct implementation of the OFFGEL strategy in gel-free approaches supported by LC-MS analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

[1] Bendixen E. The use of proteomics in meat science. Meat Sci 2005;71:138-149.

[2] Lana A, Zolla L. Proteolysis in meat tenderization from the point of view of each single protein: A proteomic perspective. J Proteomics 2016;147:85-97.

[3] Franco D, Mato A, Salgado FJ, López-Pedrouso M, Carrera M, Bravo S, Gallardo JM, Zapata C. Tackling proteome changes in the longissimus thoracis bovine muscle in response to pre-slaughter stress. J Proteomics 2015;122:73-85.

[4] Ferguson DM, Warner RD. Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants? Meat Sci 2008;80:12-19.

[5] McVeigh J, Tarrant P, Harrington M. Behavioral stress and skeletal muscle glycogen metabolism in young bulls. J Anim Sci 1982;54:790-795.

[6] Newton KG, Gill CO. The microbiology of DFD fresh meats: A review. Meat Sci 1981;5:223-232.

[7] Averós X, Martin, S, Riu, M, Serratosa, J, Gosalvez LF. Stress response of extensively reared young bulls being transported to growing-finishing farms under Spanish summer commercial conditions. Livest Sci 2008;119:174-182.

[8] Schilling MW, Suman SP, Zhang X, Nair MN, Desai MA, Cai K, Ciaramella MA, Allen PJ. Proteomic approach to characterize biochemistry of meat quality defects. Meat Sci 2017;132:131-138.

[9] Bjarnadóttir SG, Hollung K, Færgestad EM, Veiseth-Kent E. Proteome changes in bovine longissimus thoracis muscle during the first 48 h postmortem: shifts in energy status and myofibrillar stability. J Agri Food Chem 2010;58:7408-7414.

[10] Chulayo A, Muchenje V. Activities of some stress enzymes as indicators of slaughter cattle welfare and their relationship with physico-chemical characteristics of beef. Animal 2017;11:1645-1652.

[11] Wickham SL, Collins T, Barnes AL, Miller DW, Beatty DT, Stockman C, Fleming PA. Qualitative behavioral assessment of transport-naïve and transport-habituated sheep. J Anim Sci 2012;90:4523-4535.

[12] Larkins NT, Murphy RM, Lamb GD. Influences of temperature, oxidative stress, and phosphorylation on binding of heat shock proteins in skeletal muscle fibers. AM J Physiol-Cell Ph 2012;303:654-665.

[13] Xing T, Wang MF, Han MY, Zhu XS, Xu XL, Zhou GH. Expression of heat shock protein 70 in transport-stressed broiler pectoralis major muscle and its relationship with meat quality. Animal 2017;11:1-9.

[14] Corrales FJ, Santamaria E, Fernández-Irigoyen J. Técnicas electroforéticas. In: Corrales FJ, Calvete JJ, editors. Manual de Proteómica. 2014, p.57-72.

[15] Vincent D, Solomon PS. Development of an in-house protocol for the OFFGEL fractionation of plant proteins. JIOMICS 2011;1:216-225.

[16] Meisrimler C, Lüthje S. IPG-strips versus off-gel fractionation: Advantages and limits of two-dimensional PAGE in separation of microsomal fractions of frequently used plant species and tissues. J Proteomics 2012;75:2550-2562.

[17] Franchin C, Pivato M, Rattazzi M, Arrigoni G, Millioni R. OFFGEL fractionation of peptides: where is really your sample? J Chromatogr 2014;1355:278-283.

[18] Sentandreu MA, Fraser PD, Halket J, Patel R, Bramley PM. A proteomic based approach for the detection of chicken in meat mixes. J Proteome Res 2009;9:3374-3383.

[19] Surowiec I, Koistinen KM, Fraser PD, Bramley PM. Proteomic approach for the detection of chicken mechanically recovered meat. Meat Sci 2011;89:233-237.

[20] Beldarrain LR, Aldai N, Picard B, Sentandreu E, Navarro JL, Sentandreu MA. Use of liquid isoelectric focusing (OFFGEL) on the discovery of meat tenderness biomarkers. J Proteomics 2018;183:25-33.

[21] Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Oreccia P, Zardi L, Righetti PG. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 2004;25:1327-1333.

[22] Dreiza CM, Komalavilas P, Furnish EJ, Flynn CR, Sheller MR, Smoke CC, Lopes LB, Brophy CM. The small heat shock protein, HSPB6, in muscle function and disease. Cell Stress Chaperon 2010;15:1.

[23] Jia X, Hollung K, Therkildsen M, Hildrum KI, Bendixen E. Proteome analysis of early post-mortem changes in two bovine muscle types: M-longissimus dorsi and M-semitendinosis. Proteomics 2006;6:936-944.

[24] Laville E, Sayd T, Morzel M, Blinet S, Chambon C, Lepetit J, Renand G, Hocquette JF. Proteome changes during meat aging in tough and tender beef suggest the importance of apoptosis and protein solubility for beef aging and tenderization. J Agr Food Chem 2009;57:10755-10764.

[25] Hamm R. Postmortem breakdown of ATP and glycogen in ground muscle: A review. Meat Sci 1077;1:15-39.

[26] Mymrikov EV, Seit-Nebi AS, Gusev NB. Large potentials of small heat shock proteins. Physiol Rev 2011;91:1123-1159.

[27] Ouali A, Herrera-Mendez CH, Coulis G, Becila S, Boudjellal A, Aubry L, Sentandreu MA. Revisiting the conversion of muscle into meat and the underlying mechanisms. Meat Sci 2006;74:44-58.

[28] Beere HM. 'The stress of dying': The role of heat shock proteins in the regulation of apoptosis. J Cell Sci 2004;117:2641-2651.

[29] Jia X, Ekman M, Grove H, Færgestad EM, Aass L, Hildrum KI, Hollung K. Proteome changes in bovine longissimus thoracis muscle during the early postmortem storage period. J Proteome Res 2007;6:2720-2731.

[30] Rogalla T, Ehrnsperger M, Preville X, Kotlyarov A, Lutsch G, Ducasse C, Paul C, Wieske M, Arrigo AP, Buchner J, Gaestel M. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. J Biol Chem 1999;274:18947-18956.

[31] Honikel KO. Conversion of muscle to meat. In: Jensen WK, Devine CE, Dikeman M, editors. Encyclopedia of Meat Sciences, vol 1. Academic Press/Elsevier; 2004, p.318-323.

[32] Silva JA, Patarata L, Martins C. Influence of ultimate pH on bovine meat tenderness during ageing. Meat Sci 1999;52:453-459.

[33] Pearce KL, Rosenvold K, Andersen HJ, Hopkins DL. Water distribution and

mobility in meat during the conversion of muscle to meat and ageing and the impacts on fresh meat quality attributes — A review. Meat Sci 2011;89:111-124.

[34] Anderson MJ, Lonergan SM, Huff-Lonergan E. Myosin light chain 1 release from myofibrillar fraction during postmortem aging is a potential indicator of proteolysis and tenderness of beef. Meat Sci 2012;90:345-351.

[35] Ciaramella MA, Nair MN, Suman SP, Allen PJ, Schilling MW. Differential abundance of muscle proteome in cultured channel catfish (Ictalurus punctatus) subjected to ante-mortem stressors and its impact on fillet quality. Comp Biochem Phys D 2016;20:10-18.

[36] Picard B, Berri C, Lefaucheur L, Molette C, Sayd T, Terlouw C. Skeletal muscle proteomics in livestock production. Brief Funct Genomics 2010;9:259-278.

[37] Kültz D. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. J Exp Biol 2003;206:3119-3124.

[38] Ouali A, Gagaoua M, Boudida Y, Becila S, Boudjellal A, Herrera-Mendez CH, Sentandreu MA. Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. Meat Sci 2013;95:854-870.

[39] Rosa AF, Moncau CT, Poleti MD, Fonseca LD, Balieiro JCC, Silva SLE, Eler, JP. Proteome changes of beef in Nellore cattle with different genotypes for tenderness. Meat Sci 2018;138:1-9.

[40] Morzel M, Terlow C, Chambon C, Micol D, Picard B. Muscle proteome and meat eating qualities of *Longissimus thoracis* of "Blonde d´Aquitaine" young bulls: A central role of HSP27 isoforms. Meat Sci 2008;78:297-304.

[41] Carvalho ME, Gasparin G, Poleti MD, Rosa AF, Balieiro JCC, Labate CA, Nassu RT, Tullio RR, Regitano LCA, Mourão GB, Coutinho LL. Heat shock and structural proteins associated with meat tenderness in Nellore beef cattle, a Bos indicus breed. Meat Sci 2014;96:1318-1324.

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2 | APPENDIX II PUBLICATION II

Proteomics 20 (2020), 2000012

Characterization of the myofibrillar proteome as a way to better understand differences in bovine meats having different ultimate pH values[2]

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Abstract

Influence of ultimate pH (pHu) on the occurrence of defective meats known as *Dark*, *Firm* and *Dry* (DFD) meats was studied through a proteomic approach of the myofibrillar muscle fraction in beef cattle at early post-mortem times. The myofibrillar sub-proteome of *Longissimus thoracis et lumborum* muscle from twelve 24h post-mortem loin samples from Asturiana de los Valles x Friesian yearling bulls previously classified into two groups of six samples according to their pH values (normal, pHu < 6.0 and high, pHu ≥ 6.0) was analysed. Fractionation/enrichment of muscle samples was carried out by combining OFFGEL fractionation in the pH range 4-7 followed by SDS-PAGE of the retrieved liquid fractions. Four protein bands satisfactorily discriminated between meat samples with normal and high pHu. These bands were quantified by image analysis, and further identified by liquid chromatography-mass spectrometry as desmin, pyruvate kinase, myosin light chain and myosin heavy chain-1 and -2. Coupling OFFGEL and SDS-PAGE separation with MS provided detailed and reproducible myofibrillar protein profiles that eased comparison among the sample groups assayed. This makes feasible to identify biomarkers capable to better understand pre-slaughter stress condition susceptible to give DFD meats with high pHu values.

Keywords

LC-MS ● OFFGEL Fractionation ● Pre-Slaughter Stress ● SDS-PAGE ● Ultimate pH

2.1 INTRODUCTION

Pre-slaughter stress (PSS) condition is one of the most important issues that must be considered before slaughter, although its multi-factorial origin hinders its study. Thus, we can find intrinsic (i.e. physiology, age, sex and genetics) and extrinsic (i.e. ambient temperature, handling activities, human presence, conditions and duration of transport and lairage, feed/water deprivation at abattoir, etc) factors affecting PSS condition. When inadequate animal management is practiced before slaughter, animals can lead to depletion of muscle glycogen reserves[1-3] in reaction to acute stress through the increase hormones secretion (cathecolamines, cortisol, adrenaline, etc.). Low glycogen content in muscle modifies glycolytic metabolism after slaughter resulting in less lactic acid production and high ultimate pH (pHu), leading to a defect known as dark cutting beef or *Dark*, *Firm* and *Dry* (DFD) meat. This phenomenon causes significant economic losses to meat industry due to the poor quality of affected products, characterized by undesirable alterations in tenderness during aging, higher water holding capacity (WHC) or fast spoilage by microbial contamination[4]. DFD carcasses costs around 55 and 20 million dollars per annum for Australian[5] and British[6] industry, respectively, and may affect to a range of 6 to 50% of carcasses, depending of seasonal and geographical variation[7]. However, there is no official information about incidence of this problem in Spain, but the available data indicate that it may affect approximately 14% [8] of bovine carcasses.

Australia, which is one of the countries with the highest incidence of dark cutting beef, considerers that meat with a high pHu (>5.7) is typically dark in colour and known as DFD[7]. Moreover FAO reported that a pHu higher than 6.2 indicates that the animal was stressed, injured or diseased prior to slaughter[9]. Therefore, measurement of final pH during the conversion of muscle into meat is a highly important factor for meat industry[10] and it is used the most as routine practice to control the occurrence of defective meats. However, some studies have reported the main factors contributing to dark cutting meat and the controversial use of pHu to detect it[11]. A high pHu meat is related to pre-slaughter stress condition but not in all cases this gives rise to DFD meat[12]. The discovery of reliable protein biomarkers related to stress response in farm animals has been proposed to assist in better explain occurrence of defective meats[13]. However, proteomic approaches addressing this problem in beef cattle are based on the study of total protein extract [10, 14, 15]. Alternatively, we have recenlty proposed an innovative proteomic approach based on liquid isoelectric focusing (OFFGEL) for the specific fractionation/enrichment of the sarcoplasmic fraction hyphenated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and LC-MS analysis[16]. Although it is known that sarcoplasmic proteins (stress/defence proteins and metabolic enzymes) are the ones mainly related to stress, the number of studies reporting changes occurring on myofibrillar proteome are scarce and

deserves further investigation. The idea to go deep into the study of myofibrillar sub-proteome is supported by the fact that texture properties are notably altered in DFD meats with respect to normal ones. In that respect, Mahmood et al. studied proteome differences in the myofibrillar extract of normal and DFD meats from heifer and steer carcasses at 4 days post-mortem[17]. Here we have carried out, for the first time, the study of the myofibrillar sub-proteome of both normal and high pHu bovine meats from a commercial slaughter house at early post-mortem times (24h) to try to elucidate main biochemical pathways responsible of pHu and texture changes that can give rise to DFD meats and potential early predictors of this condition. Myofibrillar proteins were carefully extracted and exhaustively characterized by a hybrid OFFGEL-SDS-PAGE fractionation/enrichment step followed by LC-MS analyses. The proposed workflow analysis hunts the elucidation of protein/peptides as confident early indicators of PSS condition.

Significance Statement

Meat industry has moves towards producing animals that are efficient feed converters, fast growing and have high lean meat content with minimum production cost. This resulted in the production of animals with higher greater muscle development and less muscle glycogen reserves, that in some cases are more susceptible to stress and consequently increasing appearance of meat quality defects in the production chain, such as dark-cutting beef. Dark cutting meats are generally linked to a low muscle glycogen content at slaughter caused by elevated glycogenolysis induced by on-farm nutrition, stress and exercise in the pre-slaughter period. This implies metabolism modifications of the post-mortem muscle resulting in an abnormal post-mortem acidification process. This defective meat with high post-mortem pH has become a significant problem in the meat industry inflicting important economic losses (55 and 20 million dollars per annum for Australian and British industry, respectively). The present proteomic investigation constitutes a straightforward strategy to reveal differences in bovine muscle for an appropriate assessment of dark cutting meat, an issue of major importance due to its implications regarding security of consumers, animal welfare and economic benefits of the meat industry.

2.2 EXPERIMENTAL SECTION

2.2.1 Sample preparation

Samples from twelve bovine animals were obtained from commercial abattoir in the north-eastern Spain and belonging to Asturiana de los Valles (AV) x Friesian breed. They were reared under intensive system (indoor fattening with concentrate meal and barley straw *ad libitum*). Muscle samples were collected from *Longissimus thoracis et lumborum* (LTL) of yearling bulls slaughtered at their 14-15 months of age according to EU regulations (Council Regulation (EC) No 853/2004 and No 1099/2009). At 24h post-mortem, approximately 10 g of muscle sample were

removed from the thirteenth rib of the left half carcass, immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Muscle samples were sorted into two different groups according to their pHu values: 'normal' samples (n=6) having pHu values below 6.0 (5.53 ± 0.14), whereas the other six samples (n=6) were classified as 'high' showing pHu values higher than 6.0 (6.56 ± 0.25). Measurements of pH were performed at the sixth rib of the LTL muscle at 24h post-mortem.

2.2.2 Protein extraction

For extraction of myofibrillar proteins, 0.5 g of each stored sample was dissolved in 4 mL of 10 mM Tris extraction buffer, pH 7.6, containing 0.25 M sucrose, 1 mM EDTA and 25 μL of protease inhibitors (P8340, Sigma-Aldrich Co., St. Louis, MO, USA), homogenized using IKA Yellow Line Di 25 homogenizer (IKA-Werke GmbH, Staufen, Germany) and centrifuged at 20,000 g for 20 min at 4 °C. The retrieved precipitate was washed once in 4 mL of extraction buffer and centrifuged under the same conditions described above. The washed precipitate was further dissolved in lysis buffer (10 mM Tris pH 7.6, 7 M urea, 2 M thiourea, 2% w/v CHAPS, 10 mM DTT) and centrifuged under the same conditions. The supernatant was filtered through glass wool and stored at -80 °C until analysis. Total protein content of samples was quantified using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to the Bradford method[18]. Bovine serum albumin standard ranging from 0.05 to 0.8 mg/mL was used to get calibration curve.

2.2.3 Protein OFFGEL fractionation

Myofibrillar proteins were separated in liquid phase based on their isoelectric point (pI) along 12 fractions using 13 cm immobilized pH gradient gel (IPG) strips (GE healthcare, Uppsala, Sweden) with a linear pH gradient in the range 4-7. Procedures for OFFGEL preparation were from Fuente-Garcia et al.[16] with some modifications to specifically fit to the characteristics of myofibrillar sub-proteome. Briefly, a volume containing 2 mg of total protein of each myofibrillar extract was considered for protein fractionation using an Agilent 3100 OFFGEL fractionator (Agilent Technologies, Palo Alto, CA, USA). IPG strips corresponding to a 12-well frame size covering the pH range 4-7 were placed onto the tray and rehydrated for 5 min adding 1.25X protein OFFGEL stock solution to each well. A total of 150 μL of myofibrillar protein extract, which were previously diluted with 1.25X protein OFFGEL stock solution, were loaded into each well. After focusing, the solution containing the proteins was collected from each well and stored at -80 °C until analysed. All analysis were done by duplicate.

2.2.4 Gel electrophoresis and image analysis

The 12 proteins fractions obtained from each sample after OFFGEL fractionation were subjected to electrophoretic separa-

tion under reducing and denaturant conditions (SDS-PAGE) as described previously[16] with some modifications. Electrophoresis of samples was performed in duplicate. All stained gel images were captured through a LAS-1000 Luminescent Image Analyzer with Intelligent Dark Box II (Fujifilm, Barcelona, Spain). After background subtraction and normalization, the volume of each band was quantitatively assessed using Gel Analyzer 2010 software (http://www.gelanalyzer.com). The molecular mass (Mr) and pI of bands were determined by co-electrophoresis of a commercial protein standard mixture ranging from 14 to 200 kDa (Bio-Rad, Hercules, CA, USA) and by their focusing position in the OFFGEL fractions, respectively.

2.2.5 *In-gel* digestion of bands

Gel bands being significantly different in abundance between the two groups (normal and high pHu) were cut out from the gel and digested with trypsin in order to identify the proteins. Briefly, excised bands were cut into pieces and washed with 50 mM ammonium bicarbonate. Bands were then dehydrated with ACN and the remained liquid was removed using a Speed-Vac concentrator. Gel pieces were digested (overnight at 37 °C) with 15 μL of trypsin solution at a 12.5 μg/mL concentration (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate (pH 8.5). After incubation, the liquid was transferred to a clean Eppendorf tube and the remaining peptides were recovered by adding ACN/0.1% TFA (50:50). This supernatant was combined with the previous one and the resulting liquid was evaporated in a Speed-Vac. Samples were then acidified with 0.1% TFA and transferred into glass vials.

2.2.6 LC-MS analysis

Samples (25 μL) were analysed using a ThermoScientific Surveyor HPLC with a cooled autosampler, quaternary pump, column oven and vacuum degasser coupled to a ThermoFinnigan LCQ Advantage ion-trap MS detector loading an electrospray ion source operating in positive ionization mode (Thermo Scientific, San Jose, CA, USA). Peptides were separated at 50 μL/min by a 150 x 0.5 mm, 4-µm particle size reverse-phase Phenomenex Jupiter Proteo column (Phenomenex Inc., Torrance, CA., U.S.A.). Chromatographic and MS conditions of analysis were from Fuente-Garcia et al.[16]. Peptide identification was done by analysis of generated MS/MS data against Uniprot KB v2017_11 (www.uniprot.org) and NCBIprot v20170428 protein databases using Mascot v2.3 as search engine with the following parameters: enzyme, trypsin; no fixed or variable modifications but "Error tolerant" option enabled; mass accuracy was set to 1.2 and 0.8 Da for MS and MS/MS analyses, respectively; The option "Mammalia" was selected as taxonomy restriction parameter; decoy option was used to estimate the false positive rates by means of False Discovery Rate threshold from 1%. Only those tentative assignments with high individual ion scores indicating

identity on extensive homology were considered as reliable results (P<0.05).

2.2.7 Statistical analysis

Ultimate pH from each meat group and image results from normal and high pHu gel bands were compared by *t*-test to find significant (P<0.05) differences using SPSS 23.0.0 software (SPSS Inc., Chicago, IL, USA). Principal component analysis (PCA) was applied for each animal and selected protein biomarkers in order to investigate the biological variability of individual samples.

Each identified protein showing statistically significant differences in abundance between normal and high pHu group was matched in agreement with their biological function using Gene Ontology term from the AmiGO website (http://amigo.geneontology.org/amigo/). Analysis of functional interactions from these identified proteins was performed using the STRING v.10.5 software in order to obtain a protein-protein interaction network. *Bos taurus* species was used as a main taxonomy constraint in all analyses.

2.3 RESULTS AND DISCUSSION

Figure 1 illustrates the reproducible protein distribution achieved in SDS-PAGE gels regarding the fractionated myofibrillar sub-proteome of normal (**1a**) and high (**1b**) pHu samples using OFFGEL technology in the pH range 4-7. Statistically significant differences ($P \leq 0.001$) of pH were observed between the two pHu groups, being mean values for normal and high pHu groups 5.53 ± 0.14 and 6.56 ± 0.25, respectively. Statistical treatment of image results from gels revealed how intensity of bands A, B and C were significantly ($P < 0.05$) higher in normal than in high pHu meats; contrary to this, intensity of band D was higher in high pHu samples (squared bands in **Figure 1**). Quantification values obtained from image analysis of these four bands are shown by **Figure 2.**

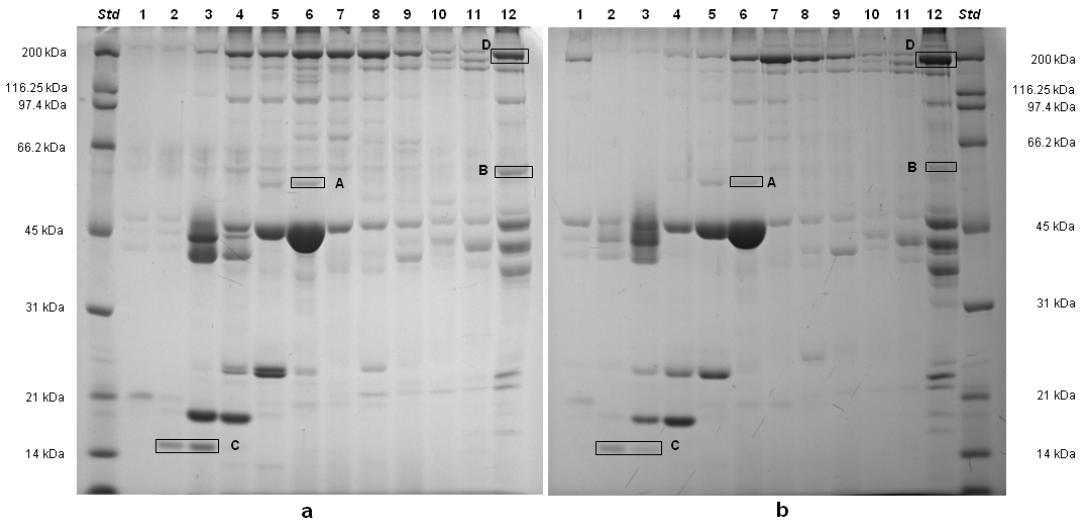

Figure 1. SDS-PAGE (12 %) separations of two representative meat samples obtained after OFFGEL enrichment/fractionation (4-7 pH range): a) normal pHu; b) high pHu. Std: Molecular weight distribution of protein standards. The complete set of gel images corresponding to all analysed samples and their duplicates are available at http://doi.org/10.20350/digitalCSIC/12508.

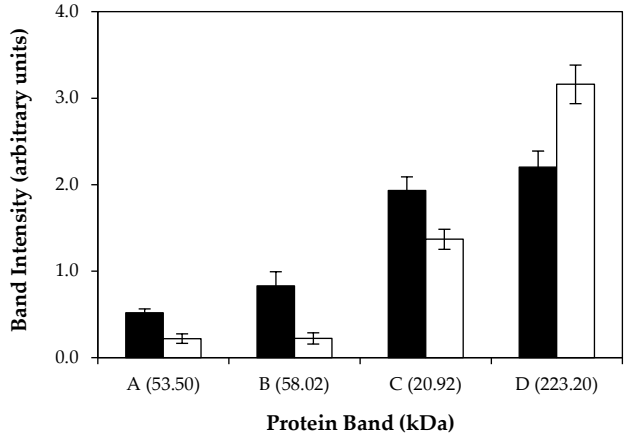

Figure 2. Image analysis quantification of bands A to D from Figure 1 corresponding to normal pHu (■, n=6) and high pHu (□, n=6) meat groups. Error bars indicate the standard error of the mean for each group.

Table 1 summarizes main results of LC-MS analysis addressing identification of proteins corresponding to bands A to D. Quantitative significant differences ($P < 0.05$) of these bands between normal and high pHu meats would allow proposing the identified proteins as reliable indicators of high pHu on bovine meats. Further qualitative information of protein assignments regarding sequence of their identified tryptic peptides is detailed in **Table S1** of supplementary material. As expected, desmin (DES) isolated in band A matched its theoretical pI with the pH value of its corresponding OFFGEL position (fraction 6; **Table 1**). Similarly, pyruvate kinase isoform X1 (PKM, theoretical pI 7.62) detected in band B was focused on fraction 12 at the highest pH range assayed (6.54 – 6.75, **Table 1**),

evidencing that a better protein fractionation could be achieved by expanding the upper experimental pH range of OFFGEL fractionation. Interestingly, LC-MS analysis revealed how myosin light chain 1/3 (MYL1) was the main protein of band C, being distributed along two neighbouring OFFGEL fractions with different pI value (**Table 1**). This can be understood considering the occurrence of post-translational modifications (i.e. phosphorylations or acetylations) that can influence the pI of this protein[19]. In any case, quantification of this protein was done by the sum of the band intensities from both aforementioned neighbouring fractions conforming band C. Efficiency of the LC-MS analysis led to identify myosin-1 (MYH1) and myosin-2 (MYH2) isoforms coexisting in band D, both sharing same pI and Mr (5.6 and ~220 kDa, respectively, **Table 1**). Again, discrepancies between theoretical pI and experimental pH value of OFFGEL fraction hosting these proteins can be explained attending to the previously mentioned post-translational protein modifications.

Table 1. LC-MS protein identification of biomarkers proposed in **Figure 2** using MASCOT search engine loading NCBIprot and Uniprot KB databases. Raw MS/MS data corresponding to LC-MS analysis of bands A–D are available at http://doi.org/10.20350/digitalCSIC/12508.

Band	Protein /Gene Identification	Theoretical/ apparent Mr (kDa)[a]	Theoretical p*I* (pH value in the OFFGEL fraction)[b]	Protein accession number[c]	Mascot Score	Protein coverage (%)	*P*-value[d]	Biological function
A	Desmin (DES)	53.50 / 45	5.21 (5.29 – 5.50)	O62654	363	23	<0.05	Structural maintenance
B	Pyruvate kinase isoform X1 (PKM)	58.02 / 51	7.62 (6.54 – 6.75)	XP_005211367	525	32	<0.05	Regulation of glycogen metabolism
C	Myosin light chain 1/3 (MYL1)	20.92 / 29	4.96 (4.46 – 4.88)	A0JNJ5	122	34	<0.05	Muscle contraction
D	Myosin-1 (MYH1)	222.85 / 220	5.57 (6.54 – 6.75)	Q9BE40	1515	19	<0.05	Muscle contraction
	Myosin-2 (MYH2)	223.18 / 220	5.63 (6.54 – 6.75)	Q9BE41	1297	17	<0.05	Muscle contraction

[a] Theoretical vs calculated molecular weight (Mr, from gel band position) of identified proteins.
[b] Theoretical pI of the protein as compared to the pH value of the OFFGEL fraction where it was focused.
[c] Accession numbers of bands A, C and D were from Uniprot KB database whereas band B corresponded to NCBIprot finding.
[d] *P*-value was estimated using the *t*-test.

The PCA displaying the two first discriminant functions explain 76.70% of the total variability and represents the factor scores of each individual (animal) and factor loadings of each selected protein (**Figure 3**). Normal and high pHu samples were clearly separated through the first component (PC1) in which normal pHu samples had positive factor scores, while high pHu samples had negative factor scores. On the other hand, PC1 show high correlation (factor loadings > 0.7) with proposed PSS protein markers being desmin, pyruvate kinase and myosin light chain related to normal pHu samples, while myosin-1 and -2 were related to high pHu samples. Therefore, the mentioned proteins proved to be highly discriminant for differentiating samples with normal pHu from those with high pHu.

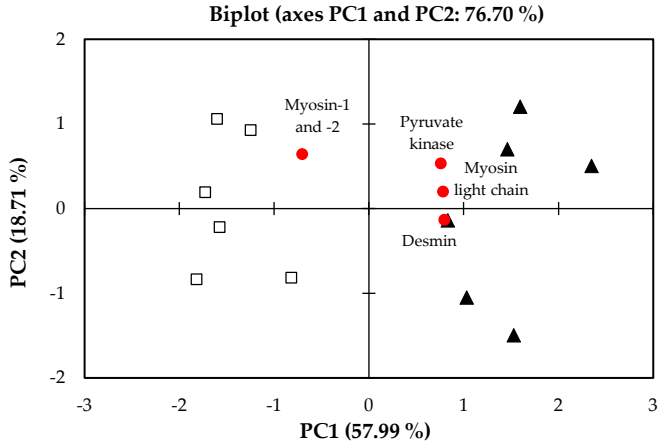

Figure 3. Animal sample and protein distribution on the two-dimensional coordinate system defined by principal component (PC) 1 and 2. Each point represents either an individual sample (▲ Normal pHu samples; □ High pHu samples) or the proposed PSS protein biomarkers (●).

Figure S1 of supplementary material illustrates the protein-protein interaction network among the identified proteins shown in **Table 1** considering their biological role in *Bos taurus* species. Logically, PKM is out of the network since the myofibrillar fraction is mainly integrated by structural (insoluble) rather than metabolism (soluble) proteins, these latter having an important role in the glycolysis pathway. Presence of soluble proteins in the myofibrillar fraction has been previously described[20]. This can be explained considering solubility changes of proteins induced by the pH reduction and high temperature occurring at early post-mortem times[21], which can cause protein denaturalization and aggregates that precipitate onto myofibrils[22]. MYL1 is located in the centre of the node and it is connected to MYH1, MYH2 and DES, which is reasonable considering that myosins are the most abundant proteins of thick filaments. Interaction of such structural proteins was reported through the study of the actomyosin complex that associates myosin light chains with myosin heavy chains and actin[23]. Additionally, some authors stated how DES connects myofibrils at Z-line level and to other

cellular structures, maintaining the structural integrity and contractility of muscle cells[24, 25]. This cytoskeletal protein can be degraded by the action of calpains during the first 24h post-mortem[26, 27], with the resulting weaker mechanical resistance and the loss of muscle cell integrity that reduce drip loss and increase WHC[28]. Moreover, μ-calpain activity seemed to hydrolyze both myosin chains[29].

In this research, meat with high pHu had a significantly lower abundance of desmin (**Figure 2**). It is known that DFD meats are positively related to tenderness and WHC at 24h post-mortem[14] due to the fact that higher pHu of meat enhances calpain activity[30-33], having DES as one of its preferred substrates[29]. Other authors found that DES was degraded more quickly in samples with lower shear force values than from samples with higher values[34]. So, our results could be understood considering those reported by previous authors who stressed that the increase of tenderness associated with desmin degradation at early post-mortem times[34] could be a result of enhanced calpain activity in meat with high pHu induced by stress[30].

Regarding the pyruvate kinase, even though several studies have stated its relevance as biomarker of tenderness[20, 35], knowledge about its role in PSS meat is scarce. As depicted in **Figure 2** (band B), abundance of PKM was significantly higher in normal compared to high pHu meat. This can be explained considering the glycogen depletion occurring in cattle under PSS condition, giving rise to a decrease of the glycolysis rate after slaughter. Some studies suggested that the phenomenon of dark cutting meat might be related to reduced concentration and/or activity of muscle glycolytic enzymes[17]. Therefore, and supporting the present results, proteins involved in the glycolytic pathway such as PKM may be downregulated by PSS.

The role of myosin light chain protein isoforms in meat tenderization is still controversial. As previously mentioned, elevated pH of DFD meat allows proteolytic enzymes to increase the hydrolysis of myofibrillar proteins such as MYL1, thus exhibiting a lower abundance with respect to normal pHu meat as shown in **Figure 2**. Some authors pointed out that both calpain and caspase system might lead to higher myofibrillar proteolysis under oxidative stress, resulting in greater myofibrillar degradation[36]. In contrast, Franco et al. described two phosphorylated fast skeletal myosin light chain 2 proteins that were specific of DFD meat, so they were proposed as PSS biomarkers in LT muscle of Rubia Gallega cattle breed[14]. In any case, further investigations are needed to better understand the evolution of myosin light chain proteins with respect to pHu variations of beef cattle.

Opposite to the above commented proteins (DES, PKM and MYL1), myosin-1 and myosin-2 isoforms showed higher abundance in high pHu samples compared to normal pHu samples (**Figure 2**). **Table 2** details the peptide differences unveiled by LC-MS analysis that led to the positive assignment of myosin isoforms -1 and -2 (also known as myosin heavy chain 2x and 2a, respectively) coexisting in band D

(**Figure 1**). The myosin isoforms present in each muscle determine the fibre type of muscle. Isoform 1 is the main component in slow-twitch fibres (type I fibres), whereas the other three isoforms (2a, 2x and 2b) are predominant in fast-twitch oxidative and/or glycolytic fibres (types IIA, IIX and IIB). Results concerning the higher content of myosin 2a and 2x isoforms in high pHu samples found in this work (**Figure 2**) could be understood considering those obtained by Ashmore and Ozawa et al., who suggested that αR fibres, also known as fast-twitch oxidative-glycolytic fibres type IIA (rich in 2a isoforms), seemed to be positively related to PSS in DFD meat[37,38]. In our case we have identified isoforms 2a and 2x, both of them present in fibres type IIA. Moreover, Young and Foote concluded that bovine muscles that are low in glycolytic capacity and high in oxidative capacity (integrated by fibres type I) are less prone to the dark-cutting condition[39]. In this sense, further investigations are required in order to understand the roles that these isoforms play in pre-slaughter stressed animals.

Table 2. Discriminant peptide sequences of myosin heavy chain isoforms from LTL muscle identified by LC-MS analysis. Amino acids in bold corresponded to those that were specific to isoform 2x or isoform 2a. All data were obtained from Uniprot KB protein database using MASCOT search engine.

Protein	Peptide sequence[a]	Observed Mr of peptide sequence (Da)	Peptide score	Nº of peptides identified	Charge
Myosin-1 (Isoform 2x)	^{417}GQTVEQV**Y**NAVGALAK^{432}	824.36	96	8	2+, 3+, 4+
	^{888}NDLQLQVQSEAD**A**LADAEER^{907}	1108.04	115	6	2+, 3+
	^{1596}IVESMQS**T**LDAEIR^{1609}	796.26	80	8	2+, 3+, 4+
Myosin-2 (Isoform 2a)	^{417}GQTVEQV**T**NAVGALAK^{432}	793.57	50	3	2+, 3+, 4+
	^{890}NDLQLQVQSEAE**G**LADAEER^{909}	1108.04	105	4	2+, 3+

[a] Superscripts indicate the position of the discriminant peptide into the protein sequence.

2.4 CONCLUDING REMARKS

Quantification/identification of different myofibrillar protein profiles of normal and high pHu meats enabled to propose desmin, pyruvate kinase isoform X1 and myosin light chain 1/3, together with myosin-1 and myosin-2 isoforms, as reliable indicators of the occurrence of ultimate high pH and PSS condition on bovine meats. These findings contribute to a better understanding of the biological mechanisms responsible for dark cutting meat. The study also supported the idea that sarcoplasmic proteins are not the only ones affected by stress at early post-mortem times, but also myofibrillar proteome is altered by this condition and deserves further investigation.

The proposed methodology using OFFGEL technology coupled to SDS-PAGE and LC-MS analysis enabled the detailed study of the myofibrilar sub-proteome of both normal and high pHu bovine meat samples at early post-mortem times. Implementation of this analysis workflow has proved to be an important step forward for comprehensive meat proteomic research in myofibrillar muscle extracts. In this line, future actions on this approach combined with other proteomic strategies will be the focus of future research.

SUPPORTING INFORMATION

Supporting Information is available from the Wiley Online Library or from the author.

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The authors declare no conflict of interest.

REFERENCES

[1] P. Tarrant, *Ir. J. Food Sci. Technol*. **1989**, *13*, 1.

[2] N. G. Gregory, T. Grandin, *Animal Welfare and Meat Science*. CABI, NY **1998**.

[3] D. M. Ferguson, R. D. Warner, *Meat Sci*. **2008**, *80*, 12.

[4] K. G. Newton, C. O. Gill, *Meat Sci.* **1981**, **5**, 223.

[5] C. G. Jose, P. McGilchrist, J. L. Perovic, G. E. Gardner, D. W. Pethick, in *61st International Congress of Meat Science and Technology*, Clemont Ferrand, France **2015**.

[6] F. Adzitey, H. Nurul, *Int. Food Res. J*. **2011**, *18*.

[7] K. M. W. Loudon, I. J. Lean, D. W. Pethick, G. E. Gardner, L. J. Grubb, A. C. Evans, P. McGilchrist, *Meat Sci.* **2018**, *144*, 110.

[8] N. Mach, A. Bach, A. Velarde, M. Devant, *Meat Sci*. **2008**, *78*, 232.

[9] P. G. Chambers, T. Grandin, G. Heinz, T. Srisuvan, *Guideness for Humane Handling, Transport and Slaughter of Livestock*, RAP

Publication, Food and Agriculture Organization of the United Nations, Thailand **2001**.

[10] M. D. Poleti, C. T. Moncau, B. Silva-Vignato, A. F. Rosa, A. R. Lobo, T. R. Cataldi, J. A. Negrão, S. L. Silva, J. P. Eler, J. C. de Carvalho Balieiro, *Meat Sci.* **2018**, *145*, 209.

[11] E. N. Ponnampalam, D. L. Hopkins, H. Bruce, D. Li, G. Baldi, A. E. Bekhit, *Compr Rev Food Sci F.* **2017**, *16*, 400.

[12] A. Apaoblaza, S. D. Gerrard, S. K. Matarneh, J. C. Wicks, L. Kirkpatrick, E. M. England, T. L. Scheffler, S. K. Duckett, H. Shi, S. L. Silva, A. L. Grant, D. E. Gerrard, *Meat Sci.* **2020**, *161*, 107996.

[13] A. Marco-Ramell, A. M. Gutiérrez, A. Velarde, J. J. Cerón, A. Bassols, in: *Proteomics in Domestic Animals: From Farm to Systems Biology*, Springer, Gewerbestrasse, Switzerland **2018**, pp. 339-360.

[14] D. Franco, A. Mato, F. J. Salgado, M. López-Pedrouso, M. Carrera, S. Bravo, M. Parrado, J. M. Gallardo, C. Zapata, *J Proteomics.* **2015**, *122*, 73.

[15] A. Mato, R. Rodríguez-Vázquez, M. López-Pedrouso, S. Bravo, D. Franco, C. Zapata, *BMC Genomics.* **2019**, *20*, 590.

[16] C. Fuente-Garcia, N. Aldai, E. Sentandreu, M. Oliván, S. García-Torres, D. Franco, C. Zapata, M. A. Sentandreu, *J Proteomics.* **2019**, *198*, 59.

[17] S. Mahmood, N. Turchinsky, F. Paradis, W. T. Dixon, H. L. Bruce, *Meat Sci.* **2018**, *137*, 47.

[18] M. Bradford, *Anal Biochem.* **1976**, *72*, 248.

[19] K. Zhu, J. Zhao, D. M. Lubman, F. R. Miller, T. J. Barder, *Anal. Chem.* **2005**, *77*, 2745.

[20] E. Laville, T. Sayd, M. Morzel, S. Blinet, C. Chambon, J. Lepetit, G. Renand, J. F. Hocquette, *J. Agric. Food Chem.* **2009**, *57*, 10755.

[21] I. Zapata, H. N. Zerby, M. Wick, *J. Agric. Food Chem.* **2009**, *57*, 4956.

[22] L. Stryer, *Biochemistry* 4th ed., WH Freeman & Company, NY **1995**.

[23] J. Bendall, *The Physiology and Biochemistry of Muscle as a Food*, University of Wisconsin Press, Madison, WI **1966**.

[24] R. Robson, M. Yamaguchi, T. Huiatt, F. Richardson, J. O'shea, M. Hartzer, W. Rathbun, P. Schreiner, L. Kasang, M. Stomer, in *Proceedings-Annual Reciprocal Meat Conf.* American Mear Science Association, IL **1981**.

[25] R. G. Taylor, G. H. Geesink, V. F. Thompson, M. Koohmaraie, D. E. Goll, *J. Anim. Sci.* **1995**, *73*, 1351.

[26] E. Huff-Lonergan, S. M. Lonergan, in *Quality attributes of muscle foods*, Springer, Gewerbestrasse, Switzerland, **1999**, pp. 229-251.

[27] M. Koohmaraie, *Biochimie* 1992, 74, 239-245.

[28] E. Huff-Lonergan, S. M. Lonergan, *Meat Sci.* **2005**, *71*, 194.

[29] R. Lametsch, P. Roepstorff, H. S. Møller, E. Bendixen, *Meat Sci.* **2004**, *68*, 515.

[30] J. A. Beltrán, I. Jaime, P. Santolaria, C. Sañudo, P. Albertí, P. Roncalés, *Meat Sci*. **1997**, *45*, 201.

[31] J. Camou, J. Marchello, V. Thompson, S. Mares, D. Goll, *J. Anim. Sci.* **2007**, *85*, 2670.

[32] T. Kendall, M. Koohmaraie, J. Arbona, S. Williams, L. Young, *J. Anim. Sci.* **1993**, *71*, 96.

[33] M. Koohmaraie, *Meat Sci.* **1994**, *36*, 93.

[34] E. Huff-Lonergan, T. Mitsuhashi, D. D. Beekman, F. C. Parrish Jr., D. G. Olson, R. M. Robson, *J. Anim. Sci.* **1996**, *74*, 993.

[35] R. Polati, M. Menini, E. Robotti, R. Millioni, E. Marengo, E. Novelli, S. Balzan, D. Cecconi, *Food Chem*. **2012**, *135*, 2052.

[36] A. J. Smuder, A. N. Kavazis, M. B. Hudson, W. B. Nelson, S. K. Powers, *Free Radical Bio Med*. **2010**, *49*, 1152.

[37] C. R. Ashmore, *J. Anim. Sci.* **1974**, *38*, 1158.

[38] S. Ozawa, T. Mitsuhashi, M. Mitsumoto, S. Matsumoto, N. Itoh, K. Itagaki, Y. Kohno, T. Dohgo, *Meat Sci*. **2000**, *54*, 65.

[39] O. Young, D. Foote, *Meat Sci*. **1984**, *11*, 159.

3 APPENDIX III PUBLICATION III

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A straightforward gel-free proteomics pipeline assisted by liquid isoelectric focusing (OFFGEL) and mass spectrometry analysis to study bovine meat proteome[3]

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Abstract

Bovine sarcoplasmic sub-proteome was studied through a straightforward gel-free pipeline supported by liquid isoelectric focusing (OFFGEL) protein fractionation coupled to liquid chromatography-mass spectrometry (LC-MS) analysis. Full-MS and data-dependent MS/MS analyses were simultaneously performed by a conventional three-dimensional ion-trap addressing targeted quantitative and untargeted qualitative research, respectively. There were unambiguously identified 47 proteins distributed along 12 OFFGEL fractions assayed. Regarding intermediate- and high-abundant peptides, bulky quantitative data processing performed by MZmine 2 freeware yielded a satisfactory linearity and coefficient of variation with r^2 in the 0.95–0.99 range and about 25%, respectively. Up to 41 peptides from 20 identified proteins were relatively quantified throughout OFFGEL fractions. This reliable, flexible and affordable gel-free proteomic approach could be readily implemented by industry to improve quality assessment of protein-based food products.

Keywords

Proteomics ● Food Proteins ● Quality Assessment ● Chromatography Analysis

3.1. INTRODUCTION

Continuous innovations in "omic" technologies move researchers towards the generation of voluminous data that needs to be appropriately refined to achieve concise conclusions (Lin et al., 2011). In the particular case of LC-based proteomics and metabolomics in "top" basic research, their current success strongly depends on the use of modern MS detectors merging, totally or partially, features such as high-mass resolution (HRMS), multiplexing capacity, dynamic range, scan speed and sensitivity (Griffiths and Wang, 2009). Uncertainties from conventional low resolution (LRMS) devices were surpassed by actual LC-HRMS hardware that greatly facilitated the design of simple analytical procedures that can also skip the chromatographic separation step through direct infusion mass spectrometry approaches (Southam et al., 2017; Lu et al., 2019). Furthermore, bundles mating modern LC-HRMS detectors and licensed software packages constitute a powerful budget-dependent technology that is not commonly implemented in food industry. Generation of valuable results using conventional, and affordable, LC-LRMS instruments demands special attention regarding sample preparation (i. e. enrichment/purification), chromatogra-phic resolution and optimization of MS settings of analysis to overcome their inherent technical limitations such as low scan rate, sensitivity, signal stability and mass resolution. Data processing also deserves special attention to ameliorate such drawbacks that greatly affect qualitative and quantitative analyses (Evans 2014; Mann and Kelleher, 2008). From this, food industry calls for straightforward, affordable, flexible, robust and reliable high-output "omic" pipelines of analysis supported by conventional LC-LRMS detection (Bu et al., 2016).

Actions addressing sample enrichment greatly help in the betterment of LC-MS sensitivity that, in the particular case of protein analysis, was traditionally guided by gel-based approaches. However, gels have intrinsic disadvantages such as the use of hazardous chemicals (i. e. poly-acrylamide), higher susceptibility to contamination, lack of reproducibility, experimental costs and manual work demanding (Monteoliva and Albar, 2004). Recent studies proposed liquid isoelectric focusing (OFFGEL) as a preliminary enrichment/fractionation step followed by SDS-PAGE and LC-MS approaches for biomarkers hunting of meat tenderness (Beldarrain et al., 2018; Fuente-García et al., 2019). Interestingly, authors emphasized the good reproducibility of the achieved OFFGEL protein profiles in addition to the simplicity of the methodology, suggesting its potential use for gel-free proteomics. To date, there are no studies dealing with the advantages of direct hyphenation of OFFGEL protein fractionation with LC-MS analysis in meat quality research (i. e. discovery of peptide biomarkers related to tenderness or detection of defective Pale Soft Exudative, and Dark Firm Dry meats).

Unlike qualitative analysis, automation in LC-MS quantitative proteomics appeared

over the last decade. It was traditionally carried out manually or using inaccessible and intricate in-house programming approaches that hindered their externalization (Wang et al., 2003). Recent popularization of open source programming languages (i. e. R environment) led to release of flexible alternatives for automated quantification (Kohlbacher et al., 2009; Taverner et al., 2012), but their implementation is limited by the need to incorporate skilled programmers into research units. In this line, instability of the signal response of former MS detectors decreases efficiency of algorithms embedded by most of the currently available data processing solutions. All those limitations together with uncertainties from LC-LRMS detection restricted former quantitative proteomic assays to some manually assisted label-free determinations (Sentandreu et al., 2010). Thus, there is a necessity for robust, easy, affordable and reliable alternatives for automated qualitative/ quantitative protein research supported by LC-LRMS approaches. The simplest strategy to carry out the automated processing of MS data is given by the classical metabolomics workflow. Large data sets are uploaded into the considered computational application loading an in-house/on-line library listing *m*/*z* and retention times of target analytes. Main constraints for positive assignments are mass and retention times tolerances but in the particular case of right peak picking MS^1 filtered peptides, uncertainties from LRMS detection result less harmful than in metabolomics research since target masses used to be above *m*/*z* 500. Then, time-aligned identified peaks are integrated and results finally merged in an exportable report. Main differences between programs are the algorithms considered for peak picking/integration of signals and the possibility of operating as search engines using on-line spectral libraries such as UniprotKB or NCBIprot in the case of proteomic approaches. However, uncertainties from LC-LRMS analysis combined with the impossibility of considering retention time of signals as a constraint negatively affect automated quantification of peptides supported by on-line databases. On the contrary, it is not critical for automated identification of proteins by search engines interrogating MS/MS breakdown patterns of their enzymatically released peptides. In any case, the number of studies addressing qualitative/quantitative gel-free meat proteomics combining OFFGEL fractionation, untargeted/ targeted LC-LRMS analysis and automated data processing by open source solutions is negligible.

This research aims at studying sarcoplasmic proteins in detail through a flexible and straightforward pipeline research. OFFGEL protein fractionation was coupled to LC-LRMS determination of tryptic peptides as an easy and flexible gel-free strategy for bulky analysis. Complementary untargeted qualitative and targeted quantitative workflow analysis combining Full-MS and data dependent MS/MS (dd-MS^2) experiments featured by a conventional three-dimensional ion-trap (3D-IT) was exhaustively evaluated.

This affordable gel-free methodology can be of great interest for food industry to

study and enhance protein quality of traded products.

3.2. MATERIALS AND METHODS

3.2.1 Chemicals

LC-MS grade ACN, TFA, formic acid (FA), EDTA, Tris, glycerol, urea, thiourea and DTT were from Scharlab (Scharlab S. L., Barcelona, Spain). Ultrapure grade water was from Millipore (EMD Millipore Co., Billerica, MA, USA). Sucrose, protease inhibitor cocktail (P8340) and ammonium bicarbonate were from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Bradford Protein Assay Kit was from Bio-Rad (Bio-Rad, Hercules, CA, USA). Immobilized pH gradient gel (IPG) strips and IPG buffer pH 3-10 were from GE healthcare (GE healthcare, Uppsala, Sweden). Modified trypsin was from Promega (Promega, Madison, WI, USA).

3.2.2 Preparation of the internal standards solution

Wild almond was from a local market (Valencia, Spain) and protein extract was obtained as proposed by Mirzapour et al. (2016) finding a resultant protein content of 10 mg/mL determined by Bradford protein assay (Bradford, 1976). Extracts were tryptically digested (3 h, 37 °C and pH 7.8) at an enzyme/substrate ratio of 1.0% (w/w) and centrifuged at 4000 *g* for 10 min at 4 °C using an Avanti J-26S XP Beckman Coulter centrifuge (Brea, CA, USA). Supernatants were 1:46 diluted with aqueous 0.1% TFA and aliquots stored at -20 °C until spiked as internal standards (ISs) into meat protein samples.

3.2.3 Sample selection

Longissimus thoracis (LT) muscle was obtained from a 18 months old Limousine veal carcass (Industrias Cárnicas Corella S.L. Venta del Aire, Teruel, Spain) immediately after slaughter and kept at 4 °C. The epimysium was carefully dissected and, at 24 h post-mortem, samples were vacuum packed in 50 g portions and stored at -80 °C until processed for protein extraction.

3.2.4 Extraction of sarcoplasmic proteins

Half a gram of meat was homogenized in 4 mL of extraction buffer (10 mM Tris pH 7.6, 1 mM EDTA, 0.25 M Sucrose) containing 25 μL of inhibitors cocktail using an Ultra-Turrax Yellow Line Di 25 (IKA-Werke GmbH, Staufen, Germany). The homogenate was centrifuged at 20,000 *g* for 20 min at 4 °C using a Beckman Coulter centrifuge (Brea, CA, USA), being the obtained supernatant filtered through a 45 μm PVDF filter and stored at -80 °C until fractionation. Total protein quantification of the extract was assessed by the aforementioned assay.

3.2.5 *Gel-free* OFFGEL fractionation/enrichment

Sarcoplasmic proteins were separated according to their isoelectric point (p*I*) in liquid phase along 12 fractions using 13 cm immobilized pH gradient gel (IPG) strips

with a linear pH gradient in the range 3-10. A volume containing 1 mg of total protein of the sarcoplasmic extract was considered for protein fractionation, bringing it up to a final volume of 2 mL with 1.25X protein OFFGEL stock solution (6% v/v glycerol, 7 M urea, 2 M thiourea, 65 mM DTT and IPG buffer pH 3-10) in order to load 150 μL of this solution on each well. Fractionation was carried out using an Agilent 3100 OFFGEL fractionator (Agilent Tech., Palo Alto, CA, USA) at 20 °C with a constant current of 50 μA to reach 20 kVh during 20 hours following the manufacturer's instructions. Fractions were separately collected by pipetting, cleaned up using OMIX 100 μL C18 pipette tips (Agilent Tech. S. L., Madrid, Spain) and recovered acetonitrile eluates completely evaporated by a SPD121P Speed Vac concentrator (Thermo Scientific, San Jose, CA, USA) previously to tryptic digestion.

3.2.6 *In-solution* trypsin digestion of the OFFGEL protein fractions

Dried fractions were rediluted in 15 μL of a sequencing grade modified trypsin solution at a 12.5 μg/mL concentration and mixed with an equal volume of 50 mM ammonium bicarbonate (pH 8.5). The mix was then incubated overnight at 37 °C with continuous gently shaking allowing digestion of proteins by trypsin. After incubation, each fraction was completely evaporated as indicated above and resuspended in 70 μL of a 0.1% TFA aqueous solution. Fifty μL of each fraction were separately mixed with 15 μL of the IS solution in Eppendorf tubes, stirred and centrifuged at 22,000 *g* for 3 min prior to be poured into LC-MS vials, constituting the OFFGEL (OFGL) sarcoplasmic fractions batch. The remaining 20 μL of each fraction were collected in the same Eppendorf tube, giving rise to a final pooled volume of 240 μL. Seventy μL of this pool were mixed with 20 μL of the IS solution in a separate Eppendorf tube, stirred, centrifuged (22,000 *g* for 3 min) and poured into a LC-MS vial, constituting the quality control (QC) batch. The remaining 170 μL of the pooled mix were used to construct the calibration curves of targeted peptides as follows: five different volumes of the mix ranged from 10 to 50 μL (in 10 μL steps) were isolately mixed in Eppendorf tubes with 10 μL of the IS solution and a variable volume of aqueous 0.1% TFA solution to reach a final volume of 60 μL. Tubes were stirred, centrifuged (22,000 *g* for 3 min) and poured into LC-MS vials constituting the calibration (CAL) batch.

3.2.7 LC-MS analysis

Chromatographic analysis was carried out in a Thermo Surveyor LC system with a cooled autosampler, quaternary pump and vacuum degasser coupled to a Thermo LCQ Advantage ion-trap analyzer loading an ESI probe operating in positive ionization mode (Thermo Scientific, San Jose, CA, USA). Peptides separation was achieved by a reverse-phase 150 x 0.5 mm, 4 μm particle size Jupiter Proteo column from Phenomenex (Phenomenex Inc., Torrance, CA, USA). Mobile phases were solvent A containing 0.1% FA (v/v) in ultrapure water and solvent B containing 0.1% FA (v/v) in ACN with the following

separation conditions: initially 0% B, held for 15 min, 0-20% B in 2 min, held for 4 min, 20-40% B in 1 min, held for 8 min, 40-100% B in 1 min, washing with 100% B for 8 min and column equilibration at initial conditions for 55 min. Injection volume was 10 μL for quality control (QC) and 25 μL for CAL and OFGL batches. Autosampler and column temperatures were 10 °C and 23 °C, respectively. Flow-rate was set to 50 μL/min.

Operating conditions of the MS device were: capillary temperature, 250 °C; normalized collision (CID) energy for MS/MS analysis, 35%; spray voltage, 4.0 kV; capillary voltage, 15.0 V. Injection method merged two scan events: Full-MS analysis of intact peptide masses in the 400-2000 m/z range followed by a dd-MS^2 experiment of most intense ions from Full-MS scan with +1 to +4 charges with the following dynamic parameters: minimum MS/MS ion intensity threshold, 1x10^5; exclusion list, 25 masses including those from spiked almond ISs and background noise from a blank injection; exclusion time, 3 min; exclusion mass width, 3 amu; repeat count for MS/MS of most intense ion, 3; repeat count duration, 0.5 min. Regarding sensitivity and scan speed of the mass analyzer the number of microscans-maximum injection time were 1-200 ms and 1-300 ms for Full-MS and dd-MS^2, respectively. Control of the LC-MS system and manual data processing was featured by a PC loading the Thermo Xcalibur v2.04 software (Thermo Sci., San Jose, CA, USA).

The LC-MS device continuously operated until completing the sample list analysis (about 40 h) to test robustness of determinations. Then, QC vial was injected at the beginning, middle and end (n=3) of the OFGL batch sequence (n=12), finalizing the sample list with the CAL batch analysis (n=5).

3.2.8 Untargeted protein identification analysis (protein mapping)

Sarcoplasmic protein were identified interrogating dd-MS^2 data from OFGL batch analysis using a licensed copy of Mascot v2.3 search engine (www.matrixscience.com) loading the UniprotKB (www.uniprot.org) and NCBIprot protein databases with the following settings: enzyme, trypsin; no fixed or variable modifications but enabled the "Error tolerant" option; mass accuracy; 1.2 and 0.8 Da for Full MS and MS/MS scans, respectively; taxonomy restriction parameter, Mammalia. The decoy option was used to estimate the false positive rates by means of False Discovery Rate (FDR) threshold 1%. Only those identifications with a protein score derived from individual ion scores indicating identity or extensive homology (p<0.05) were considered as true protein identifications.

Tryptic almond peptides of the IS solution were partially characterized as mentioned above but using "green plants" as a taxonomy restriction parameter.

3.2.9 Targeted peptide quantification analysis

Full-MS data from OFGL batch analysis was manually processed using Xcalibur to

study peak symmetry of signals corresponding to peptides that positively covered previously identified proteins. Mass tolerance considered was 0.5 Da and assignments were manually inspected by matching experimental MS/MS break-down patterns with those detailed in Mascot search. Thus, an in-house library (.csv format, **Table S1**) was built listing intact masses and respective retention times of identified sarcoplasmic peptides with acceptable peak shape. Database also listed 16 known/unknown almond peptides (IS) used to normalize quantitative results. Since peptides at *m*/*z* 903.1 and 949.2 were shared by alpha and beta enolases, their automated determination will address the achievement of a unique alpha/beta enolase assignment.

Proteomic research assisted by LC-LRMS detection conventionally addressed qualitative analysis and then, only MS/MS data used to be relevant independently of symmetry and retention time reproducibility of chromatographic peaks achieved by Full-scan analyses (i.e. Full-MS, multiple reaction monitoring and all ion fragmentation (AIF)). In contrast, success in automated quantitation strongly depends on chromatographic-related features affecting peak area integration and alignment. As an example, **Figure S1** illustrates acceptable (**S1A**) and non-acceptable (**S1B**) Full-MS signals (in gray) of ions at *m*/*z* 1045.5 and 927.7 corresponding to peptides of hemoglobin subunit beta and gluthatione-S-transferase found in OFGL fraction 9, respectively. Peak asymmetry constrained quantitative analysis since many signals were tentatively identified (see **Figure S1C** for Mascot search of ion at *m*/*z* 927.7) but no further quantified as consequence of their poor peak shape (not considered in **Table S1**). Similarly, a minimum number of scans across the chromatographic peak is needed to achieve correct area integrations of Full-scan signals. Low scan rate of affordable analyzers that can be of interest for food industry greatly compromise quality of quantifications. Such setback must be handled through the appropriate optimization of the scanning acquisition according to the width of chromatographic peaks and the number of multiplexed scan events, thus reaching a good compromise between qualitative and quantitative approaches. In our case, peak width of peptides listed in **Table S1** was rather wide (1 to 2 min) as consequence of the diffusion effects from injecting large volumes into a 0.5 mm i. d. chromatographic column. Under MS conditions assayed the Full-MS scan rate was 1 scan/1.8 sec, giving 35-60 scans/peak that was translated into the obtaining of irregularly shaped signals as consequence of loading former unstable ESI probes.

Considering the above, there was performed a preliminary screening of freely available computational solutions addressing the automated quantitative analysis of batches studied. Freeware MZmine 2 v.2.53 (https://github.com/mzmine/mzmine2/releases) was finally selected since it exhibited important advantages such as its user-friendly interface, processing of Thermo RAW data files without a preliminary format conversion (i. e. mzXML, mzML etc.), wide mass and retention time tolerances, low hardware

requirements and algorithm of analysis yielding comparable results than those from manual Xcalibur processing. In this line, other licensed and open-source alternatives such as Thermo Sieve 2.2.58 SP2 and Maven_682, respectively, achieved very unsatisfactory results (data not shown). The well-formatted customized library loaded was **Table S1** whereas automated workflow and optimized settings used are summarized in **Table S2**.

3.3 RESULTS AND DISCUSSION

Supplementary **Figure S2** displays a graphical summary of the workflow followed in this research to ease understanding of the proposed proteomic pipeline.

3.3.1 Untargeted qualitative analysis

Coverage of the exploratory proteome research of OFGL samples yielded the unambiguous identification of 47 sarcoplasmic proteins. **Table S3** shows protein distribution across the 12 OFFGEL fractions sorted by their molecular (Mr) weight and pH range assayed. In contrast to previously stated regarding **Table S1**, alpha and beta enolases were differentiated by Mascot analysis since MS/MS patterns from discriminant peptides were considered. Qualitative analysis addressed majority of identifications to *Bos taurus* species with only six exceptions (bolded in **Table S3**) since protein databases loaded did not list sequences of such proteins from bovine species. Moreover, efficiency of fractionation was evidenced when highest Mascot scores (in brackets at ID cells in **Table S3**) were generally achieved in fractions whose experimental pH range met, or was close to, the theoretical pI of positive protein assignments. Apparition of proteins in fractions with a far or neighboring pH range from their theoretical pI (i. e. glyceraldehyde-3-phosphate dehydrogenase and myoglobin, respectively) can be explained by post-translational modifications such as phosphorylations or acetylations (Franco et al., 2015; Meisrimler and Lüthje, 2012).

3.3.2 Targeted quantitative analysis of peptides

As stated above, comparable peak area integrations of peptides were reached by MZmine and Xcalibur in a preliminary screening to optimize the scan rate of the MS system. After that, a more detailed validation assessment of quantitative analysis by processing QC data was carried out.

Firstly, we must consider that customized library (**Table S1**) was built from the analysis of the OFGL batch (25 μL as injection volume), finding shorter retention times for assignments from QC injections (10 μL of injection volume). Then, automated processing of QC batch loaded such database but listing reduced retention times (-0.5 and -1.5 min for assignments up to the first 10 min of run time and from 55 min onwards, respectively). Secondly, integrated peak areas of positive assignments (sarcoplasmic peptides and ISs) from QC, CAL and OFGL analyses were normalized in this work by the closest IS eluted

(analyte/IS ratio) to relatively quantify samples. Differences in the election of IS normalizers between batches can occur since averaged retention times of assignments varied by different injection volumes assayed.

Figure S3 illustrates manual (A to C) and automated (D to F) peak area integration of low- (*m*/*z* at 1012.3, **Figures S3A** and **S3D**), medium- (*m*/*z* at 748.3, **Figures S3B** and **S3E**) and high- (*m*/*z* at 1222.3, **Figures S3C** and **S3F**) abundant peptides found in QC2 run (center of the injection sequence of OFGL batch, see LC-MS analysis section) from phosphoglycerate kinase 1, myoglobin and unknown IS proteins, respectively. Comparable results were reached by Xcalibur and MZmine data processing, finding similar results in most assignments throughout QC samples (Xcalibur vs. MZmine, **Table 1**). Automated results were IS normalized and ratios achieved led to evaluate reproducibility among QC injections (3 technical replicates). From this, there was established a confidence threshold whereby peak areas automatically integrated with values above $7x10^6$ were generally comparable with those from manual processing and yielded reproducible results with acceptable deviations (up to 25% of coefficient of variation, CV, bolded in **Table 1**). Such threshold was influenced by inherent sensitivity limitations of 3D-ITs aggravated by interferences from using low-mass resolution detection that increased its overall value. Obviously, harmfulness of such limitations was lower for signals unaffected by mass uncertainties as shown by the low-abundant phosphoglycerate kinase 1-4 peptide (**Figures S3A**-**S3D** and **Table 1**), suggesting their potential use as robust peptide biomarkers in meat quality assessment. Overall, reliability of this methodology was mostly limited to medium- and high- abundant (integrated peak area values from $7x10^6$ onwards) peptides under instrumental conditions assayed.

Table 1. Reproducibility of automated quality control (QC) batch processing.

m/z [M+H]+	Rt (min)	row identity (main ID)	[a]QC1 Xcalibur	[a]QC1 MZmine	[a]QC2 Xcalibur	[a]QC2 MZmine	[a]QC3 Xcalibur	[a]QC3 MZmine	[b]IS normalizer	[c]QC 1 norm.	[c]QC 2 norm.	[c]QC 3 norm.	Norm. mean	Norm. SD	[d]CV (%)
519.2	**1.96**	**IS, Almond (unknown)-1**	**2.66E+07**	**2.47E+07**	**2.42E+07**	**2.16E+07**	**2.40E+07**	**2.33E+07**	**IS, Almond (unknown)-2**	**1.0007**	**0.9595**	**1.2313**	**1.0638**	**0.1465**	**13.8**
941.4	**1.99**	**beta enolase-1**	**1.15E+07**	**1.24E+07**	**1.45E+07**	**1.49E+07**	**1.67E+07**	**1.57E+07**	**IS, Almond (unknown)-1**	**0.5027**	**0.6896**	**0.6730**	**0.6218**	**0.1034**	**16.6**
968.2	2.02	Galectin 1-1	1.90E+06	2.61E+05	1.96E+06	8.80E+05	1.98E+06	4.74E+05	IS, Almond (unknown)-1	0.0106	0.0406	0.0203	0.0239	0.0153	64.3
629.2	2.03	Myoglobin- 2	6.24E+06	5.41E+06	2.01E+06	1.54E+06	4.47E+06	2.44E+06	IS, Almond (unknown)-1	0.2195	0.0713	0.1047	0.1319	0.0777	59.0
978.6	2.35	Actin-1	1.51E+06	8.66E+05	6.27E+06	2.35E+06	6.66E+06	5.12E+06	IS, Pru 2 partial-3	0.0144	0.0425	0.0997	0.0522	0.0435	83.4
1002.4	2.39	Serum albumin	2.24E+06	5.00E+05	4.72E+06	1.75E+06	2.10E+06	6.40E+05	IS, Pru 2 partial-3	0.0083	0.0317	0.0125	0.0175	0.0125	71.4
727.5	2.46	Protein/nucleic acid deglycase DJ 1-1	9.36E+05	6.27E+05	2.32E+06	1.26E+06	9.62E+05	3.65E+05	IS, Pru 2 partial-3	0.0104	0.0229	0.0071	0.0135	0.0083	61.9
955.7	**2.51**	**IS, Pru 2 partial-3**	**6.03E+07**	**6.03E+07**	**5.45E+07**	**5.52E+07**	**5.25E+07**	**5.14E+07**	**IS, Almond (unknown)-1**	**2.4452**	**2.5520**	**2.2038**	**2.4003**	**0.1784**	**7.4**
748.3	**2.77**	**Myoglobin-3**	**8.20E+06**	**8.03E+06**	**1.07E+07**	**1.15E+07**	**9.19E+06**	**8.63E+06**	**IS, Almond (unknown)-2**	**0.3259**	**0.5097**	**0.4358**	**0.4238**	**0.0925**	**21.8**
954.4	2.95	Triosephosphate isomerase-1	4.81E+06	5.05E+06	7.62E+06	6.48E+06	8.40E+06	7.47E+06	IS, Almond (unknown)-2	0.2048	0.2874	0.3774	0.2899	0.0863	29.8
593.1	**2.96**	**IS, Almond (unknown)-2**	**2.53E+07**	**2.46E+07**	**2.34E+07**	**2.26E+07**	**1.98E+07**	**1.98E+07**	**IS, Prunin 1 precursor-3**	**0.7904**	**0.7047**	**0.6302**	**0.7084**	**0.0802**	**11.3**
1162.8	**3.13**	**IS, Prunin 1 precursor-3**	**2.87E+07**	**3.12E+07**	**2.90E+07**	**3.20E+07**	**2.91E+07**	**3.00E+07**	**IS, Pru 2 partial-3**	**0.5171**	**0.5795**	**0.5848**	**0.5605**	**0.0377**	**6.7**

Continued in the next page . . .

Table 1 – Continued

m/z [M+H]$^+$	Rt (min)	row identity (main ID)	[a]QC1 Xcalibur	[a]QC1 MZmine	[a]QC2 Xcalibur	[a]QC2 MZmine	[a]QC3 Xcalibur	[a]QC3 MZmine	[b]IS normalizer	[c]QC 1 norm.	[c]QC 2 norm.	[c]QC 3 norm.	Norm. mean	Norm. SD	[d]CV (%)
1055.2	**3.78**	**IS, Pru 2 partial-1**	**9.35E+07**	**9.60E+07**	**9.29E+07**	**9.77E+07**	**9.43E+07**	**9.47E+07**	**IS, Almond (unknown)-3**	**2.8764**	**2.7249**	**2.8472**	**2.8162**	**0.0803**	**2.9**
734.3	4.00	Phosphoglycerate kinase 1-1	3.53E+06	1.45E+06	2.19E+06	9.26E+05	6.25E+06	2.86E+06	IS, Almond (unknown)-1	0.0588	0.0428	0.1229	0.0748	0.0424	56.6
1245.4	**4.35**	**IS, Almond (unknown)-3**	**3.39E+07**	**3.34E+07**	**3.63E+07**	**3.59E+07**	**3.43E+07**	**3.33E+07**	**IS, Prunin 1 precursor-3**	**1.0712**	**1.1197**	**1.1074**	**1.0994**	**0.0252**	**2.3**
718.6	**4.71**	**IS, Prunin 1 precursor-1**	**1.06E+07**	**1.00E+07**	**1.08E+07**	**1.10E+07**	**8.91E+06**	**9.42E+06**	**IS, Almond (unknown)-3**	**0.3003**	**0.3079**	**0.2833**	**0.2972**	**0.0126**	**4.2**
1026.3	**5.19**	**IS, Almond (unknown)-4**	**2.72E+07**	**2.76E+07**	**3.11E+07**	**3.08E+07**	**3.01E+07**	**2.96E+07**	**IS, Prunin 1 precursor-1**	**2.7556**	**2.7877**	**3.1383**	**2.8939**	**0.2123**	**7.3**
770.6	**5.68**	**Triosephosphate isomerase-2**	**1.19E+07**	**1.17E+07**	**1.19E+07**	**1.18E+07**	**1.24E+07**	**1.22E+07**	**IS, Almond (unknown)-4**	**0.4234**	**0.3830**	**0.4139**	**0.4067**	**0.0211**	**5.2**
595.0	**63.04**	**IS, Prunin 2 precursor partial-2**	**7.30E+06**	**7.26E+06**	**8.05E+06**	**7.81E+06**	**8.53E+06**	**8.72E+06**	**IS, Prunin 2 precursor partial-1**	**0.2921**	**0.2612**	**0.3871**	**0.3135**	**0.0656**	**20.9**
687.0	**63.09**	**IS, Prunin 2 precursor, partial-1**	**2.43E+07**	**2.49E+07**	**2.86E+07**	**2.99E+07**	**2.11E+07**	**2.25E+07**	**IS, Almond (unknown)-8**	**1.0541**	**1.1547**	**0.9195**	**1.0428**	**0.1180**	**11.3**
893.6	**63.10**	**Creatine kinase M- 3**	**1.21E+07**	**1.27E+07**	**1.46E+07**	**1.49E+07**	**1.20E+07**	**1.25E+07**	**IS, Prunin 2 precursor partial-1**	**0.5096**	**0.4991**	**0.5526**	**0.5205**	**0.0283**	**5.4**
1056.4	**63.39**	**Phosphoglycerate kinase 1-2**	**9.25E+06**	**9.21E+06**	**1.00E+07**	**1.01E+07**	**8.76E+06**	**8.81E+06**	**IS, Prunin 2 precursor partial-1**	**0.3707**	**0.3375**	**0.3908**	**0.3663**	**0.0269**	**7.3**
1123.8	63.50	Actin-2	4.97E+06	3.08E+06	3.42E+06	2.80E+06	5.52E+06	5.30E+06	IS, Prunin 2 precursor partial-1	0.1238	0.0938	0.2350	0.1509	0.0744	49.3
876.6	64.51	Malate deshydrogenase-1	3.52E+06	1.49E+06	3.84E+06	3.69E+05	2.86E+06	1.91E+06	IS, Pru 2 partial-2	0.0804	0.0199	0.1132	0.0712	0.0474	66.5

Continued in the next page . . .

Table 1 – Continued

m/z [M+H]⁺	Rt (min)	row identity (main ID)	[a]QC1 Xcalibur	[a]QC1 MZmine	[a]QC2 Xcalibur	[a]QC2 MZmine	[a]QC3 Xcalibur	[a]QC3 MZmine	[b]IS normalizer	[c]QC 1 norm.	[c]QC 2 norm.	[c]QC 3 norm.	Norm. mean	Norm. SD	[d]CV (%)
992.2	**64.53**	**Phosphoglycerate kinase 1-3**	**7.05E+06**	**7.48E+06**	**6.97E+06**	**6.24E+06**	**7.07E+06**	**6.31E+06**	**IS, Pru 2 partial-2**	**0.4045**	**0.3360**	**0.3733**	**0.3712**	**0.0343**	**9.2**
879.1	**64.90**	**IS, Pru 2 partial-2**	**1.87E+07**	**1.85E+07**	**1.88E+07**	**1.86E+07**	**1.68E+07**	**1.69E+07**	**IS, Prunin 2 precursor partial-1**	**0.7444**	**0.6217**	**0.7498**	**0.7053**	**0.0725**	**10.3**
1317.9	**64.95**	**IS, Almond (unknown)-8**	**2.21E+07**	**2.36E+07**	**2.45E+07**	**2.59E+07**	**2.34E+07**	**2.45E+07**	**IS, Pru 2 partial-2**	**1.2744**	**1.3930**	**1.4505**	**1.3727**	**0.0898**	**6.5**
903.1	**65.15**	**alpha/beta enolase-3**	**1.78E+07**	**1.71E+07**	**1.73E+07**	**1.69E+07**	**1.61E+07**	**1.71E+07**	**IS, Pru 2 partial-2**	**0.9249**	**0.9092**	**1.0128**	**0.9490**	**0.0558**	**5.9**
1012.3	**65.57**	**Phosphoglycerate kinase 1-4**	**4.41E+06**	**4.06E+06**	**4.88E+06**	**4.20E+06**	**5.58E+06**	**4.65E+06**	**IS, Pru 2 partial-2**	**0.2192**	**0.2259**	**0.2755**	**0.2402**	**0.0307**	**12.8**
743.3	**65.73**	**Triosephosphate isomerase-3**	**3.79E+06**	**3.01E+06**	**3.12E+06**	**3.42E+06**	**2.71E+06**	**2.60E+06**	**IS, Pru 2 partial-2**	**0.1630**	**0.1839**	**0.1549**	**0.1673**	**0.0150**	**9.0**
1292.9	66.98	Protein/nucleic acid deglycase DJ 1-3	2.76E+06	2.81E+06	5.08E+06	4.88E+06	4.45E+06	2.08E+06	IS, Almond (unknown)-5	0.1649	0.2518	0.1089	0.1752	0.0720	41.1
754.7	**67.05**	**Creatine kinase M-2**	**4.20E+06**	**3.15E+06**	**5.10E+06**	**4.68E+06**	**3.45E+06**	**2.78E+06**	**IS, Almond (unknown)-5**	**0.1846**	**0.2412**	**0.1456**	**0.1905**	**0.0481**	**25.3**
802.1	**67.08**	**Triosephosphate isomerase-4**	**1.03E+07**	**1.04E+07**	**1.06E+07**	**1.00E+07**	**1.10E+07**	**1.03E+07**	**IS, Almond (unknown- 5**	**0.6098**	**0.5159**	**0.5403**	**0.5553**	**0.0488**	**8.8**
1045.8	**68.16**	**Hemoglobin subunit beta**	**3.57E+06**	**3.98E+06**	**5.13E+06**	**4.73E+06**	**3.91E+06**	**3.30E+06**	**IS, Almond (unknown)-5**	**0.2333**	**0.2443**	**0.1729**	**0.2168**	**0.0384**	**17.7**
1275.5	68.70	Heat shock prot beta6-2	5.40E+06	6.96E+06	6.31E+06	5.43E+06	4.84E+06	1.46E+06	IS, Almond (unknown- 5	0.4080	0.2801	0.0765	0.2549	0.1672	65.6
1288.4	**68.72**	**IS, Almond (unknown) 5**	**1.63E+07**	**1.71E+07**	**1.70E+07**	**1.94E+07**	**1.67E+07**	**1.91E+07**	**IS, Almond (unknown)-6**	**0.1861**	**0.2053**	**0.1997**	**0.1970**	**0.0099**	**5.0**

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Table 1 – Continued

m/z [M+H]+	Rt (min)	row identity (main ID)	[a]QC1 Xcalibur	[a]QC1 MZmine	[a]QC2 Xcalibur	[a]QC2 MZmine	[a]QC3 Xcalibur	[a]QC3 MZmine	[b]IS normalizer	[c]QC 1 norm.	[c]QC 2 norm.	[c]QC 3 norm.	Norm. mean	Norm. SD	[d]CV (%)
850.8	**68.81**	**Heat shock prot beta6-1**	**3.88E+06**	**3.51E+06**	**3.19E+06**	**2.66E+06**	**4.59E+06**	**3.33E+06**	**IS, Almond (unknown)-5**	**0.2058**	**0.1373**	**0.1744**	**0.1725**	**0.0343**	**19.9**
1088.8	**69.23**	**IS, Prunin 1 precursor 2**	**3.00E+07**	**2.95E+07**	**3.24E+07**	**3.42E+07**	**2.98E+07**	**3.07E+07**	**IS, Almond (unknown)-5**	**1.7287**	**1.7668**	**1.6055**	**1.7003**	**0.0843**	**5.0**
863.2	**69.31**	**Galectin 1-2**	**6.25E+06**	**5.21E+06**	**6.27E+06**	**5.69E+06**	**6.01E+06**	**6.03E+06**	**IS, Prunin 1 precursor-2**	**0.1766**	**0.1663**	**0.1967**	**0.1799**	**0.0155**	**8.6**
1236.4	**70.28**	**IS, Almond (unknown)-6**	**9.19E+07**	**9.16E+07**	**9.32E+07**	**9.44E+07**	**9.62E+07**	**9.57E+07**	**IS, Prunin 1 precursor-2**	**3.1079**	**2.7570**	**3.1191**	**2.9947**	**0.2059**	**6.9**
1139.3	**72.39**	**Glyceraldehyde-3-phosphate dehydrogenase**	**2.83E+07**	**3.13E+07**	**3.32E+07**	**3.38E+07**	**3.22E+07**	**3.55E+07**	**IS, Almond (unknown)-7**	**0.6787**	**0.6301**	**0.7209**	**0.6766**	**0.0454**	**6.7**
1222.3	**73.22**	**IS, Almond (unknown)-7**	**4.45E+07**	**4.61E+07**	**5.39E+07**	**5.36E+07**	**4.85E+07**	**4.93E+07**	**IS, Almond (unknown)-6**	**0.5027**	**0.5677**	**0.5152**	**0.5285**	**0.0345**	**6.5**

In bold: Assignments with averaged normalized results exhibiting CVs up to 25%.

[a]Peak area integration from manual (Xcalibur) and automated (MZmine) processing of Full MS data (scan event 1, see LC-MS analysis section for full description).

[b]Normalization of automated data through the closest internal standard (IS) eluted to the considered analyte (analyte/IS).

[c]Normalized automated results from ratios defined in b.

[d]CV: coefficient of variation expressed as %.

Upon defining validation boundaries, next step was to test linearity of those sarcoplasmic peptides that exhibited acceptable results in the QC experiment (15, bolded in **Table 1**). Automated quantitative analysis of CAL batch (**Table 2**) yielded 18 calibration curves of bovine peptides and included those from triosephosphate isomerase-1, actin-2 and Protein/nucleic acid deglycase DJ 1-3 that were close to reach acceptable results in the preliminary QC study (25%<CV<50%, **Table 1**) finding, as expected, that retention times of assignments met those listed in **Table S1**. Linearity achieved was rather good in all cases (r^2 ranged between 0.95 and 0.99) as shown by **Table 2** and **Figure S4**. Among these meaningful results, triosephosphate isomerase-1, actin-2 and Protein/nucleic acid deglycase DJ 1-3 peptides confirmed the relevance of reaching the confidence threshold in their peak area integrations (**Table 2**). Similarly to LC-MS-based metabolomics, peptides with lower experimental masses (about m/z <850) had the weakest results ($r^2 \approx 0.95$) since they were more prone to deviations exerted by LRMS detection.

Results from pooled samples (QC and CAL batches) may suggest feasibility of direct quantitative peptide analysis of raw (unpurified/unfractionated) protein extracts once completing the preliminary qualitative approach. Further research is needed about the potential use of this methodology for routine analysis.

Table 2. [a]Robustness of automated relative quantitation of calibration (CAL) batch analysis and linearity of [b]peptides assayed.

[M+H]+ *m/z*	Rt (min)	Peptide	[c]A Peak area	[c]B Peak area	[c]C Peak area	[c]D Peak area	[c]E Peak area	[d]IS normalizer	[e]A norm	[e]B norm	[e]C norm	[e]D norm	[e]E norm	[f]R^2
519.1	2.4	IS, Almond (unknown)-1	6.75E+07	7.92E+07	8.34E+07	7.28E+07	7.32E+07	IS, Almond (unknown)-2	1.1340	1.1852	1.0015	1.2291	1.1544	
941.4	2.8	Beta enolase-1	5.20E+06	1.70E+07	2.23E+07	3.30E+07	3.73E+07	IS, Pru 2 partial-3	0.0330	0.1063	0.1430	0.1932	0.2164	0.9820
955.6	2.9	IS, Pru 2, partial-3	1.57E+08	1.60E+08	1.56E+08	1.71E+08	1.72E+08	IS, Almond (unknown)-1	2.3320	2.0173	1.8697	2.3463	2.3559	
748.3	3.3	Myoglobin-3	6.68E+06	1.73E+07	4.01E+07	3.67E+07	5.40E+07	IS, Almond (unknown)-2	0.1122	0.2586	0.4817	0.6198	0.8524	0.9823
593.1	3.4	IS, Almond (unknown)-2	5.95E+07	6.69E+07	8.33E+07	5.92E+07	6.34E+07	IS, Prunin 1 precursor-3	0.9594	0.8044	0.8647	0.5948	0.6267	
954.2	3.5	Triosephosphate isomerase-1	4.94E+06	1.07E+07	2.03E+07	3.06E+07	3.51E+07	IS, Prunin 1 precursor-3	0.0796	0.1287	0.2106	0.3073	0.3469	0.9906
1162.8	3.6	IS, Prunin 1 precursor-3	6.20E+07	8.31E+07	9.63E+07	9.96E+07	1.01E+08	IS, Pru 2 partial-3	0.3941	0.5200	0.6176	0.5830	0.5867	
1055.2	4.3	IS, Pru 2, partial-1	2.27E+08	2.91E+08	3.21E+08	3.19E+08	3.40E+08	IS, Almond (unknown)-3	4.1006	3.9728	4.0688	3.9871	4.1140	
1245.3	4.9	IS, Almond (unknown)-3	5.55E+07	7.31E+07	7.90E+07	8.01E+07	8.27E+07	IS, Prunin 1 precursor-3	0.8938	0.8798	0.8198	0.8047	0.8174	
718.5	5.6	IS, Prunin 1 precursor-1	3.12E+07	4.02E+07	3.94E+07	3.93E+07	3.84E+07	IS, Almond (unknown)-3	0.5624	0.5494	0.4985	0.4909	0.4651	
1026.2	5.6	IS, Almond (unknown)-4	5.95E+07	7.02E+07	8.08E+07	8.18E+07	1.09E+08	IS, Prunin 1 precursor-1	1.9069	1.7469	2.0521	2.0807	2.8311	
770.5	5.9	Triosephosphate isomerase-2	1.34E+07	2.37E+07	4.41E+07	5.60E+07	6.88E+07	IS, Prunin 1 precursor-1	0.4311	0.5895	1.1196	1.4237	1.7895	0.9896
595.0	64.3	IS, Prunin 2 precursor, partial-2	1.47E+07	2.12E+07	2.75E+07	2.45E+07	2.94E+07	IS, Prunin 2 precursor partial-1	0.1897	0.2457	0.2619	0.2620	0.3112	

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Table 2 – Continued

[M+H]+ *m/z*	Rt (min)	Peptide	[c]A Peak area	[c]B Peak area	[c]C Peak area	[c]D Peak area	[c]E Peak area	[d]IS normalizer	[e]A norm	[e]B norm	[e]C norm	[e]D norm	[e]E norm	[f]R^2
686.9	64.4	IS, Prunin 2 precursor, partial-1	7.73E+07	8.62E+07	1.05E+08	9.34E+07	9.44E+07	IS, Almond (unknown)-7	1.6241	1.5153	1.5734	1.4898	1.5323	
893.6	64.4	Creatine kinase M-3	1.13E+07	2.43E+07	4.16E+07	4.66E+07	6.91E+07	IS, Prunin 2 precursor partial-1	0.1466	0.2813	0.3960	0.4989	0.7324	0.9852
1123.7	64.7	Actin-2	4.08E+06	9.68E+06	2.36E+07	2.65E+07	3.75E+07	IS, Prunin 2 precursor partial-1	0.0527	0.1123	0.2242	0.2838	0.3968	0.9769
1056.3	65.0	Phosphoglycerate kinase 1-2	4.68E+06	1.75E+07	2.93E+07	3.86E+07	4.28E+07	IS, Prunin 2 precursor partial-1	0.0605	0.2025	0.2794	0.4130	0.4534	0.9826
992.1	65.8	Phosphoglycerate kinase 1-3	5.04E+06	1.53E+07	2.27E+07	2.83E+07	3.63E+07	IS, Almond (unknown)-8	0.1057	0.2691	0.3406	0.4512	0.5893	0.9925
1318.0	66.0	IS, Almond (unknown)-8	4.76E+07	5.69E+07	6.68E+07	6.27E+07	6.16E+07	IS, Pru 2, partial-2	1.1985	1.0910	1.1096	1.1273	1.4162	
879.1	66.0	IS, Pru 2, partial-2	3.97E+07	5.22E+07	6.02E+07	5.56E+07	4.35E+07	IS, Prunin 2 precursor partial-1	0.5137	0.6049	0.5728	0.5954	0.4608	
903.1	66.4	Alpha/Beta-enolase-3	1.63E+07	2.69E+07	6.11E+07	8.23E+07	1.02E+08	IS, Almond (unknown)-8	0.3423	0.4723	0.9156	1.3126	1.6509	0.9825
1012.2	66.7	Phosphoglycerate kinase 1-4	5.39E+06	8.86E+06	1.85E+07	2.35E+07	2.69E+07	IS, Almond (unknown)-8	0.1132	0.1558	0.2769	0.3748	0.4375	0.9888
743.0	67.1	Triosephosphate isomerase-3	2.82E+06	6.06E+06	1.40E+07	1.54E+07	2.06E+07	IS, Almond (unknown)-8	0.0593	0.1065	0.2095	0.2460	0.3351	0.9862
1292.8	68.2	Protein/nucleic acid deglycase DJ 1-3	2.37E+06	5.77E+06	1.20E+07	1.78E+07	2.34E+07	IS, Almond (unknown) 5	0.0710	0.1251	0.1908	0.2957	0.3306	0.9885
802.1	68.2	Triosephosphate isomerase-4	1.26E+07	1.88E+07	4.19E+07	4.55E+07	7.92E+07	IS, Almond (unknown)-5	0.3761	0.4067	0.6689	0.7560	1.1202	0.9456

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Table 2 – Continued

[M+H]⁺ *m/z*	Rt (min)	Peptide	[c]A Peak area	[c]B Peak area	[c]C Peak area	[c]D Peak area	[c]E Peak area	[d]IS normalizer	[e]A norm	[e]B norm	[e]C norm	[e]D norm	[e]E norm	[f]R^2
754.6	68.3	Creatine kinase M-2	2.99E+06	8.92E+06	1.47E+07	1.77E+07	2.37E+07	IS, Almond (unknown)-5	0.0894	0.1934	0.2352	0.2938	0.3352	0.9543
1045.7	69.5	Hemoglobin subunit beta	1.53E+06	4.34E+06	8.66E+06	1.23E+07	1.90E+07	IS, Almond (unknown)-5	0.0457	0.0941	0.1383	0.2038	0.2687	0.9901
1288.3	69.9	IS, Almond (unknown)-5	3.34E+07	4.61E+07	6.27E+07	6.02E+07	7.07E+07	IS, Almond (unknown)-6	0.2006	0.1897	0.2097	0.2006	0.2329	
850.7	70.0	Heat shock protein beta 6-1	2.95E+06	4.80E+06	1.40E+07	1.59E+07	2.64E+07	IS, Almond (unknown)-5	0.0882	0.1040	0.2240	0.2645	0.3734	0.9723
1088.8	70.3	IS, Prunin 1 precursor-2	5.48E+07	8.78E+07	1.02E+08	1.06E+08	1.32E+08	IS, Almond (unknown)-5	1.6396	1.9033	1.6344	1.7638	1.8723	
863.1	70.4	Galectin-1-2	3.02E+06	8.76E+06	2.13E+07	2.13E+07	3.61E+07	IS, Prunin 1 precursor-2	0.0552	0.0997	0.2080	0.2009	0.2727	0.9574
1236.3	71.6	IS, Almond (unknown)-6	1.66E+08	2.43E+08	2.99E+08	3.00E+08	3.04E+08	IS, Prunin 1 precursor-2	3.0399	2.7693	2.9182	2.8258	2.2935	
1139.2	72.7	Glyceraldehyde-3-phosphate dehydrogenase	1.67E+07	3.78E+07	7.35E+07	9.03E+07	1.25E+08	IS, Almond (unknown)-6	0.1002	0.1556	0.2461	0.3009	0.4106	0.9913
1222.2	74.7	IS, Almond (unknown)-7	6.98E+07	1.13E+08	1.46E+08	1.47E+08	1.79E+08	IS, Almond (unknown)-6	0.4195	0.4641	0.4892	0.4909	0.5894	

[a]From reproducibility of ISs response thorough samples.
[b]Peptides from quality control (QC) analysis with coefficients of variation (CVs) up to 50% (**Table 1**).
[c]Results from automated peak area integration. Pooled volumes (μL) assayed: A, 10; B, 20; C, 30; D, 40; E, 50.
[d]Normalization of data by the closest internal standard (IS) eluted to the considered analyte (analyte/IS). Ratios proposed for ISs normalization were from QC analysis (**Table 1**).
[e]Normalized results from ratios defined in b.
[f]Linear correlation coefficient from y=mx representation (full graphic details illustrated by **Figure S4**).

The final step of the proposed proteomics pipeline was to perform the automated quantitative analysis of OFGL batch. As a constraint, integrated peak areas below $7x10^6$ were discarded to ensure reliability of determinations. **Table S4** shows direct automated peak area integration and IS normalized results of 41 sarcoplasmic peptides found in OFFGEL fractions studied. Then, normalized ratios for each identified peptide were divided by the lowest value reached by same assignment across pH ranges assayed (**Table S5**) to calculate the fold change variation. This representation facilitated the elucidation of representative peptides (bolded in **Table S5**) from identified proteins according to their presence in fractions. As an example, we have creatine kinase M with three quantitative peptides (**Table S1**) but among them, peptide at *m*/*z* 893.8 was present in all detectable fractions (1, 7, 8, 9, 10, 11 and 12) addressing the identification of the parent protein. In contrast, peptides at *m*/*z* 998.3 and 754.7 appeared in only five (7, 8, 9, 10 and 11) and three (7, 8 and 9) of those fractions, respectively. Summation of the fold change of peptides belonging to the same parent protein detailed in **Table S5** enabled visualization of the OFFGEL distribution of 20 muscle sarcoplasmic proteins, not peptides, achieved by the targeted quantitative approach (**Table 3A**). Results (sorted by molecular weight) were comparable with those regarding same proteins (**Table 3B**) previously characterrized by the untargeted qualitative study and listed in **Table S3**. Interestingly, normalized protein abundances shown by **Table 3A** generally followed the same trend than Mascot scores in **Table 3B**, suggesting that higher protein abundances were translated into higher scores.

Compared to **Table S3**, number of proteins listed in **Table 3A** must be lower since many of identified peptides by Mascot analysis had a poor chromatographic response, thus hindering quantitative analysis as previously mentioned. Moreover, automated quantitative processing of OFGL data was also constrained by the integrated peak area confidence threshold considered, which evidently compromised determination of low abundant peptides (i. e. absence of myoglobin peptides in OFFGEL fraction 1 in **Table 3A**). Improving chromatographic features of signals (through narrower and better-shaped peaks) and/or using LC-HRMS approaches that greatly raise sensitivity and selectivity of analyses can surpass such restriction. In contrast, apparition of peptides in OFGL experiment not previously mentioned by the QC study (i. e. at *m*/*z*s 907.5 and 911.6 from phosphoglucomutase-1 and histidine triad nucleotide-binding protein 1, respectively, **Table S4** and **Table 3A**) demonstrated efficiency of the OFFGEL fractionation as a protein enrichment strategy.

Independently of the different experimental conditions assayed to analyze batches, well-established ratios among ISs must remain constant all time. To test such assumption, **Table S6** summarizes overall robustness of the automated quantitative analysis since ISs in QC (3 technical replicates), CAL (5 technical replicates) and OFGL (12 technical replicates) approaches were equally normalized through pairs initially proposed in QC

study (**Table 1**). In general, averaged ratios from different batches were rather comparable and confirmed reliability of the methodology proposed as shown by global CVs achieved (up to 20% in most cases, **Table S6**) considering 20 technical replicates.

Table 3. OFFGEL muscle sarcoplasmic protein distribution achieved by targeted MZmine quantitative (A) and untargeted Mascot qualitative (B) approaches.

	[a]**Targeted MZmine relative quantitative distribution**											**A**
Mr (Da)	Protein characterized	FR 1	FR 2	FR 3	FR 4	FR 5	FR 6	FR 7	FR 8	FR 9	FR 10	FR 11	FR 12
71196	Heat shock cognate 71 kDa				2.0								
69248	Serum albumin					2.7	1.0						
61551	Phosphoglucomutase							1.0					
47066	Alpha/beta-enolase	1.0					1.4	3.8	15.8	37.9	26.3	17.7	14.0
44509	Phosphoglycerate kinase 1									2.2	2.0	8.4	7.0
42962	Creatine kinase M	10.7						3.0	18.8	41.4	13.4	9.3	7.1
42024	Actin			9.6	31.9		1.0					2.7	
36415	Malate deshydrogenase					1.0	11.0	3.6					
35845	Glyceraldehyde 3 phosphate dehydrogenase						1.4	1.0	2.2	4.5	20.1	32.9	32.9
28667	Phosphoglycerate mutase										1.0	2.0	6.8
26673	Triosephosphate isomerase						4.4	12.4	53.2	6.9	2.0		
25051	Peroxi redoxin 6						3.9	1.0					
22379	Heat shock protein beta 1						1.0						
21650	Adenylate kinase isoenzyme							1.0			2.4	2.9	2.6
20023	Protein/nucleic acid deglycase DJ 1						3.1	3.4	1.0				
17458	Heat shock protein beta 6					7.0	34.8	8.8	1.0				
17067	Myoglobin							2.0	18.6	22.7	20.8	6.9	1.9
15944	Hemoglobin subunit beta									3.6	4.0	1.9	1.0
14734	Galectin 1		1.0	21.3	3.0								
13770	Histidine triad nucleotide binding protein							1.0					

Continued in the next page . . .

Table 3 - Continued

	[b]**Untargeted MASCOT qualitative score distribution**												**B**
Mr (Da)	Protein characterized	FR 1	FR 2	FR 3	FR 4	FR 5	FR 6	FR 7	FR 8	FR 9	FR 10	FR 11	FR 12
71196	Heat shock cognate 71 kDa				67								
69248	Serum albumin					175	69						
61551	Phosphoglucomutase							42.0					
47066	[c]Alpha/beta-enolase	b 67					a 61	a 164 + b 150	b 228	b 351	b 219	b 225	b 342
44509	Phosphoglycerate kinase 1										98	258	253
42962	Creatine kinase M	66						104	328	417	184	176	42
42024	Actin			198	196			33				36	
36415	Malate deshydrogenase					30	213	89					
35845	Glyceraldehyde 3 phosphate dehydrogenase	49					64	74	44	56	371	285	356
28667	Phosphoglycerate mutase											34	95
26673	Triosephosphate isomerase						276	278	524	396	89	31	
25051	Peroxi redoxin 6						142	38					
22379	Heat shock protein beta 1				53	31	50						
21650	Adenylate kinase isoenzyme							37			118	66	156
20023	Protein/nucleic acid deglycase DJ 1						36	72	37				
17458	Heat shock protein beta 6					28	44						
17067	Myoglobin	39						59	119	628	421	78	154
15944	Hemoglobin subunit beta								43	194	129	120	63
14734	Galectin 1		137	137	157								
13770	Histidine triad nucleotide binding protein							117					

[a] Results from summation of the fold change of peptides belonging to the same parent protein detailed in **Table S5**.
[b] Results from Mascot scores detailed in **Table S3**.
[c] Alpha (a) and beta (b) enolases fully distinguished by the untargeted qualitative analysis (**Table S3**).

3.4 CONCLUSIONS

The efficiency of the proposed straightforward gel-free pipeline analysis to perform reliable qualitative/quantitative proteomic approaches was demonstrated supported by conventional LC-MS analysis. Protein enrichment carried out by OFFGEL fractionation directly hyphenated to optimized LC-LRMS analysis greatly facilitated sampling procedure and improved sensitivity in the detection of tryptic peptides derived from muscle sarcoplasmic proteins.

Implementation of this robust, flexible, reliable and affordable LC-MS proteomic-based methodology is extremely easy and can promote the creation of new insights in proteome research of foodstuffs by industry to obtain higher quality products.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

Beldarrain LR., Aldai N, Picard B, Sentandreu, E, Navarro, JL, Sentandreu, MA (2018). Use of liquid isoelectric focusing (OFFGEL) on the discovery of meat tenderness biomarkers. *Journal of Proteomics* 183: 25–33.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.

Bu X, Regalado EL, Hamilton SE, Welch CJ. (2016). The emergence of low-cost compact mass spectrometry detectors for chromatographic analysis. *TrAC Trends in Analytical Chemistry* 82: 22–34.

Evans AM, Bridgewater BR, Liu Q, Mitchel MW, Robinson RJ, Dai H, et al. (2014). High Resolution Mass Spectrometry Improves Data Quantity and Quality as Compared to Unit Mass Resolution Mass Spectrometry in High-Throughput Profiling *Metabolomics: open access* 4:132.

Franco D, Mato A, Salgado FJ, López-Pedrouso M, Carrera M, Bravo S, Parrado M, Gallardo JM, Zapata C. (2015). Tackling proteome changes in the longissimus thoracis bovine muscle in response to pre-slaughter stress. *Journal of Proteomics* 122: 73–85.

Fuente-Garcia C, Aldai N, Sentandreu E, Oliván M, García-Torres S, Franco D, Zapata C, Sentandreu MA. (2019). Search for proteomic biomarkers related to bovine pre-slaughter stress using liquid isoelectric focusing (OFFGEL) and mass spectrometry. *Journal of Proteomics* 198: 59–65.

Griffiths WJ and Wang Y. (2009). Mass spectrometry: from proteomics to metabolomics and lipidomics. *Chemical Society Reviews* 38: 1882–1896.

Kohlbacher O, Reinert K, Gröpl C, Lange E, Pfeifer N, Schulz-Trieglaff O, Sturm M. (2007). TOPP—the OpenMS proteomics pipeline. *Bioinformatics* 23: e191–e197.

Lin K, Kools H, de Groot PJ, Gavai AK, Basnet RK, Cheng F, Wu J, Wang X, Lommen A, Hooiveld GJEJ, Bonnema G, Visser RGF, Muller MR, Leunissen JAM. (2011). MADMAX – Management and analysis database for multiple ~omics experiments. *Journal of Integrative Bioinformatics* 8: 59–74.

Lu X, Lodi A, Konopleva M, Tiziani S. (2019). Three-Dimensional Leukemia Co-Culture System for In Vitro High-Content Metabolomics Screening. *SLAS DISCOVERY: Advancing the Science of Drug Discovery* 24: 817–828.

Mann M and Kelleher NL. (2008). Precision proteomics: The case for high resolution and high mass accuracy. *Proceedings of the National Academy of Sciences of the United States of America* 105(47): 18132–18138.

Meisrimler CN and Lüthje S. (2012). IPG-strips versus off-gel fractionation: Advantages and limits of two-dimensional PAGE in separation of microsomal fractions of frequently used plant species and tissues. *Journal of Proteomics* 75: 2550–2562.

Mirzapour M, Rezaei K, Sentandreu MA, Moosavi-Movahedi AA. (2016). In vitro antioxidant activities of hydrolysates obtained from Iranian wild almond (*Amygdalus scoparia*) protein by several enzymes. *International Journal of Food Science & Technology* 51: 609–616.

Monteoliva L and Albar JP. (2004). Differential proteomics: An overview of gel and non-gel based approaches. *Briefings in Functional Genomics* 3: 220–239.

Sentandreu MA, Fraser PD, Halket J, Patel R, Bramley PM. (2010). A Proteomic-Based Approach for Detection of Chicken in Meat Mixes. *Jornal of Proteome Research* 9: 3374–3383

Southam AD, Weber RJM, Engel J, Jones MR, Viant MR. (2017). A complete workflow for high-resolution spectral-stitching nanoelectrospray direct-infusion mass-spectrometry-based metabolomics and lipidomics. *Nature Protocols* 12: 310–328.

Taverner T, Karpievitch YV, Polpitiya AD, Brown JN, Dabney AR, Anderson GA, Smith RD. (2012). DanteR: an extensible R-based tool for quantitative analysis of -omics data. *Bioinformatics* 28: 2404–2406.

Wang W, Zhou H, Lin H, Roy S, Shaler TA, Hill LR, Norton S, Kumar P, Anderle M, Becker CH. (2003). Quantification of Proteins and Metabolites by Mass Spectrometry without Isotopic Labeling or Spiked Standards. *Analytical Chemistry* 75: 4818–48

4 | APPENDIX IV
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Proteomic pipeline for biomarker hunting of defective bovine meat assisted by liquid chromatography-mass spectrometry analysis and chemometrics[4]

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Abstract

A wide variety of factors prior to slaughter may affect the stress status of beef cattle, giving rise to well-known 'dark-cutting' defective meats characterised by a high ultimate pH (pHu). To understand the underlying mechanisms of pHu fluctuations in beef cattle there was studied the proteome changes caused by pre-slaughter stress through a *gel-free* proteomic approach. Comparative peptidomic analysis was carried out on 12 loin samples at 24h post-mortem from *Longissimus thoracis et lumborum* bovine muscle of crossbred animals, previously sorted into two different groups according to their pHu values: normal (pHu < 6.0) and high (pHu ≥ 6.0). Tryptic peptides from direct protein extracts were approached by combining untargeted (intact mass, MS^1) and targeted (Selected Reaction Monitoring, SRM) quantitative LC-MS assays followed by chemometric analysis. Seventeen peptide biomarkers belonging to 10 different proteins appropriately discriminated sample groups assayed. Results may promote the use of this simple and effective methodology towards the creation of new insights in meat quality research.

Significance: The significance of this study was the optimization of an affordable straightforward *gel-free* proteomic approach addressing the differentiation of the muscle sub-proteome of normal and high pHu meat samples. This strategy allowed the study of tryptic peptides from direct meat protein extracts by combining untargeted MS^1 and targeted SRM quantitative assays performed by conventional LC-MS detection. Affordability, simplicity and robustness of this methodology can facilitate its readily implementation in routine protocols for quality assessment of meat.

Keywords

Pre-Slaughter Stress ● High pHu Meat ● *Gel-Free* Analysis ● Targeted Quantitative Analysis • Peptide Biomarkers • Muscle Proteome

4.1 INTRODUCTION

Pre-slaughter stress (PSS) response in cattle is one of the main negative modulators of meat quality attributes. Animal stressing promotes apparition of defective meats such as dark-cutting meat (also known as dark, firm and dry, DFD) having a characteristic high ultimate pH (pHu) that is commonly rejected by consumers due to their undesirable colour/flavour and short shelf life [1]. Seasonal and geographical peculiarities as well as animal and meat handling practices have an important role on the occurrence of defective carcasses, ranged between 6 and 50% of total carcasses [2], causing significant losses to meat industry [3,4]. Scientific studies can favour understanding of PSS condition aiming to minimize occurrence of faulty meat and to satisfy current high-quality demands of consumers and traders. Several studies were conducted to elucidate the fundamental basis of high pHu meats to develop efficient post-harvest strategies that can improve their colour and value [5]. Unfortunately, investigation on PSS is hindered by its multi-factorial origin involving intrinsic (i. e. genetics, breed, age, sex) and extrinsic (i. e. animal housing, feeding, transportation, lairage) variables [6]. PSS response induces the secretion of hormones such as catecholamines, cortisol and adrenaline that lead depletion of glycogen reserves of muscle to produce energy for its contraction and relaxation before slaughter [6-8]. In glycogen-depleted muscle fibres, the substrate availability for post-mortem anaerobic glycolysis is reduced, limiting the production of lactic acid that is translated into the occurrence of defective meat with high pHu values. Determination of meat pHu is critical for industry [9] since authorities consider worldwide that values higher than 6.0 measured at 24 h post-mortem are intimately associated to PSS animals [10] and DFD meats [2]. However, usefulness of pHu assessment is compromised since high values do not necessarily guarantee apparition of true DFD meats [1].

New insights addressing the search of new stress indicators are needed to understand biochemical mechanisms underlying PSS condition and to overcome uncertainties from classic pHu determinations in meat [1]. Further advances will positively contribute to better understand all aspects involving generation of animal welfare guidelines and good meat industry practices [1]. Assessment of PSS response can be excellently approached by proteomic strategies monitoring protein/enzyme groups altered during post-mortem metabolism of meat from stressed animals. Surprisingly, only few recent proteomic studies addressed changes induced by PSS on the muscle proteome [9,11-18]. Achieved results strongly depended on gel-based methodologies that provided an excellent resolving power, although still having important limitations such as the poor representation of low abundant proteins, the use of hazardous chemicals (i. e. acrylamide), inaccurate quantitative image analysis and lack of reproducibility and automation [19]. Alternatively, Sentandreu et al. (2021) recently proposed a more sustainable *gel-free* proteomic

pipeline supported by liquid isoelectric focusing (OFFGEL) protein fractionation coupled to LC-MS exploratory analysis to study the bovine proteome [20]. Enrichment and purification provided by OFFGEL fractionation greatly facilitated protein mapping and peptide quantification of bovine sarcoplasmic extracts despite of using a conventional three-dimensional ion-trap (3D-IT). Advantages shown by OFFGEL fractionation in untargeted analysis can be overshadowed by its time-demanding sample preparation (about 20 h) when used in routine analysis of targeted analytes. This compromises its implementation in standardized quality control protocols in food industry.

Alternatively to efficient but costly high-resolution (LC-HRMS) shotgun proteomics mainly used in basic research, targeted LC-MS strategies based on selected reaction monitoring/multiple reaction monitoring (SRM/MRM) full-scan analysis can greatly simplify routine analysis of known peptides [21]. Main advantages of SRM/MRM approach are the affordability of the MS technology requested (mainly low-resolution, LC-LRMS, ion-trap and triple-quadrupole, QQQ, devices), its high sensitivity and specificity that can avoid tedious enrichment/purification steps (i. e. SDS-PAGE gels and OFFGEL fractionation) commonly used in the preparation of protein samples before analysis. Surprisingly, there is a lack of MS-based analytical methodologies covering the study of the proteome associated to defective high pHu meats that could be easily implemented for quality evaluation in food industry.

Main goal of this research was the study of the sarcoplasmic proteome of normal and high pHu bovine meat extracts through an innovative *gel-free* LC-MS proteomic strategy coupled with chemometrics aiming at biomarker hunting of meats with different pHu. Samples were analyzed by a conventional 3D-IT featuring a preliminary MS^1 and MS/MS untargeted approach to search potential biomarker candidates followed by a targeted SRM assay to accurately test their discrimination efficiency. Chemometrics combined principal component analysis (PCA) of preliminary MS^1 data with parametric and non-parametric statistical approaches of SRM results from direct protein extracts. Once identified, peptides showing discrimination capacity can be straightforwardly determined in crude protein extracts by targeted SRM analysis.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

LC-MS grade Formic Acid (FA), ACN, TFA and 0.45 μm PVDF filters were from Scharlab (Scharlab S. L., Barcelona, Spain). Ultrapure grade water was from Millipore (EMD Millipore Co., Billerica, MA, USA). Bradford Protein Assay Kit was from Bio-Rad (Bio-Rad, Hercules, CA, USA).

4.2.2 Preparation of the internal standards (IS) solution

A wild almond protein extract obtained from a local market in Valencia (Spain) was prepared according to [22] with a

resultant protein content of 10 mg/mL determined by the Bradford assay kit [23]. The extract was tryptically digested (3 h, 37 °C and pH 7.8) at an enzyme/substrate ratio of 1.0% (w/w) and centrifuged at 4000 *g* for 10 min at 4 °C using an Avanti J-26S XP Beckman Coulter centrifuge (Brea, CA, USA). Supernatants were 1:46 diluted with aqueous 0.1% TFA and aliquots stored at -20 °C until spiked as IS into samples assayed.

4.2.3 Sample selection

Twelve bovine muscle samples belonging to Asturiana de los Valles x Friesian breed were collected from a commercial abattoir in the northern Spain. Animals were reared under intensive system (indoor fattening with concentrate meal and barley straw *ad libitum*). They were slaughtered and dressed at the age of 15 months (yearling bulls) according to EU regulations (Council Regulation (EC) No 853/2004 and No 1099/2009). At 24 h post-mortem, approximately 10 g of *Longissimus thoracis et lumborum* (LTL) muscle sample from the 13th rib was excised from the left half of each carcass. The epimysium was carefully dissected and samples were immediately frozen in liquid nitrogen and stored at -80 °C until processed for protein extraction. Muscle samples were classified in two different groups according to their pHu values: a) normal pHu samples (NORMAL, n=6), having pHu values below 6.0 (in our case 5.53 ± 0.14), and b) high pHu samples (HIGH, n=6) with pHu values higher than 6.0 (in our case 6.56 ± 0.25). Measurements of pHu were performed at the sixth rib of the LTL muscle at 24h post-mortem using a penetration electrode (CRISON pH/mV-meter 506, CRISON Instruments S. A., Spain).

4.2.4 Preparation of samples

4.2.4.1 Extraction of sarcoplasmic proteins

It was carried out following the procedure proposed by Fuente-García et al. (2019) [12] with modifications. Briefly, half a gram of muscle sample was homogenized in 4 mL of extraction buffer (10 mM Tris pH 7.6 containing 0.25 M sucrose, 1 mM EDTA and 25 μL of protease inhibitors), centrifuged at 20,000 *g* for 20 min at 4 °C and the supernatant filtered through a 0.45 μm PVDF filter, constituting the soluble protein extract. Those extracts (100 μL each) were cleaned up through OMIX 100 μL C18 pipette tips (Agilent Tech. S. L., Madrid, Spain) and the recovered ACN eluates completely evaporated by a SPD121P Speed Vac concentrator (Thermo Scientific, San Jose, CA, USA) previously to tryptic digestion.

4.2.4.2 In-solution trypsin digestion of soluble protein extracts

Dried samples were rediluted in 15 μL of a sequencing grade modified trypsin solution at a 12.5 μg/mL concentration and mixed with an equal volume of 50 mM ammonium bicarbonate (pH 8.0). The mix was incubated overnight at 37 °C with continuous gently shaking allowing protein digestion. After incubation, samples were completely evaporated

using Speed Vac and finally re-suspended in 80 μL of a 0.1% TFA aqueous solution, respectively.

Fifty μL of resuspended samples were spiked with 15 μL of the IS solution, stirred, centrifuged and poured into LC-MS vials conforming the normal and high pHu replicates batch (NORMAL/HIGH-RPL). The remaining extract volumes (30 μL) were pooled in either normal or high pHu groups (6 replicates per group giving a final volume of 180 μL), constituting the calibration batch (NORMAL/HIGH-CAL) as follows: five different volumes of the pool ranging from 10 to 50 μL (in 10 μL steps) were individually mixed in Eppendorf tubes with 10 μL of the IS solution and a variable volume of aqueous 0.1% TFA solution to reach a final volume of 60 μL. Tubes were stirred, centrifuged (22,000 *g* for 3 min) and poured into LC-MS vials. All samples were stored at -80 °C until LC-MS analysis.

4.2.5 LC-MS analysis

Chromatographic analysis was performed in a Thermo Surveyor LC system equipped with a cooled autosampler, quaternary pump and vacuum degasser coupled to a Thermo LCQ Advantage ion-trap mass analyzer loading an ESI probe operating in positive ionization mode (Thermo Scientific, San Jose, CA, USA). Separation of peptides was achieved by a reverse-phase 150 x 0.5 mm i. d., 4 μm particle size Jupiter Proteo column from Phenomenex (Phenomenex Inc., Torrance, CA, USA) using 0.1% FA (v/v) in ultrapure water as solvent A and 0.1% FA (v/v) in ACN as solvent B. Separation conditions were: initially 0% B, held for 15 min; 0-20% B in 2 min, held for 4 min; 20-40% B in 1 min, held for 8 min; 40-100% B in 1 min, washing with 100% B for 8 min and column equilibration at initial conditions for 55 min. Injection volume and flow-rate were 25 μL and 50 μL/min, respectively. Temperatures were set to 10 °C and 23 °C for autosampler and column, respectively.

A preliminary untargeted qualitative/quantitative peptide approach of NORMAL/HIGH-RPL batch merging full-MS^1 and data dependent MS/MS (dd-MS^2) experiments was performed using the same instrumental conditions proposed by [20]. Briefly, general settings of analysis were: capillary temperature, 250 °C; spray voltage, 4.0 kV; capillary voltage, 15.0 V; considered *m*/*z* range for MS^1 analysis, 400-2000. Specific MS/MS parameters were set at: charge of peptides for MS/MS analysis, +1 to +4; normalized CID energy, 35%; minimum MS/MS ion intensity threshold, $1x10^5$; exclusion list, 25 masses from spiked ISs and background noise from a blank injection; exclusion time, 3 min; exclusion mass width, 3 amu; repeat count for MS/MS of most intense ion, 3; repeat count duration, 0.5 min; number of microscans-maximum injection time were 1-200 ms and 1-300 ms for MS^1 and dd-MS^2 experiments, respectively.

This first approach was followed by a targeted quantitative analysis of NORMAL/HIGH-RPL and NORMAL/HIGH-CAL batches to test discrimination capacity of preliminary biomarker candidates multiplexed full-MS^1 and SRM experiments operating under the same aforementioned general conditions. Tailo-

red settings conducting SRM research were the following: normalized CID energy, 35%; number of SRM fragments (transitions) monitored per peptide precursor, 3; number of microscans, 1; maximum injection time, 200 ms.

Control of the LC-MS device and manual data processing was featured by a PC loading the Thermo Xcalibur v2.04 software (Thermo Sci, San Jose, CA, USA).

4.2.5.1 Processing of preliminary Full-MS¹ and dd-MS² data

Processing of full-MS¹ and dd-MS² data was carried out as suggested by [20]. Briefly, quantitative MS¹ analysis was performed by MZmine 2 v.2.53 (https://github.com/mzmine/mzmine2/releases) loading the previously released library by authors (**Table S1**) regarding peptides derived from sarcoplasmic proteins. Relative MS¹ quantification of peptides was normalized considering peak area ratios of signals with their closest eluted IS (**Table S1**). Interrogation of MS/MS data from tryptic peptides used Mascot v2.3 as search engine (www.matrixscience.com) loading the UniprotKB (www.uniprot.org) and NCBIprot protein databases. Decoy option was enabled to estimate false positive rates by means of False Discovery Rate threshold 1%. Those assignments with a protein score derived from individual ion scores indicating identity or extensive homology ($P \leq 0.05$) were considered as true protein identifications.

4.2.5.2 Processing of targeted SRM data

Untargeted MS¹ quantitative approach (discussed below) yielded the list of preliminary peptide biomarkers according to their coarse discrimination features (very high, moderate and none), constituting the SRM libraries (**Tables S2A and S2B**). Such databases collect the tentative identification of potential peptide biomarkers (according to their protein of origin), their intact mass (*m*/*z* of precursors), *m*/*z* of three specific SRM fragments from their breakdown patterns and the windowed SRM scan event segments according to the retention time of peptides provided by the preliminary untargeted MS¹ analysis. Selection of SRM fragments aimed at maximizing sensitivity and specificity of the analysis, performing a manual inspection of dd-MS² data from results provided by Mascot research. Further technical considerations about the election of SRM settings are detailed in **supplementary file 1**.

Automated analysis of SRM data was carried out through the freely available Thermo Scientific FreeStyle v.1.6 software (https://thermo.flexnetoperations.com/). Optimization of FreeStyle settings (**Table S3**) was manually approached by Xcalibur since both solutions share the same algorithm of analysis (ICIS) yielding comparable results. Both FreeStyle and Xcalibur alternatives generated one single SRM chromatographic peak corresponding to the automatic summation of the all three full-scan MS/MS signals (integrated peak areas) belonging to the same peptide precursor (**Tables S2A** and **S2B**).

Relative SRM quantifications were normalized according to peak area ratios of targeted peptides with selected ISs (**Tables S2A** and **S2B**).

4.2.6 Statistical analysis

PCA was applied to full-MS^1 data from NORMAL/HIGH-RPL batch aiming at the creation of the preliminary list of potential peptide biomarkers subsequently determined by the SRM approach. Same PCA strategy was followed to study the animal sample distribution according to normalized SRM results from NORMAL/HIGH-RPL batch. Variables with communality values over 0.6 were included in the model and rotated principal components (PCs, extracted by Varimax analysis) explaining >5% of the variance were selected.

Least square means (LSM) of each dependent variable (peptide) was calculated using the normalized peak areas of NORMAL/HIGH-RPL batch and compared through parametric *t*-test and Mann-Whitney non-parametric tests. Significant level was declared at $P \leq 0.05$. The usual experimental type I error rate of $\alpha = 0.05$ was controlled using the Benjamini-Hochberg adjustment.

Descriptive statistic and conventional statistical test (parametric and non-parametric) was done using SPSS 25.0.0 software (New York, NY, USA).

Relative change (RC) coefficient was estimated in NORMAL/HIGH-RPL batch to assess the peptide quantitative change in normal and high pHu groups. This coefficient was calculated using LSM values (1) of peptides as follows:

$$RC = dLSM \,/\, |LSM_{High}| \quad (1)$$

in where *dLSM* is a measure of the differential normalized peak areas between the high and normal pHu group. *RC* coefficient ranges between -1.0 and +1.0 and takes a zero value in an absence of any intensity change.

Comprehensive analysis of proteomics data was performed by Perseus software v. 1.6.5.0 (http://www.perseus-framework.org) featuring hierarchical clustering [24]. Matrix data from NORMAL/HIGH-RPL batch was previously 2x log transformed and missing values were replaced by random numbers that were drawn from a normal distribution. Hierarchical clustering of peptides (rows) and biological replicates (columns) from each group assayed (normal and high pHu) was used to design a heat map representation of the clustered matrix using Euclidean distance for both row and column trees.

4.2.7 Bioinformatic analysis

In functional analysis, nomenclature used for quantified peptides from targeted SRM analysis of the NORMAL/HIGH-RPL batch was referred to their proteins of origin. Among proteins studied, only those exhibiting significant differences ($P \leq 0.05$) between normal and high pHu meat samples were considered for functional classification using Gene Ontology (GO) slim terms by means of AmiGO webside (http://amigo.geneontology.org/amigo/). Information about protein-protein inter-

actions was provided by String v.10.5 freeware (https://string-db.org) and results were uploaded into the open source platform Cytoscape [25] to elucidate protein networks. *Bos taurus* species was selected in all steps of the functional analysis performed.

4.3 Results and discussion

LC-MS files (mzML format) of preliminary full-MS^1 analysis of NORMAL/HIGH-RPL batch and targeted SRM files (mzML format and native RAW extension for Thermo FreeStyle analysis) from replicates and calibration batches are freely available at: https://digital.csic.es/handle/10261/221963.

4.3.1 Preliminary full-MS^1 screening for coarse election of potential peptide biomarkers

PCA analysis of normalized full-MS^1 results (total number of signals relatively quantified was 32) from NORMAL/HIGH-RPL batch (n=12) featured the initial screening of feasible biomarkers. **Table S4** presents factor loadings, amino acid sequence and charge of quantified peptides associated to the first six rotated PCs that explained 95.89% of the variance. Among those 32 signals, final selection of preliminary biomarker candidates was featured according to high factor loadings achieved (above 0.6, absolute value) in PC1, PC2 or PC3 (explaining 30.90, 25.69 and 13.54% of variance, respectively). As a result, 24 peptides (bolded in **Table S4**) populated the definitive list of potential peptide biomarkers that were subsequently studied in detail by SRM analysis. Myoglobin-1, triosephosphate isomerase-1, malate dehydrogenase-1, triosephosphate isomerase-3 and protein/nucleic acid deglycase DJ 1-3 were excluded because they showed poor signal values. Despite their poor results, phosphoglycerate mutase 2 and malate dehydrogenase-2 were exceptionally considered for further research since both peptides were referred to proteins not yet considered by other candidates. Total number of different proteins assayed according to the targeted SRM peptidomic approach was 14.

4.3.2 Targeted SRM quantitative analysis of selected peptide biomarkers

Fig. S1 illustrates advantages of targeted SRM analysis (SRM 1) under conditions assayed over untargeted MS^1 detection both featured by conventional 3D-IT detection in NORMAL/HIGH-CAL samples. Very clearly, accuracy of determinations provided by SRM assay led to overcome uncertainties from LRMS (see differences from A-B MS^1 analyses with respect to their C-D SRM counterparts in the study of LGALS1-1 and HSPB6-2 peptides, respectively, see **Table 1** for nomenclature description).

It must be highlighted that from the 24 biomarker candidates proposed by the preliminary MS^1 approach (**Tables S2A** and **S2B**), only one at *m*/*z* 992.2 (**Table S2A**) had an erratic behaviour and was discarded for further SRM research.

Table 1 represents LSM from normalized quantitative SRM results of peptides determined in NORMAL/HIGH-RPL batch. Seventeen peptides showed signifi-

cant statistical differences ($P \leq 0.05$) between groups assayed, finding higher values in high pHu samples. Interestingly, peptide representing HSPB6-1 (*m*/*z* at 850.7), previously considered to be exclusive of high pHu meats [12], was observed even in low abundance in several (3 of 6 replicates) normal meat samples, evidencing usefulness of SRM analysis compared to less specific LC-LRMS[1] untargeted alternatives. It was also noticeable that SRM peptides at *m*/*z* 734.3, 770.5, 1275.5, 863.2, 893.0 and 1597.5 were exclusively found in all high pHu replicates studied.

Table 1. SRM 1 and 2 results (LSM ± SEM, arbitrary units) from normal (n=6) and high (n=6) pHu protein samples (NORMAL/HIGH-RPL batch) of LTL bovine muscle.

Peptide ID[a]	*m*/*z* [M + H]$^+$	RT	Normal	High	SEM	*P*-value[b]	Acronym[c]
Myoglobin-2	629.4	2.2	0.175	0.273	0.025	0.043	**MB**-2
Galectin 1-1	968.5	2.4	0.00626	0.0168	0.00266	0.016	**LGALS1**-1
Actin-1	978.6	2.5	0.0178	0.0665	0.0093	0.006	**ACTA1**-1
beta enolase-1	941.7	2.7	0.0992	0.235	0.0287	0.007	**ENO3**-1
Phosphoglycerate kinase 1-1	734.3	2.7	nd	0.0357	0.0047	0.001	**PGK1**-1
beta-enolase-2	1296.3	3.0	0.00694	0.00815	0.00150	ns	**ENO3**-2
Phosphoglucomutase 1	907.5	3.2	0.0276	0.0469	0.0064	ns	**PGM1**
Triosephosphate isomerase-2	770.5	4.8	nd	0.0285	0.0126	0.001	**TPI1**-2
Creatine kinase M-1	998.3	5.1	0.110	0.238	0.033	0.009	**CKM**-1
alpha/beta-enolase-3	903.2	63.3	0.488	0.673	0.068	ns	**ENO1**
Triosephosphate isomerase-4	802.1	66.1	0.245	0.473	0.051	0.012	**TPI1**-4
Creatine kinase M-2	754.8	66.2	0.0556	0.142	0.0257	0.016	**CKM**-2
Creatine kinase M-3	893.5	66.3	0.0647	0.194	0.0270	0.005	**CKM**-3
Actin-2	1123.5	66.8	0.00603	0.0211	0.00398	0.010	**ACTA1**-2
Phosphoglycerate kinase 1-2	1056.4	67.6	0.351	0.470	0.033	ns	**PGK1**-2
Phosphoglycerate mutase 2	821.6	67.8	0.282	0.377	0.040	ns	**PGAM2**
Phosphoglycerate kinase 1-4	1012.2	68.4	0.0604	0.102	0.0097	0.010	**PGK1**-4
Adenylate kinase isoenzyme 1	1031.5	69.0	0.0333	0.0406	0.0023	ns	**AK1**
Heat shock prot b6-1	850.7	71.5	nq	0.187	0.071	0.001	**HSPB6**-1
Heat shock prot b6-2	1275.5	71.9	nd	0.0688	0.0240	0.001	**HSPB6**-2
Galectin 1-2	863.2	72.1	nd	0.00608	0.00224	0.001	**LGALS1**-2
Malate deshydrogenase-2	893.0	72.3	nd	0.00259	0.00045	0.001	**MDH1**
Heat shock prot b1	1597.5	73.2	nd	0.0140	0.0041	0.001	**HSPB1**

Least Square Means were calculated using absolute intensity values from each group of samples (normal and high pHu meats).

LSM, Least Square Means, SEM, Standard Error of the Mean; nd, not detected (results constrained by the condition of finding considered SRM peptide in all six replicates belonging to the same sample group); nq, not quantified; ns, not significant.

[a] Peptide identification.

[b] Statistical differences were declared at $P \leq 0.05$.

[c] Entire acronym corresponds to the peptide name (the part highlighted **in bold** refers to the protein).

Figs. S2A and **S2B** illustrate calibration curves achieved by NORMAL/HIGH-CAL analyses, respectively. Dilution of pooled samples greatly compromised the obtaining of reliable signals from low abundant peptides, thus only those curves

with at least four calibration steps into **Figs. S2A** and **S2B** were considered. Results evidenced how better results in terms of number of signals (23 for HIGH-CAL *vs* 16 for NORMAL-CAL) and linearity were achieved by high pHu samples. Moreover, CAL batch analysis also confirmed that *m*/*z* at 734.3, 770.5, 1275.5, 863.2, 893.0 and 1597.5 exclusively belonged to HIGH-CAL sample group as previously shown by HIGH-RPL. In any case, NORMAL/HIGH-CAL assay demonstrated robustness of SRM determinations and confirmed reliability of results from the replicates batch study.

Fig. 1 illustrates differences in quantitative peptide change from normal to high pHu RPL groups. All SRM peptides assayed were highly related to high pHu groups since RC coefficients took positive values. Considering RC coefficient, the aforementioned peptides at *m*/*z* 734.3, 770.5, 1275.5, 863.2, 893.0 and 1597.5 plus peptide at *m*/*z* 850.7 underwent the strongest quantitative change (RC = +1.0) and certified their exclusiveness to high pHu sample groups. Only those peptides not showing significant statistical differences in the analysis of the replicates batch (*m*/*z* 1296.3, 907.5, 903.2, 1056.4, 821.6 and 1031.5, **Table 1**) had noticeable RC deviations as shown by **Fig. 1**.

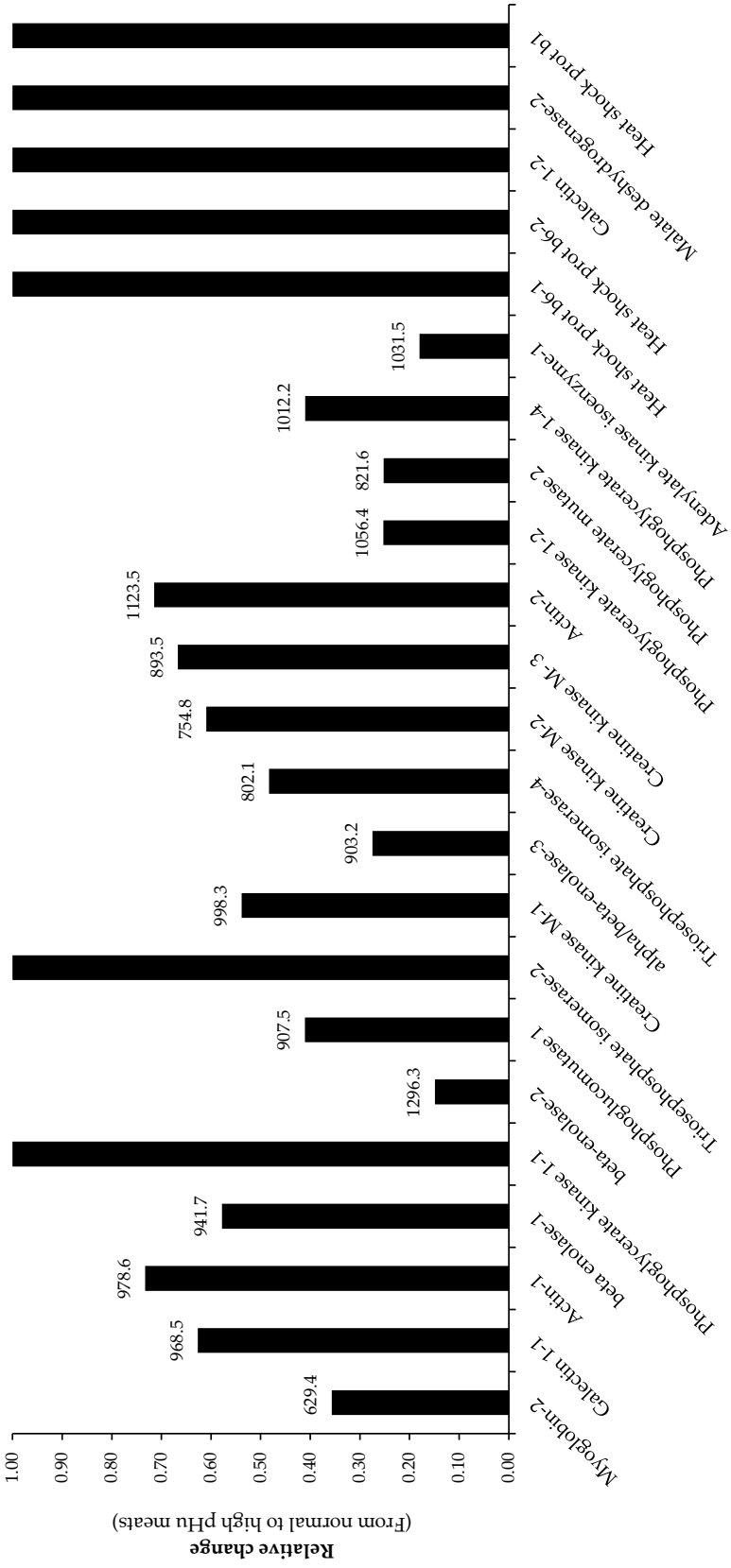

Fig. 1. Quantitative change between high and normal pHu meat samples of RPL batch was assessed by the RC coefficient. Nomenclature of peptides is shown in **Table 1**.

A PCA was performed to explain peptide and samples distribution from SRM assay. **Table 2** collects the first four rotated PCs (that explained 90.80% of variance) and associated variables. From 23 peptides populated in **Tables S2A** and **S2B** (signal at *m*/*z* 992.2 was discarded as previously mentioned), 13 showed high factor loadings (≥ 0.7) with PC1 (53.11% explained variance). ENO3-2 and PGK1-2 were highly correlated (factor loading ≥ 0.7) with PC2 (20.62% of explained variance) and PC3 (11.03% of explained variance), respectively. Remaining peptides were distributed among PC1-PC2, PC1-PC3, PC1-PC4, PC2-PC3 and PC1-PC2-PC3 and exhibited medium factor loadings (≈0.4 - 0.7).

Table 2. Rotated factor loadings of SRM quantified peptides extracted from the PCA applied to normal (n=6) and high (n=6) pHu replicate batch (NORMAL/HIGH-RPL).

Peptides assayed	[a]*m*/*z*	Principal components and explained variance (%)			
		1 (53.113%)	2 (20.621%)	3 (11.034%)	4 (6.021%)
Myoglobin-2	629.4	0.673			0.579
Galectin 1-1	968.5	0.843			
Actin-1	978.6	0.806			-0.456
beta enolase-1	941.7	0.784	-0.483		
Phosphoglycerate kinase 1-1	734.3	0.698			-0.431
beta-enolase-2	1296.3		0.788	-0.406	
Phosphoglucomutase 1	907.5	0.745			
Triosephosphate isomerase-2	770.5	0.608	-0.625	-0.460	
Creatine kinase M-1	998.3	0.649	-0.647		
alpha/beta-enolase-3	903.2	0.608	-0.614		
Triosephosphate isomerase-4	802.1	0.755	-0.507		
Creatine kinase M-2	754.8	0.596	-0.695		
Creatine kinase M- 3	893.5	0.900			
Actin-2	1123.5	0.790	0.578		
Phosphoglycerate kinase 1-2	1056.4	0.550		0.736	
Phosphoglycerate mutase 2	821.6	0.448	-0.570	0.400	
Phosphoglycerate kinase 1-4	1012.2	0.805			
Adenylate kinase isoenzyme-1	1031.5	0.666		0.498	
Heat shock prot b6-1	850.7	0.815			
Heat shock prot b6-2	1275.5	0.823			
Galectin 1-2	863.2	0.871	0.458		
Malate dehydrogenase-2	893.0	0.885			
Heat shock prot b1	1597.5	0.818			-0.441

Only variables with communality values ≥ 0.600 (absolute value) in any of the four principal components were included and factor loadings ≤ 0.400 (absolute value) were eliminated from table.
[a]*m*/*z* expressed as $[M+H]^+$.

Fig. 2 shows PCA representation depicting the meat samples distribution yielded according to the intensities of these 23 peptides in the two-dimensional coordinate system defined by PC1 and PC2. Normal and high pHu replicates were

clearly separated by PC1 in which high pHu samples had positive factor scores in contrast to negative factor scores shown by their normal counterparts.

Conversion of individual regulation levels of peptides into heat maps with hierarchical clustering (**Fig. 3**) eased further comparison of LTL muscle proteome between normal and high pHu biological replicates. Overall, peptides were up-regulated in high pHu samples with the exception of those derived from ENO3-2, AK1, PGM1, ENO1 and PGK1-2, which showed minimal regulation changes between meat groups studied (similarly than previously shown by statistical analysis summarized by **Table 1**). Elucidation of peptide biomarkers must be performed among those showing higher regulation differences from **Fig. 3**. Thus, peptides derived from HSPB1, ACTA1-1, HSPB6-1, HSPB6-2, TPI1-2, PGK1-1, CKM-3, CKM-1 and ENO3-1 confirmed their usefulness as meat discriminants (as suggested by statistical results from **Table 1**). Furthermore, SRM analysis of unenriched extracts evidenced exclusiveness of MDH1 (*m/z* 893.0) and LGALS1-2 (*m/z* 863.2) to high pHu meat group. However, since both peptides had a very similar down-regulated shade (**Fig. 3**) it can be assumed their little contribution to meat differentiation.

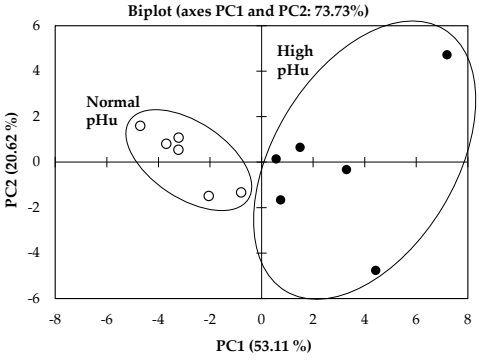

Fig. 2. Normal (○) and high pHu (●) meat samples distribution on the two dimensional coordinate system defined by the principal component (PC) 1 and 2.

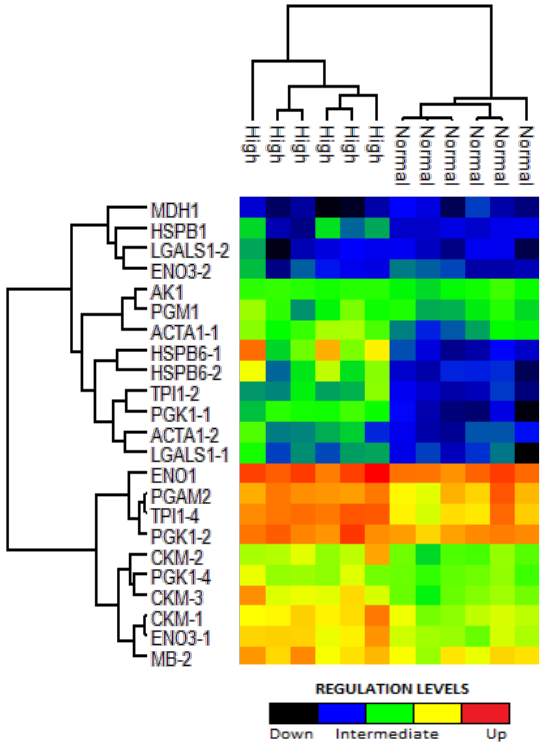

Fig. 3. Heatmap showing the peptide intensity from each biological replicate. Nomenclature of peptides shown in **Table 1**.

4.3.3 Biological and functional analysis

After the quantitative SRM research, next step was the biological explanation of the proteome changes between normal and high pHu meat groups. This goal was achieved through the study of SRM results from NORMAL/HIGH-RPL batch that yielded 17 peptide biomarkers showing significant differences among groups assayed (**Table 1**). These peptide biomarkers corresponded to TPI1, MDH1, ENO3, CKM, MB, ACTA1, HSPB6, HSPB1, PGK1 and LGALS1 proteins.

Analysis of GO slim terms showed that most of these proteins participate in different biological process such carbohydrate metabolism (TPI1, MDH1, ENO3 and PGK1), abiotic stimulus response (CKM, MB and PGK1) and biological regulation (ACTA1, HSPB1, PGK1, MB and LGALS1) having different molecular functions such as binding (all proteins except MDH1) and catalytic activity (TPI1, MDH1, PGK1, ENO3 and LGALS1). They can be found in different locations such as cytoplasmic (TPI1, MDH1, ENO3, HSPB1 and ACTA1) and extracellular (HSPB6, PGK1, CKM and LGALS1) regions.

An interaction network of proteins with different ($P \leq 0.05$) intensity levels could help to understand the impact of PSS on muscle proteome of bovine cattle. Studied proteins were clustered into a single interaction network according to GO terms with the exception of the non-interactive LGALS1 (**Fig. 4**). TPI1 had the highest number of interacting neighbours with strong associations between PGK1, ENO3 and MDH1 (0.999, 0.958 and 0.824 of score, respectively). They were displayed in the centre of the network playing an essential role in the interaction complex since they linked metabolism proteins and other interacting proteins. TPI1 was the only one that connected glycolytic enzymes with proteins involved in stress processes, showing an intermediate interaction with HSPB1 (score, 0.407) due to their participation in both cellular processes. Moreover, PGK1 and ENO3 were highly and intermediately associated with ACTA1 (score, 0.803) and CKM (score, 0.643), respectively. ACTA1, which was the only identified protein implicated in muscle structural-contractile regulation, and CKM, which catalyses the transfer of phosphate between ATP and various phosphogens, were both intermediately (score, 0.499) and strongly (score, 0.801) linked to MB, respectively. This latter is involved in oxygen transport chain, closing the interaction network.

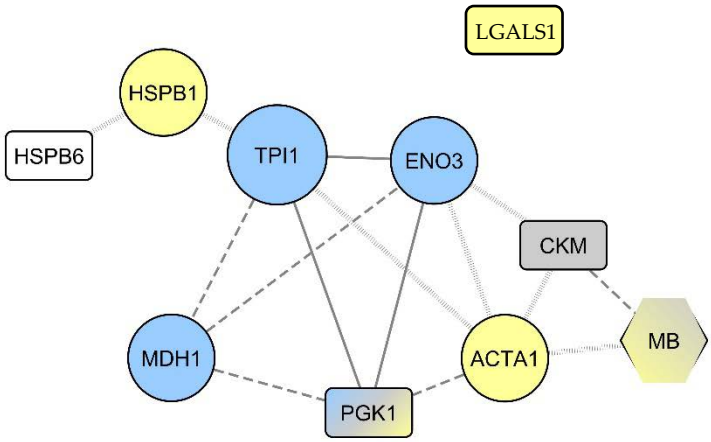

Fig. 4. Protein-protein interaction network of proteins showing significant statistical differences ($P \leq 0.05$) between normal and high pHu groups. Nomenclature of proteins shown in **Table 1**. Network nodes represent proteins, the fill colour and the shape represents the biological process (blue, carbohydrate metabolic process; grey, response to abiotic stimulus; yellow, biological regulation and white, non-defined) and the cellular component (circle, cytoplasmic part; round rectangle, extracellular region and hexagon, non-defined) in GO slim terms from AmiGO, respectively. Edges represent protein-protein functional associations and line type denotes the association strength according to threshold confidence: (—) highest, ≥ 0.9; (– –) high, 0.7 - 0.9; (‖ ‖) medium, 0.4 - 0.7.

Central proteins TPI1, PGK1, ENO3 and MDH1 are suggested to play a key role in PSS response since they participate in the carbohydrate metabolism, generation of precursor metabolites and energy pathways. It is widely described that PSS is associated with a depletion of muscle glycogen stores prior to slaughter, causing a reduction in substrate availability for the anaerobic glycolysis post-mortem [6]. Although association between pre-slaughter stress and muscle glycogen depletion has been extensively studied in ruminants, the same cannot be said for the association between PSS and post-mortem glycolytic rate [26]. TPI1, an enzyme that reversibly catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, was also up-regulated in DFD samples as reported by Franco et al. (2015) [11]. They suggested that augmented levels of that enzyme might lead to higher WHC and tenderness values in DFD meat, reinforcing again these main features of dark-cutting meats. Contrary to this, other authors indicated lower band intensity of TPI1 in animals subjected to social stress [17]. According to PGK1, which reversibly catalyses the transfer of a phosphate group from 1,3-biphosphoglycerate to ADP producing 3-phosphoglycerate and ATP, only Diaz et al. [17] related this protein to PSS response, finding higher levels on non-stressed animals. However, the co-migration of PGK1 with CKM in SDS-PAGE makes difficult to understand their results. In contrast, they also revealed a higher presence of ENO3 in the muscle of animals intensively managed and considered more susceptible to stress [17]. Similarly, other authors stated that phosphorylated

isoform of ENO3 was only identified in DFD meat samples [13]. Once again, this enzyme play essential role in both glycolysis and gluconeogenesis pathways, being the responsible for catalysing the reversible conversion of 2-phospho-glycerate to phosphoenolpyruvate.

The last enzyme displayed in the centre of the network was MDH1 (**Fig. 4**), having higher concentration in high pHu meats. In this sense, MDH1, which is involved in glucose production when glycogen is not available [27], was only found in the high pHu samples as reported other authors [28]. Contrarily, Poleti et al. (2018) found greater abundance of MDH1 in normal pHu meats [9]. Apart from its catalytic role in carbohydrate metabolism, MDH1 is also involved in tricarboxylic acid cycle (TCA) though the reversible catalysis of malate to oxaloacetate to generate ATP and NADH. TCA cycle is the final common pathway for the oxidation of the fuel molecules, accompanied by the production of energy and reduction equivalents (NADH and $FADH_2$). Since high pHu meats show less glycogen stores prior to slaughter, the muscle cells need to use other alternative post-mortem energy sources for restoring ATP levels such as TCA cycle. This would explain overabundance of this enzyme in high pHu samples. Meanwhile, in dark cutting beef, more NADH leads to higher oxygen consumption [18] and metmyo-globin (metMb) reducing activity [29], which are inherent biochemical properties that influence meat colour. Current know-ledge is that NADH and other substrates such as malate can also reduce MetMb to form deoxymyoglobin (DeoxyMb), giving rise to a darker colour and limiting brown or metMb formation. In this line, several studies have reported that a higher muscle pHu can decrease oxidative changes and increase the activity of several enzymes involved in MetMb reducing activity and oxygen consumption [29,30]. Overall, these four proteins are enzymes that belong to carbohydrate metabolism, which suggests that energy demands caused by PSS response may provoke increase of glycolysis or other alternative energy sources, enhancing the activity of these specific enzymes that are directly related to ATP production. On the other hand, literature also indicated that carcass producing abnormal dark-cutting meat (pHu ≤ 5.8) may have reduced rates of glycolysis at early post-mortem times, giving rise to low concentration of energy-related proteins [14].

The interaction between glycolytic enzy-mes, such as ENO3, with other processes regenerating ATP highlights the presence in the sarcomere of protein complexes involved in energy production (e.g. CKM) [31]. As previously stated, stressed animals have limited glucose reserves such as glycogen, therefore the most immediate way for restoring ATP levels in muscle involves other alternative sources such as phosphocreatine phosphate. CKM plays a central role in tissue energy transduction with fluctuating energy demands through the reversibly interconversion of phospho-creatine and ADP to creatine and ATP. In our study, this enzyme showed higher levels in the high pHu group, which agrees with results reported by other authors [13,17]. Under extreme living conditions, oxygen-storage of CKM as well as MB may play an essential role maintaining the

energy state of some muscles such as heart [32]. In post-mortem muscle, there is competition for the available oxygen between mitochondria and MB. If mitochondria are active there will be limited oxygenation of myoglobin, resulting in darker meat due to predominant DeoxyMb [5,18]. Furthermore, MB is not denatured at high pH, which facilitates an aerobic metabolism at meat surface remaining iron in the ferrous state and thus affecting meat colour [33]. These facts could be attributed to the higher MB concentration found in the high pHu group, giving rise to dark-cutting meats.

The rest of proteins depicted in the network of **Fig. 4** take part in biological regulation processes. On one hand, ACTA1 was strongly bounded to PGK1 since both participate in cell differentiation. As previously stated, ACTA1 was the only protein involved in muscle/tissue structure development, as well as LGALS1. It is noteworthy to mention that ACTA1 has a poor aqueous solubility to be extracted within sarcoplasmic proteins [34-36]. However, some authors reported that high pHu values of DFD meats enhanced its solubility, turning more noticeable its presence in the soluble extracts of stressed animals [12]. This is in accordance to results obtained by Mahmood et al. (2018) since they found actin up-regulation in DFD samples [14]. In this line, HSPB1 and HSPB6, were only found in high pHu meat samples in accordance to previous studies [12]. These proteins are involved in stress response, actin stability and apoptotic signalling pathways. Under stressful conditions, they play a major role as essential molecular chaperones interacting with damaged proteins to preserve their function [37,38]. PSS animals may have a great abundance of HSPs to avoid activation of apoptosis signalling pathways, hindering the activity of caspases. Taking into account its protective role, the overabundance of HSPs could also explain the up-regulation of ACTA1 in high pHu samples, then contributing to meat tenderness. However, there is a controversy regarding HSPs and tenderness due to the fact that high levels of these proteins were also related to either increased [39,40] or decreased [41] tenderness. In this regard, Mato et al. [13] found that HSPB1 and HSPB6 were differentially phosphorylated in DFD and normal meats, suggesting that their roles may depend on their phosphorylation status. Finally LGALS1, which remains out of the network with no interaction with the others has been described to participate in a wide range of biological processes including development, regulation of inflammation, cell proliferation and apoptosis [42]. Nevertheless, relevance of this protein in apoptosis is highly controversial and contradictory findings are reported in the literature as revealed by Ouali et al. [43]. Therefore, the role of LGALS1 in post-mortem muscle and PSS response still remains unclear and deserves further investigations.

4.4 CONCLUSION

The *gel-free* proteomic strategy presented in this work demonstrated its usefulness to study differences in muscle proteome of bovine cattle. Appropriate optimization of

a conventional 3D-IT eased targeted and untargeted qualitative/quantitative analysis of samples through MS^1, $ddMS^2$ and SRM approaches. Chemometric analysis of quantitative LC-MS data yielded accurate results to differentiate normal from high pHu sample groups through 17 peptide biomarkers belonging to 10 different proteins (TPI1, MDH1, ENO3, CKM, MB, ACTA1, HSPB6, HSPB1, PGK1 and LGALS1). Most relevant proteins of this group participate in carbohydrate metabolism (TPI), energy production (CKM) and stress response (HSPs) biological processes.

Higher efficiency in terms of limited sample preparation, affordability and quality of results exhibited by the straightforward analysis of soluble protein extracts can facilitate the implementation of this methodology in routine meat quality research.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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References

[1] E.N. Ponnampalam, D.L. Hopkins, H. Bruce, D. Li, G. Baldi, A.E. Bekhit, Causes and contributing factors to "dark cutting" meat: Current trends and future directions: A review, Compr. Rev. Food Sci. Food Saf. 16 (2017) 400-430.

[2] K.M.W. Loudon, I.J. Lean, D.W. Pethick, G.E. Gardner, L.J. Grubb, A.C. Evans, et al., On farm factors increasing dark cutting in pasture finished beef cattle, Meat Sci. 144 (2018) 110-117.

[3] C.G. Jose, P. McGilchrist, J.L. Perovic, G.E. Gardner, D.W. Pethick, The economic impact of dark cutting beef in Australia, 2015.

[4] F. Adzitey, H. Nurul, Pale soft exudative (PSE) and dark firm dry (DFD) meats: Causes and measures to reduce these incidences - a mini review, Int. Food Res. J. 18 (2011) 11-20.

[5] R. Ramanathan, M.C. Hunt, A.R. English, G.G. Mafi, D.L. VanOverbeke, Effects of aging, modified atmospheric packaging, and display time on metmyoglobin reducing activity and oxygen consumption of high-pH beef, Meat and Muscle Biology 3, 2019.

[6] P.V. Tarrant, Animal behaviour and environment in the dark-cutting condition in beef-a review, Irish J. Food Sci. Tec. (1989) 1-21.

[7] D.M. Ferguson, R.D. Warner, Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants? Meat Sci. 80 (2008) 12-19.

[8] N.G. Gregory, T. Grandin, Animal welfare and meat science, CABI Pub, 1998.

[9] M.D. Poleti, C.T. Moncau, B. Silva-Vignato, A.F. Rosa, A.R. Lobo, T.R. Cataldi, et al., Label-free quantitative proteomic analysis reveals muscle contraction and metabolism proteins linked to ultimate pH in bovine skeletal muscle, Meat Sci. 145 (2018) 209-219.

[10] P.G. Chambers, T. Grandin, G. Heinz, T. Srisuvan, Guidelines for humane handling, tansport and slaughter of livestock, 2001.

[11] D. Franco, A. Mato, F.J. Salgado, M. López-Pedrouso, M. Carrera, S. Bravo, et al., Tackling proteome changes in the longissimus thoracis bovine muscle in response to pre-slaughter stress, J. Proteomics, 122 (2015) 73-85.

[12] C. Fuente-Garcia, N. Aldai, E. Sentandreu, M. Oliván, S. García-Torres, D. Franco, et al., Search for proteomic biomarkers related to bovine pre-slaughter stress using liquid isoelectric focusing (OFFGEL) and mass spectrometry, J. Proteomics, 198 (2019) 59-65.

[13] A. Mato, R. Rodríguez-Vázquez, M. López-Pedrouso, S. Bravo, D. Franco, C. Zapata, The first evidence of global meat phosphoproteome changes in response to pre-slaughter stress, BMC Genomics, 20 (2019) 590.

[14] S. Mahmood, N. Turchinsky, F. Paradis, W.T. Dixon, H.L. Bruce, Proteomics of dark cutting longissimus thoracis muscle from heifer and steer carcasses, Meat Sci. 137 (2018) 47-57.

[15] A. Marco-Ramell, A.M. Gutiérrez, A. Velarde, J.J. Cerón, A. Bassols, The Use of Proteomics to Study Biomarkers of Stress and Welfare in Farm Animals, Proteomics in Domestic Animals: from Farm to Systems Biology, Springer, 2018, pp. 339-360.

[16] C. Fuente-Garcia, E. Sentandreu, N. Aldai, M. Oliván, M.Á. Sentandreu, Characterization of the myofibrillar proteome as a way to better understand differences in bovine meats having different ultimate pH values, Proteomics 20 (2020) 2000012.

[17] F. Díaz, A. Díaz-Luis, V. Sierra, Y. Diñeiro, P. González, S. García-Torres, et al., What functional proteomic and biochemical analysis tell us about animal stress in beef?, J. Proteomics 218 (2020) 103722.

[18] F. Kiyimba, S.D. Hartson, J. Rogers, D.L. VanOverbeke, G.G. Mafi, R. Ramanathan, Changes in glycolytic and mitochondrial protein profiles regulates postmortem muscle acidification and oxygen consumption in dark-cutting beef, J. Proteomics 232 (2021) 104016.

[19] C. Abdallah, E. Dumas-Gaudot, J. Renaut, K. Sergeant, Gel-based and gel-free quantitative proteomics approaches at a glance, Int. J. Plant Genom. (2012) 494572.

[20] E. Sentandreu, C. Fuente-García, J.L. Navarro, M.A. Sentandreu, A straightforward gel-free proteomics pipeline assisted by liquid isoelectric focusing (OFFGEL) and mass spectrometry analysis to study bovine meat proteome, Food Sci. Technol. Int. 27 (2021) 112-122.

[21] W. Wu, R. Dai, E. Bendixen, Comparing SRM and SWATH methods for quantitation of bovine muscle proteomes, J. Agric. Food Chem. 67 (2019) 1608-1618.

[22] M. Mirzapour, K. Rezaei, M.A. Sentandreu, A.A. Moosavi-Movahedi, In vitro antioxidant activities of hydrolysates obtained from Iranian wild almond (A mygdalus scoparia) protein by several enzymes, Int J. Food Sci. Tech. 51 (2016) 609-616.

[23] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities

of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[24] S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M.Y. Hein, T. Geiger, et al. The Perseus computational platform for comprehensive analysis of (prote) omics data, Nat. methods 13 (2016) 731.

[25] P. Shannon, A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, et al., Cytoscape: a software environment for integrated models of biomolecular interaction networks, Genome Res. 13 (2003) 2498-2504.

[26] D.M. Ferguson, D.E. Gerrard, Regulation of post-mortem glycolysis in ruminant muscle, Anim. Prod. Sci. 54 (2014) 464-481.

[27] G.C. Hung, C.R. Brown, A.B. Wolfe, J. Liu, H.L. Chiang, Degradation of the gluconeogenic enzymes fructose-1,6-bisphosphatase and malate dehydrogenase is mediated by distinct proteolytic pathways and signaling events, J. Biol. Chem. 279 (2004) 49138-49150.

[28] S. Wu, X. Luo, X. Yang, D.L. Hopkins, Y. Mao, Y. Zhang, Understanding the development of color and color stability of dark cutting beef based on mitochondrial proteomics, Meat Sci. 163 (2020) 108046.

[29] J. Tang, C. Faustman, T.A. Hoagland, R.A. Mancini, M. Seyfert, M.C. Hunt, Postmortem oxygen consumption by mitochondria and its effects on myoglobin form and stability, J. Agric. Food Chem. 53 (2005) 1223-1230.

[30] A.R. English, K.M. Wills, B.N. Harsh, G.G. Mafi, D.L. VanOverbeke, R. Ramanathan, Effects of aging on the fundamental color chemistry of dark-cutting beef, J. Anim. Sci. 94 (2016) 4040-4048.

[31] T. Merkulova, M. Lucas, C. Jabet, N. Lamandé, J. Rouzeau, F. Gros, et al., Biochemical characterization of the mouse muscle-specific enolase: developmental changes in electrophoretic variants and selective binding to other proteins, Biochem. J. 323 (1997) 791-800.

[32] F. Wu, D.A. Beard, Roles of the creatine kinase system and myoglobin in maintaining energetic state in the working heart, BMC Syst. Biol. 3 (2009) 22.

[33] D. Mouzo, R. Rodriguez-Vázquez, J.M. Lorenzo, D. Franco, C. Zapata, M. López-Pedrouso, Proteomic application in predicting food quality relating to animal welfare. A review, Trends Food Sci. Technol. 99 (2020) 520-530.

[34] S.G. Bjarnadóttir, K. Hollung, E.M. Færgestad, E. Veiseth-Kent, Proteome changes in bovine longissimus thoracis muscle during the first 48 h postmortem: shifts in energy status and myofibrillar stability, J. Agric. Food Chem. 58 (2010) 7408-7414.

[35] E. Laville, T. Sayd, M. Morzel, S. Blinet, C. Chambon, J. Lepetit, et al., Proteome changes during meat aging in tough and tender beef suggest the importance of apoptosis and protein solubility for beef aging and tenderization, J. Agric. Food Chem. 57 (2009) 10755-10764.

[36] X. Jia, K. Hollung, M. Therkildsen, K.I. Hildrum, E. Bendixen, Proteome analysis of early post-mortem changes in two bovine muscle types: M. longissimus dorsi and M. semitendinosis, Proteomics 6 (2006) 936-944.

[37] N.T. Larkins, R.M. Murphy, G.D. Lamb, Influences of temperature, oxidative stress, and phosphorylation on binding of heat shock proteins in skeletal muscle fibers, Am. J. Physiol-Cell Ph. 303 (2012) C654-C665.

[38] T. Xing, M.F. Wang, M.Y. Han, X.S. Zhu, X.L. Xu, G.H. Zhou, Expression of heat

shock protein 70 in transport-stressed broiler pectoralis major muscle and its relationship with meat quality, Animal 11 (2017) 1599-1607.

[39] M. Morzel, C. Terlouw, C. Chambon, D. Micol, B. Picard, Muscle proteome and meat eating qualities of Longissimus thoracis of "Blonde d'Aquitaine" young bulls: A central role of HSP27 isoforms, Meat Sci. 78 (2008) 297-304.

[40] M.E. Carvalho, G. Gasparin, M.D. Poleti, A.F. Rosa, J.C.C. Balieiro, C.A. Labate, et al., Heat shock and structural proteins associated with meat tenderness in Nellore beef cattle, a Bos indicus breed, Meat Sci. 96 (2014) 1318-1324.

[41] A. Ouali, C.H. Herrera-Mendez, G. Coulis, S. Becila, A. Boudjellal, L. Aubry, et al., Revisiting the conversion of muscle into meat and the underlying mechanisms, Meat Sci. 74 (2006) 44-58.

[42] B. Zhou, Y. Long, G. Song, Q. Li, Z. Cui, Molecular characterization of the lgals1 gene in large scale loach Paramisgurnus dabryanus, Gene 577 (2016) 65-74.

[43] A. Ouali, M. Gagaoua, Y. Boudida, S. Becila, A. Boudjellal, C.H. Herrera-Mendez, et al., Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved, Meat Sci. 95 (2013) 854-870.

5 APPENDIX V PUBLICATION V

Food Science and Technology International (2021), in press, 1-8

Optimization of a fluorogenic assay to determine caspase 3/7 activity in meat extracts[5]

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Abstract

Usefulness of general-purpose fluorogenic assay kits to determine caspase 3/7 activity of biological extracts is highly compromised in meat-based samples due to their scarce enzyme concentration. In the present work, a straightforward protocol is presented with two main purposes: 1) to enhance sensitivity of the fluorogenic approach addressing caspase 3/7 activity in tissues showing scarce enzyme concentration such as skeletal muscle, and 2) to reduce/economize the volume of employed reagents. The enzyme extraction procedure, peptide substrate, dithiothreitol concentration and detection settings were appropriately optimized for use in microtiter-plate fluorometers. As a result, low to high enzyme activity extracts (from 10,000 to 260,000 relative fluorescence units) can be measured under developed sampling and experimental conditions. The fact that enzyme reactions took place in 96-microtiter well plates reduces the consumption of chemical compounds when analysing a high number of samples, thus contributing to environment sustainability.

Keywords

Caspase 3/7 Activity ● Ac-DEVD-AMC Peptide Substrate ● Apoptosis ● Method Development ● Meat Extracts ● Rapid Assays

5.1 INTRODUCTION

Caspases are cysteine-dependent peptideses involved in apoptosis (caspases 3, 6, 7, 8, 9, 10 and 12) and inflammation processes (caspases 1, 4, 5, 11 and 13) by selectively cleaving C-terminal aspartic acid residues of peptides/proteins. Apoptotic caspases can be further classified according to their point of entry into the cell death pathway as initiator (caspases 8, 9, 10 and 12) and executioner (caspases 3, 6 and 7) caspases (McIlwain et al., 2013). Assessment of apoptotic caspases is important in the study of several cellular process. They are mainly synthesized as inactive zymogens (pro-caspases) and their activation is driven by a wide range of internal and/or external stimuli such as stress. Stress activates pro-apoptotic members of the Bcl-2 protein family, favoring cytochrome c-dependent formation of the caspase 9 apoptosome complex (Grilo and Mantalaris, 2019), triggering executioner caspases (3 and 7) and giving rise to the onset of cell death processes (Taylor et al., 2008). Assessment of caspase activity in skeletal muscle has been traditionally carried out during the early post-mortem period as related to the study of metabolic responses associated with intrinsic hypoxia/ischemia. This confers a potential contribution of caspase action to post-mortem proteolysis occurring during meat tenderization (Kemp et al., 2006; Ouali et al., 2006).

Assay kits for the fluorogenic detection of peptide substrates with cleavable fluorophores based on the 7-amido-4-methylcoumarin (AMC) or 7-amino-4-trifluoromethylcoumarin probe mainly allow measurement of caspase activity of cell-based samples (Gurtu et al., 1997). This strategy was commonly used in meat research for the simultaneous elucidation of caspase 3/7 activity (Cao et al., 2013; Zhang, J. et al., 2017). However, low sensitivity of traded kits have their biological application restricted to the characterization of purified enzymes, primary cell cultures or fresh tissue samples with high caspase activity, showing some limitations in those cases with scarce enzymatic presence such as in skeletal muscle (Wang et al., 2005). Such sensitivity drawbacks mainly arise from inappropriate optimization of the fluorogenic instrumental conditions of analysis and sample preparation. Caspase substrates based on rhodamine dye were proposed to overcome aforementioned limitations (Wang et al., 2005), but elevated costs of rhodamine-based assays can compromise their use in the analysis of high number of samples. Then, the development of affordable, sensitivity-enhanced and low reagent consumption methods is needed to elucidate activity of caspases 3/7 in meat extracts.

The objective of the present study was the development of an economic, sustainable and highly sensitive fluorogenic assay tailored for the accurate measurement of caspase 3/7 activity in extracts obtained from tissues with low enzyme concentration. The methodology proposed address substrate concentration optimization, sample preparation and fluorogenic detection conditions. Validation was performed by comparison with a commercial kit also based on the hydrolysis of Ac-DEVD-AMC

substrate. This study assayed several refinements of the fluorescence assay originally proposed by Stennicke and Salvesen (1999) for determining caspase 3 activity through the monitoring of the enzymatic release of AMC group from Ac-DEVD-AMC peptide substrate (Stennicke and Salvesen, 1999). Fluorogenic detection confers a 100-fold higher sensitivity than spectrophotometric approaches, addressing assessment of caspase activity and offering a wide range of customizable improvements (Stennicke and Salvesen, 1999).

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

Substrate N-Acetyl-Asp-Glu-Val-Asp-7-Amido-4-methyl coumarin (Ac-DEVD-AMC) was supplied by ATT Bioquest (Sunnyvale, CA, USA). Sucrose, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), sodium salt, phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail (P8340), $MgCl^2$, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and polyoxyethylene lauryl ether 30% (Brij 35®, CAS 9002-92-0) were supplied by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Tris-(hydroxymethyl)-aminomethane, ethylenediaminetetraacetic acid (EDTA), KCl, dimetyl sulfoxide (DMSO) dithiothreitol (DTT) and NaCl were supplied by Scharlab (Scharlab S.L., Sentmenat, Barcelona, Spain).

5.2.2 Meat samples

Meat samples were collected from *Longissimus thoracis et lumborum* muscle of crossbred yearling bulls from a local slaughterhouse. At 24h post-mortem, approximately 10 g of muscle sample were removed from the thirteenth rib of the left half carcass, immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

5.2.3 Optimization of the protein extraction buffer

For sarcoplasmic protein extraction, 0.5 g of each stored sample were homogenized with different extraction buffers using an IKA Yellow Line Di 25 homogenizer (IKA-Werke GmbH, Staufen, Germany). Four different extraction buffers were assayed (**Table S1**) to optimize protein extraction. Buffer A was selected since it is a common buffer normally employed for the sarcoplasmmic protein extraction from muscle tissue (Jia et al., 2006; Melody et al., 2004). The other three are commonly used to perform caspase extraction from intracellular membrane (Cao et al., 2013; Huang et al., 2014). General assay conditions used for the optimization of protein extraction buffer were the same as for the final optimized assay. Buffer D was finally chosen to perform the rest of refinements. Homogenates were centrifuged at 20,000 *g* for 20 min at 4 °C using a Beckman Coulter centrifuge (Brea, CA, USA). Supernatants were filtered through 0.45 μm polyvinylidene difluoride syringe filter (Merck KGaA, Darmstadt, Germany) and stored at -80 °C until analysed.

5.2.4 Optimization of the experimental conditions for fluorogenic determination of caspase 3/7 activity

In this work, the following experimental solutions were used:

- Reaction buffer: Final composition of selected reaction buffer was 0.29 M sucrose, 0.1 M NaCl, 50 mM HEPES, 0.1% CHAPS and 1 mM EDTA. Solution was adjusted to pH 7.5 with 50% HCl.

- Substrate and DTT stock solutions: A substrate stock solution was prepared dissolving 5 mg of commercial Ac-DEVD-AMC substrate in 1.48 mL of DMSO with a final concentration of 5 mM and kept at -20 °C until use. Similarly, a DTT stock solution was prepared dissolving 154 mg of DTT in 20 mL of the reaction buffer and then stored in aliquots at -80 °C until analysed.

Fluorogenic determination of caspase 3/7 activity

As stated in the introduction, the present study undertook several refinements from the original fluorescence assay of Stennicke and Salvesen (1999) with the aim to enhance sensibility, flexibility, linearity and affordability of the caspase assay. As abovementioned, different extraction buffers (**Table S1**), together with DTT and enzyme-substrate conditions (**Table S2**) and values of main instrumental settings (excitation/emission wavelengths and gain multiplier, **Table S3**) were tested for method optimization.

For the different assayed conditions, the caspase enzyme reaction took place in a 96-microtiter-well plate following this procedure: sarcoplasmic extracts were pre-incubated for 30 min at 37 °C in the presence of DTT as reducing agent. Enzyme reaction started with the addition of the substrate solution (zero time) and fluorescence intensity was monitored every 2 min over 30 min at 37 °C using a CLARIOstar microplate fluorometer (BMG LABTECH GmbH, Ortenberg, Germany). Analyses were performed in triplicate and results were expressed as mean values of Relative Fluorescence Units (RFU) from the three analytical replicates in the fluorescence range from 0 to 260,000 RFU. Additionally, three biological replicates were used and standard deviations and coefficients of variation (CV) calculated in order to validate the assay.

5.2.5 Comparison between the optimized method with a commercial fluorogenic assay of caspase 3/7 activity

Final optimized protocol described in this work was compared with a commercial kit previously used by Huang et al. (2014) to measure caspase 3/7 activity in meats extracts based on the same substrate (Ac-DEVD-AMC). Experimental conditions of the commercial kit were the same as those indicated by the manufacturer. Comparison was done in terms of sensibility, linearity and accuracy.

5.3 RESULTS AND DISCUSSION

5.3.1 Effect of the extraction buffer on enzyme activity determination

Although it is uncommonly studied, optimization of the protein extraction procedure is critical to determine caspase 3/7 activity in tissues with low activity levels such as skeletal muscle. In this regard, a preliminary enzyme activity approach of samples obtained by different extraction buffers (**Table S1**) was carried out.

No enzyme activity was found in those samples extracted with Buffer A (**Figure 1**). The main reason for this null response can be attributed to the presence of the protease inhibitor cocktail normally present in these buffers with the aim to inhibit any protease action. On the contrary, the presence of 1 mM of PMSF in Buffers B, C and D did not seem to affect caspase activity because it inhibits serine peptidases but not cysteine peptidases such as caspases (Ishisaka et al., 1998). The lack of Brij 35® in buffer A may have also contributed to this absence of signal, since the use of non-ionic surfactants in lysis buffers to solubilize membrane proteins has been reported to optimize protein extraction (Chakrabarty et al., 2004; Lin and Guidotti, 2009). As reported in previous studies, a lower volume of extraction Buffer B, C and D was used to carry out caspase extraction (Cao et al., 2013; Chen et al., 2011; Zhang et al., 2017). Higher extraction volumes (Buffer A) are widely used to dilute samples in order to avoid the occurrence of fluorescence interferences due to meat compounds. However, we did not observe any interference in fluorescence when assaying buffers B-D, and thus we set 2 mL as the final volume to perform the extraction. The main difference among these three buffers is the presence or absence of $MgCl_2$ and/or KCl. In the literature, some authors reported either the use or not use of these compounds for caspase extraction (Chen et al., 2011; Chen et al., 2015; Huang et al., 2014). In our case, maximum caspase activity was reached with buffer D (**Figure 1**). Therefore, this buffer, which contains less chemical compounds, was selected to get the final optimized assay conditions.

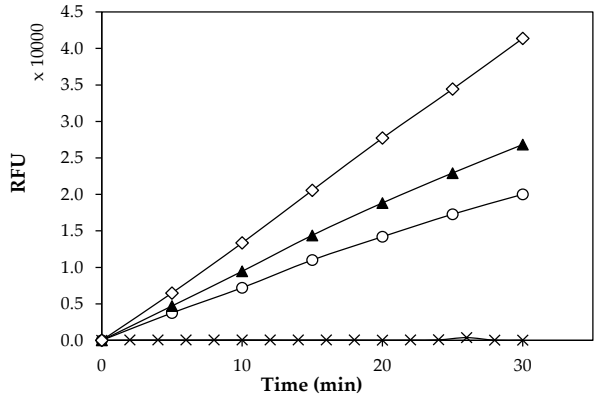

Figure 1. Effect of extraction buffer (**Table S1**) on caspase 3/7 activity: (x) Buffer A; (○) Buffer B (▲) Buffer C; and (◊) Buffer D (final optimized buffer).

5.3.2 Effect of substrate concentration on enzyme activity determination

As stated in M & M section, protein extraction was finally performed using buffer D as selected in the pre-trial. **Figure 2** shows caspase 3/7 activity of meat samples varying in final substrate concentrations (mM) and sample:substrate ratios (R-) (**Table S2**). The highest caspase 3/7 activity was achieved at 0.083 mM and R-1:1 after 46 min of reaction at 37 °C. However, such experimental conditions were discarded to avoid saturation of the fluorescence response in samples showing higher enzymatic activity, thus limiting the dynamic range of the proposed methodology. Since R- had a great influence on sensitivity (see R-1:1 vs. R-3:5) and considering the costs when processing high number of samples, an intermediate substrate concentration of 0.042 mM, R-1:1 and 30 min of reaction time was included into the definitive optimized methodology conditions. Previous studies reported that 0.01 mM of substrate would be enough to determine activity of purified caspases 3, 6, 7, and 8 expressed in *E. coli* (Stennicke and Salvesen, 1999), but as previously mentioned, determination of low activity levels in meat extracts required higher substrate concentrations to perform reliable determinations.

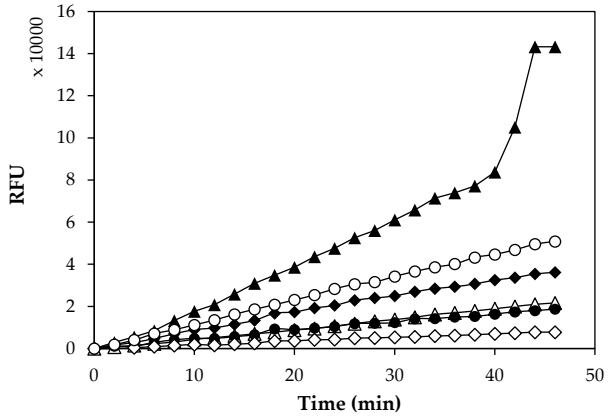

Figure 2. Effect of substrate concentration (mM) and extract:substrate ratio (R-) on caspase 3/7 activity: (Δ) 0.021 mM (R-1:1); (▲) 0.083 mM (R-1:1); (◊) 0.025 mM (R-3:5); (●) 0.05 mM (R-3:5); (♦) 0.1 mM (R-3:5) and (○) final optimized conditions (0.042 mM and R-1:1).

5.3.3 Effect of DTT concentration and sample dilution on enzyme activity determination

Figure 3 illustrates the effect of both DTT concentration in the reaction mixture and sample dilution (1:2; 1:4 and undiluted sarcoplasmic extracts) on caspase 3/7 activity. This was analysed exclusively using the optimized substrate concentration selected in the previous trial (full set of conditions is recorded in **Table S2**). The DTT requirement for either protein extraction or caspase activity assay is extensively reported in the literature (Chen et al., 2015; Kemp et al., 2009). In our work, DTT was only used in the reaction mixture to avoid the use of unnecessary chemical compounds, since the reducing

action of DTT during protein extraction would be lost in extracts stocked for long time. As it can be observed, hydrolysis of Ac-DEVD-AMC was not altered by DTT concentration used in any of the assayed conditions, suggesting that SH groups of caspases were in their reduced forms. In any case, a final concentration of 4 mM DTT in the reaction mixture was selected for enzyme pre-activation and preservation against potential catalytic cysteine oxidation (Stennicke and Salvesen, 1997) during assay preparation in agreement to previous studies (Huang et al., 2014; Zhang et al., 2013).

Different sarcoplasmic extract dilutions were assayed in this work to study possible interferences of other non-enzymatic components with the assay. The obtained results evidenced that there were no interferences neither in the undiluted extracts nor in 1:2 and 1:4 diluted extracts. However, due to the notably low caspase activity in meat extracts, undiluted extracts should be preferably used to measure caspase activity. This is in accordance to the general working conditions reported in previous works (Cao et al., 2013; Chen et al., 2011).

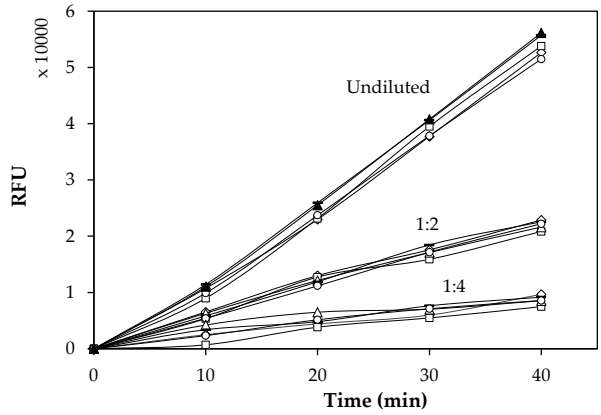

Figure 3. Effect of DTT concentration and sample dilution (undiluted, 1:2 and 1:4) on caspase 3/7 activity: (◊) 16 mM; (□) 8 mM; (Δ) 4 mM; (—) 1 mM; (○) 0 mM and (▲) final optimized conditions (undiluted sample with 4 mM DTT). Samples were assayed at R-1:1 extract/substrate ratio.

5.3.4 Influence of instrumental settings in the fluorogenic enzyme activity determination

Sensitivity limitations of the fluorogenic caspase assay can be improved by appropriate optimized conditions of the detection settings that are detailed in **Table S3**. Enzyme activity was only detected (**Figure 4**) at excitation and emission wavelengths of 380-15 and 460-20 nm, respectively, in accordance to previous results in meat extracts using the same fluorogenic peptide substrate (Cao et al., 2013; Huang et al., 2014). Furthermore, **Figure 4** also indicates how maximal fluorogenic response was achieved at the maximum amplification gain assayed (1500).

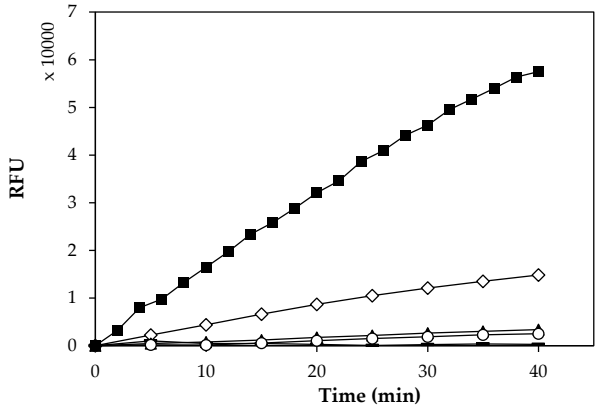

Figure 4. Effect of instrumental conditions on caspase 3/7 activity: (—) 330-20 and 385-30 nm; Gain: 600; (○) 339-15 and 439-30 nm; Gain: 750; (▲) 350-15 and 440-20 nm; Gain: 800; (◊) 380-15 and 460-20 nm; Gain: 1200 and (■) final optimized conditions (380-15 and 460-20 nm; Gain: 1500).

5.3.5 Precision of the assay and comparison of the developed method with a commercial kit

As shown in **Table 1**, the reproducibility was good either within or between-run, obtaining CV below 5% and 14%, respectively. Higher CV were found between-run due to the fact that the caspase 3/7 activity undergoes some fluctuations over long storage times (almost 600 days) being its activity 14% less than the initial one; however, those variations are negligible at short storage times.

Table 1. Precision data and reproducibility of the assay for caspase 3/7 activity using Ac-DEVD-AMC as substrate.

	Fluorescence Intensity (RFU)	SD (RFU)	CV (%)
Within-run (n=3)			
A	22252	357	2
B	33201	947	3
C	31529	594	2
Between-run (n=3)			
A	22096	2950	13
B	34960	2818	8
C	30796	2722	9

Three different samples were used for caspase 3/7 activity (A, B and C).

Validation of the developed protocol for determining caspase 3/7 activity in meat extracts was carried out by comparing the fluorescence response with that of a commercial assay based on the hydrolysis of the same fluorogenic substrate (Ac-DEVD-AMC). **Figure 5** illustrates the results obtained comparing our developed procedure with a commercial kit employed by Huang et al. (2014). Linear regression analysis of data showed higher correlation values ($R^2 > 0.99$) for two different muscle caspase enzyme levels under our optimized methodology conditions compared

with that obtained by the commercial kit ($0.9547 < R^2 < 0.9831$). The linearity of the commercial kit was compromised, being this reflected in lower correlation values, especially in the sample with lower caspase activity (**Figure 5B**). Fluorescence values were quite similar between the two methods in the sample yielding higher caspase 3/7 activity (**Figure 5A**). Those differences were more evident when caspase 3/7 activity was lower (**Figure 5B**). In this line, it can be observed that our optimised assay offers an overall better response as compared to the commercial protocol.

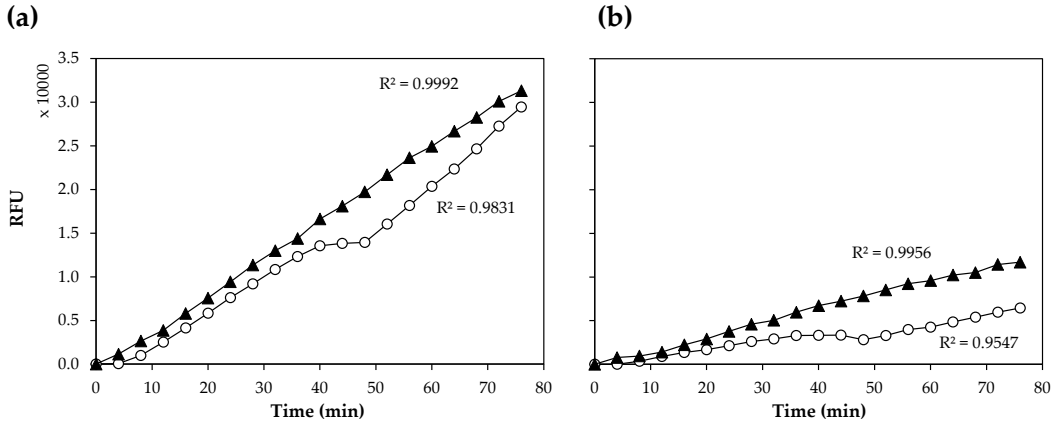

Figure 5. Comparison between the optimized method of the present work (▲) and a commercial caspase 3/7 assay kit (○) in terms of linearity, sensitivity and reliability in the measurement of two meat samples representative of higher (a) and lower (b) caspase activity levels.

These results confirmed the high sensitivity and linearity of our proposed method, being able to discriminate samples yielding low caspase 3/7 activity over long incubation times. Moreover, it is worth noting that our proposed methodology noticeably reduced chemical expenditure and therefore, the environmental impact of its implementation.

5.4 CONCLUSIONS

Linearity of the fluorogenic assay to measure low caspase 3/7 activities in meat/muscle extracts was greatly improved by the optimized and easy-to-use protocol described in this work as compared with a commercial kit. Reaction and instrumental conditions proposed here make possible the study of a wide range of tissue samples independently of their intrinsic caspase 3/7 activity and biological origin. The use of a 96-microtiter-well format loading a customized substrate concentration enable the affordable analysis of a high number of meat extracts. This flexible, reliable, economic, sustainable and straightforward protocol can be an interesting alternative to current caspase assay kits, especially in the study of those tissues such as skeletal muscle yielding low caspase activity levels.

DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest.

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REFERENCES

Cao J, Ou C, Zou Y, Ye K, Zhang Q, Khan MA, et al. (2013). Activation of caspase-3 and its correlation with shear force in bovine skeletal muscles during postmortem conditioning. *Journal of Animal Science* 91: 4547-4552.

Chakrabarty D, Hazra P, Chakraborty A and Sarkar N. (2004). Dynamics of solvation and rotational relaxation in neutral Brij 35 and Brij 58 micelles. *Chemical Physics Letters* 392: 340-347.

Chen L, Feng XC, Lu F, Xu XL, Zhou GH, Li QY, et al. (2011). Effects of Camptothecin, Etoposide and Ca2+ on Caspase-3 Activity and Myofibrillar Disruption of Chicken during Postmortem Ageing. *Meat Science* 87: 165-174.

Chen L, Feng X, Zhang Y, Liu X, Zhang W, Li C, et al. (2015). Effects of Ultrasonic Processing on Caspase-3, Calpain Expression and Myofibrillar Structure of Chicken during Post-Mortem Ageing. *Food Chemistry* 177: 280-7.

Grilo AL and Mantalaris A. (2019). Apoptosis: A Mammalian Cell Bioprocessing Perspective. *Biotechnology Advances* 37: 459-475.

Gurtu V, Kain SR and Zhang G. (1997). Fluorometric and Colorimetric Detection of Caspase Activity Associated with Apoptosis. *Analytical Biochemistry* 251: 98-102.

Huang F, Huang M, Zhang H, Guo B, Zhang D and Zhou G. (2014). Cleavage of the Calpain Inhibitor, Calpastatin, during Postmortem Ageing of Beef Skeletal Muscle. *Food Chemistry* 148: 1-6.

Ishisaka R, Utsumi T, Yabuki M, Kanno T, Furuno T, Inoue M, et al. (1998). Activation of caspase-3-like protease by digitonin-treated lysosomes. *FEBS Letters* 435: 233-236.

Jia X, Hildrum KI, Westad F, Kummen E, Aass L and Hollung K. (2006). Changes in enzymes associated with energy metabolism during the early post mortem period in longissimus thoracis bovine muscle analyzed by proteomics. *Journal of Proteome Research* 5: 1763-1769.

Kemp CM, King DA, Shackelford SD, Wheeler TL, Koohmaraie M. (2009). The caspase proteolytic system in callipyge and normal lambs in longissimus, semimembranosus, and infraspinatus muscles during postmortem storage. *Journal of Animal Science* 87: 2943-2951.

Kemp CM, Bardsley RG and Parr T. (2006a). Changes in caspase activity during the postmortem conditioning period and its relationship to shear force in porcine longissimus muscle. *Journal of Animal Science* 84: 2841-2846.

Kemp CM, Parr T, Bardsley RG and Buttery PJ. (2006b). Comparison of the Relative Expression of Caspase Isoforms in Different Porcine Skeletal Muscles. *Meat Science* 73: 426-431.

Lin S and Guidotti G. (2009). Purification of Membrane Proteins. In *Methods in*

enzymology Vol. 463. Cambridge, MA: Academic Press, pp. 619-629.

McIlwain DR, Berger T and Mak TW. (2013). Caspase functions in cell death and disease. *Cold Spring Harbor Perspectives in Biology* 5: a008656.

Melody JL, Lonergan SM, Rowe LJ, Huiatt TW, Mayes MS and Huff-Lonergan E. (2004). Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. *Journal of Animal Science* 82: 1195-1205.

Ouali A, Herrera-Mendez CH, Coulis G, Becila S, Boudjellal A, Aubry L, et al. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Science* 74: 44-58.

Stennicke HR and Salvesen GS. (1997). Biochemical characteristics of caspases-3, -6, -7, and -8. *The Journal of Biological Chemistry* 272: 25719-25723.

Stennicke HR and Salvesen GS. (1999). Caspases: preparation and characterization. *Methods* 17: 313-319.

Taylor RC, Cullen SP and Martin SJ. (2008). Apoptosis: controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology* 9: 231.

Wang Z, Liao J and Diwu Z. (2005). N-DEVD-N′-morpholinecarbonyl-rhodamine 110: novel caspase-3 fluorogenic substrates for cell-based apoptosis assay. *Bioorganic & Medicinal Chemistry Letters* 15: 2335-2338.

Zhang J, Yu Q, Han L, Chen C, Li H and Han G. (2017). Study on the apoptosis mediated by cytochrome c and factors that affect the activation of bovine longissimus muscle during postmortem aging. *Apoptosis: An International Journal on Programmed Cell Death* 22: 777-785.

Zhang X, Pan D, Cao J and Wu Z. (2013). Changes in the Major Caspases Involved in Cytoskeletal Degradation of Goose Muscle during Prolonged Aging. *Food Research International* 51: 603-610.

6 | APPENDIX VI PUBLICATION VI

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Caspase activity in *post mortem* muscle and its relation to cattle handling practices[6]

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Abstract

BACKGROUND: Animal handling practices are one of the factors majorly affecting animal metabolism prior to slaughter. This phenomenon increases the occurrence of meat quality defects such as dark cutting-beef, causing high economical losses in the meat industry. Under this framework, the assessment of apoptosis onset in *post mortem* muscle was proposed as a novel approach to reveal biochemical characteristics in several Spanish bovine breeds (Asturiana de los Valles, Retinta and Rubia Gallega) managed under different production systems (intensive vs semi-extensive) and transport/lairage conditions (mixing vs not mixing with unfamiliar animals). To do so, the activities of initiator caspase 9 and executioner caspases 3/7 were determined in *Longissimus thoracis et lumborum* muscle at three early *post mortem* times (2, 8 and 24 h).

RESULTS: Breed effect and transport/lairage conditions were the most relevant factors that influenced both caspase activities over *post mortem* time, showing Rubia Gallega breed a completely different behaviour compared to Asturiana de los Valles and Retinta breeds. Moreover, it is postulated that apoptosis cascade is initiated via the activation of caspase 9 under hypoxic or metabolic stress followed by the activation of executioner caspases 3/7.

CONCLUSIONS: Assessment of apoptosis on *post mortem* muscle can be a novel approach to study the influence of animal handling on muscle metabolism and *post mortem* cell death and its consequences on meat quality traits.

Keywords

Caspases ● Cell Death ● Apoptosis ● Animal Management ● *Bos taurus* ● Meat Quality

6.1 INTRODUCTION

Over the years, meat quality research has been focused on the study of several proteolytic enzyme groups, physical state of myofibrillar proteins, fiber structure, intramuscular fat and other quality attributes such as color or water holding capacity [1] in order to understand meat tenderness over aging time. In this sense, the relevance of apoptosis processes was not considered until Herrera-Mendez et al. [2] proposed cell death as one of the first events triggered during the conversion of muscle into meat. Since then, several researchers have focused on the study of metabolic responses associated with intrinsic hypoxia/ischemia that could contribute to *post mortem* proteolysis occurring during meat tenderization [3,4]. However, these studies did not investigate any relationship that can occur between *ante mortem* factors influencing animal metabolism and *post mortem* cell death. Inadequate animal management prior to slaughter could trigger a stress response through the increase of hormone secretion (catecholamines, cortisol, adrenaline, etc), leading to the depletion of muscle glycogen reserves [5-7]. This reduction of *ante mortem* glycogen storage will modify the glycolytic metabolism in *post mortem* muscle, resulting in less lactic acid production thus impairing a decrease of ultimate pH meat to normal values (≈ 5.5). This promotes the occurrence of several meat defects such as dark cutting or DFD (*dark, firm and dry*) meat that causes significant economic losses in the meat industry [8]. Therefore, a careful study of *ante mortem* factors related to cattle (mainly breed, sex, age) and their handling procedures (mainly feeding systems, transportation, lairage and slaughter conditions) may ease the understanding of those biochemical pathways more related to meat quality traits.

Caspases constitute a family of cysteine-dependent peptidases that play an essential role in apoptosis (caspases 3, 6, 7, 8, 9, 10 and 12) and inflammatory response system (caspases 1, 4, 5, 11 and 13). Apoptotic caspases can be further classified according to their point of entry into the cell death pathway: initiator caspases (caspases 8, 9 10 and 12) and executioner caspases (caspases 3, 6 and 7). These enzymes are mainly synthesized as inactive zymogens (pro-caspases), and internal and/or external stimuli initiate a series of controlled reactions that ultimately lead to cell death. The extrinsic pathway initiates apoptosis through the activation of extracellular receptors that will finally activate initiator caspase 8. Meanwhile, the intrinsic pathway is activated by internal stimuli (metabolic and/or hypoxic stress) leading to the permeability of the outer mitochondrial membrane, favouring cytochrome-C release and the formation of the caspase 9 apoptosome complex [9,10]. This activates executioner caspases 3 and 7 that will proceed with cell dismantling [11]. Concerning activation of these enzymes in relation to stress, previous studies have found a higher caspase 1 activity in mice when animals were stressed [12] and activation of caspase 3 in Zebrafish by heat shock in response to stress [13]. In this line, some authors found a higher expression of the

caspase 3 large subunit in DFD beef samples at 24 h *post mortem* times [14].

In the present study, and for the first time, the study of apoptosis as a way to understand the influence of pre-slaughter handling practices on biochemical muscle characteristics of several bovine breeds is proposed. The research investigated the activity of executioner caspases 3/7 and initiator caspase 9 over time (2, 8, and 24 h *post mortem*) in loin samples of three Spanish beef cattle breeds managed under different feeding systems (intensive *vs* semi-extensive) and transport/lairage (mixed *vs* non-mixed with unfamiliar animals) conditions.

6.2 MATERIALS AND METHODS

6.2.1 Animal handling

A total of ninety (n = 90) male calves from three native Spanish bovine breeds were used: 29 Asturiana de los Valles (**AV**), 33 Retinta (**RE**) and 28 Rubia Gallega (**RG**). Calves were reared under two different production systems, intensive (**I**) and semi-extensive (**E**), following the typical procedures of each region and breed.

AV calves were suckled by their mothers under grazing conditions. They were weaned at 8 months of age and supplemented with concentrate *ad libitum* in winter period. At 10-12 months, calves were finished under one of the two systems for a period of 100 days until they were slaughtered at 13-15 months: (I) 18 yearling bulls were managed indoors and fed 8 kg/head/day of concentrate and 2 kg/head/day of barley straw, and (E) 11 yearling bulls were managed outdoors grazing and complemented with 3.5 kg/head/day of concentrate. The concentrate was composed of 84% barley meal, 10% soya meal, 3% fat, 3% minerals, vitamins and oligoelements, and pasture was mainly composed of ryegrass (*Lolium perenne*) and clover (*Trifolium repens*).

RE calves were suckled by their mothers under grazing conditions until they were weaned at 6 months. Later, calves were reared under one of the two systems until they were slaughtered at 13-14 months: (I) 13 yearling bulls were managed indoors and fed 8 kg/head/day of concentrate and 2 kg/head/day of barley straw, and (E) 20 yearling bulls were managed outdoors grazing and complemented with 4.0 kg/head/day of concentrate. The concentrate was composed of 51% corn meal, 34% barley meal, 8% soya meal, 3%fat, 4% minerals, vitamins and oligoelements, and pasture was mainly composed of ryegrass (*Lolium perenne* and *Lolium rigidum*) and clover (*Trifolium repens*).

Management of RG calves was slightly different as they are commercially slaughtered at a younger age (10 months) compared to the other studied breeds. Under the intensive system (I), 12 RG calves were weaned at 1.5 months and managed indoors with concentrate and barley straw *ad libitum*. The other 16 RG calves (E) were suckled by their mothers under grazing conditions mainly perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) and supplemented with 3.5 kg/head/day of concentrate and barley

straw until 7 months. Then, they were finished with concentrate *ad libitum* for 100 days. The concentrate was composed of 40% corn meal, 28% barley meal, 21% toasted soybean flour, 4% palmist extraction flour, 2% palm oil, 2% calcium carbonate, 2% fatty acid calcium salt and 1% sodium chloride.

At the time of slaughter, animals were transported in a lorry with natural ventilation and space allowance of 2.5 m^2 per animal. Within each breed and production system, half of the animals were mixed with unfamiliar individuals during transport and further lairage at abattoir installations prior to slaughter (I: 8 AV, 7 RE and 6 RG; E: 5 AV, 12 RE and 6 RG), whereas the other half were not mixed with other animals (I: 10 AV, 6 RE and 6 RG, E: 6 AV, 8 RE and 10 RG). At arrival to the abattoirs, animals were located in lairage pens and were slaughtered 30 min after arrival to avoid additional stress.

Animals were slaughtered in commercial abattoirs of each region following safety and welfare conditions according to European Union regulations (Council Regulation (EC) No 853/2004 and No 1099/2009). The average carcass weights for each breed were 297.5 ± 59.1 kg for AV, 314.5 ± 23.3 kg for RE and 244.9 ± 39.0 kg for RG.

6.2.2 Sample collection

At 2 h *post mortem*, approximately 30 g of *Longissimus thoracis et lumborum* (**LTL**) muscle sample was taken at 13th rib level from the left-half carcass of each animal. Subsamples of 10 g were immediately frozen in liquid nitrogen and stored in Falcon tubes at -80 °C until further analysis. The rest of muscle samples (20 g) were transported to the laboratory under refrigeration conditions (4 °C) and sub-samples of 10 g were snap-frozen in liquid nitrogen and stored at -80 °C in Falcon tubes after 8 and 24 h *post mortem* time.

At 24 h *post mortem*, ultimate pH measurements (**pHu**) were taken at the 6th rib level using a penetration electrode (CRISON pH/mV-meter 506, CRISON Instruments SA, Spain).

6.2.3 Extraction of sarcoplasmic proteins

Half gram of each LTL muscle sampled at different *post mortem* times (2, 8 and 24 h) was homogenized in 2 mL of extraction buffer containing 10 mM HEPES, pH 7.5, 0.1% v/v Brij 30%, 10% w/v sucrose, 1 mM EDTA and 1 mM PMSF using an Ika ultra-Turrax device (Yellow Line Di 25 model, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The homogenate was centrifuged at 20,000 x *g* for 20 minutes at 4 °C (Beckman Coulter Inc., Indianapolis, ID, USA), the supernatant filtered through 0.45 μm PVDF syringe filter and stored at -80 °C until further analyses.

6.2.4 Determination of caspase 9 and 3/7 activities

Sarcoplasmic protein extracts (50 μL) were poured each in a well of a 96-well microtiter plate followed by the addition of 20 μL of a 24 mM DTT solution. Microplate was pre-incubated for 30 min at 37 °C and the enzyme reaction was initiated by

adding 50 μL of 0.1 mM of Ac-LEHD-AMC (caspase 9) or Ac-DEVD-AMC (caspases 3/7) substrate dissolved in HEPES-CHAPS buffer (10% w/v sucrose, 100 mM NaCl, 50 mM HEPES pH.7.5, 0.1% w/v CHAPS and 1 mM EDTA). Fluorescence intensity (expressed as Relative Fluorescence Units) was measured using a CLARIOstar microplate fluorometer (BMG LABTECH GmbH, Ortenberg, Germany) for 30 min at 37 °C in 2 min intervals using excitation and emission wavelengths of 360 ± 15 and 480 ± 20 nm, respectively. Three technical replicates were done for each assay.

6.2.5 Statistical analysis

Data were subjected to ANOVA using Linear Mixed Model procedures of SPSS statistical software (version 26.0, New York, USA) considering the individual animal as a subject and carcass weight as a covariate in the model. Caspase activity (9 and 3/7) data were analyzed using breed, production system and transport as fixed factors and *post mortem* time as a repeated measure factor. Production system was nested within breed and transport was nested within production system and breed. For muscle pHu, the effect of *post mortem* time was excluded from the model. The parameters of the model were estimated using the restricted maximum likelihood method and the Huynh-Feldt matrix was selected for the repeated measures covariance structure following the Akaike information criterion. Additionally, least square means of dependent variables for the levels of fixed factors breed and *post mortem* time were compared using the lest significant difference (LSD) test. Significance level was declared at $P \leq 0.05$.

6.3 RESULTS AND DISCUSSION

Results of statistical significances of studied factors and binary interactions are reported in **Table 1**. The covariate (carcass weight) included in the mixed model did not affect significantly any of the variables studied (pHu and caspase activities; $P > 0.05$) but it was maintained in the models as adjustments were improved. In the present study, all carcasses presented normal pHu values (below 5.9), but these values were significantly affected by breed ($P \leq 0.001$) while the effects of the production system and transport type were not significant. RE breed showed significantly higher values (5.64 ± 0.12) compared to the other two breeds (5.49 ± 0.06 for AV and 5.55 ± 0.14 for RG), and this could be related to its excitable temperament [15]. In the present study, other indicators apart from pHu, have been considered to evaluate the biochemical characteristics of *post mortem* muscle and final meat quality. In this sense, measurements of caspase 9 and 3/7 activities were considered as an interesting and innovative approach taking into account that animals suffering higher stressing conditions would yield higher apoptosis levels compared to animals managed under less stressful conditions. It is worth considering, however, that caspase 9 and 3/7 activities can vary depending on *post mortem* time. Therefore, these activities were evaluated at 2, 8 and

24 h *post mortem* to determine the caspase enzyme kinetics at early *post mortem* times as a key factor to better understand the biochemical characteristics of meat on the considered bovine breeds.

Table 1. Statistical significances of studied factors and binary interactions.

Effects and interactions	pH[a]	Caspase 9	Caspases 3/7
Breed	***	*	ns
Prod. system(Breed)	ns	ns	ns
Transport(Prod. system(Breed))	ns	ns	**
PM Time	-	*	*
PM Time x Breed	-	***	***
PM Time x Prod. system(Breed)	-	ns	ns
PM Time x Transport(Prod. system(Breed))	-	*	**

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant ($P > 0.05$).

[a] Post mortem (PM) time was not included as split plot factor in the mixed model.

The activity of caspase 9 was significantly affected by breed ($P \leq 0.05$) and *post mortem* time ($P \leq 0.05$). However, evolution of caspase 9 activity over time depended on breed ($P \leq 0.001$) and the transport utilized within each production system and breed ($P \leq 0.05$; **Table 1**). In **Figure 1,** the interaction between breed and *post mortem* time on caspase 9 activity has been depicted. In RG breed, a different behavior of caspase 9 activity over time can be clearly observed, showing significantly higher activities at 2 h and lower activities at 24 h *post mortem* compared to AV and RE breeds. Intermediate and statistically not different values among breeds were observed at 8 h *post mortem*. It is assumed that caspases are activated immediately after animal exsanguination, showing the highest activity values at early *post mortem* times and then decreasing over time [2,3,16-18]. In this sense, as previously reported in the literature [19-21], this pattern was only observed in RG breed. In contrast, the low caspase 9 activity observed in RE and AV breed at early *post mortem* times could be indicative of higher stress levels reached before slaughter. This would trigger activation of other cell defense mechanisms such as synthesis of heat shock proteins (**HSPs**), which has been widely described for their anti-apoptotic role [22,23]. They interact with active caspases hindering their function and, consequently, slow down the cellular death process. This could explain the lower levels of caspase 9 activity determined early *post mortem* for RE and AV. In the scientific literature, breed effect and its relation to excitable temperament and stress susceptibility has been reviewed for several species [24] and previously reported for RE breed [15], but there is no information available concerning the non-aggressive behavior described of AV and RG [25,26]. Low caspase 9 activity in AV breed can be explained considering that it is a double muscle breed [27] caused by myostatin gene mutation, giving rise to an increase in the number of muscle fibers. Hypertrophied animals may be more susceptible to stress for their regularly limited mobility and reduced muscle capillary density, myoglobin content and lung weight as compared to normal cattle[28]. In this line, other authors also found the same caspase pattern in

callipyge lambs [18], showing low caspase activity at early *post mortem* times and then increasing over time reaching the maximum at 24 h. In any case, further research would be necessary to understand the different behavior of caspase 9 activity between breeds and their relationship with stress events.

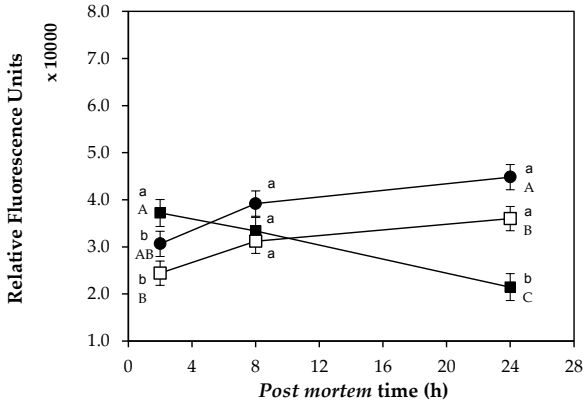

Figure 1. Interaction between breed and *post mortem* time factors for caspase 9 activity. Least square means and standard error of the means have been represented. Different capital letters indicate significant differences among breeds and different lower case letters indicate significant differences among *post mortem* times ($P \leq 0.05$). ●, Asturiana de los Valles; □, Retinta; ■, Rubia Gallega.

The interaction between transport/lairage and *post mortem* time effects has been depicted in **Figure 2**. While AV and RE breeds behaved with a similar increasing pattern over time and no differences between mixed and non-mixed animals (**Figure 2A** and **2B**), a very different pattern was observed for RG breed (**Figure 2C**), reinforcing the idea of the preponderant role played by bovine breed in caspase activity. Initially (2 h), RG breed yielded slightly higher caspase activity values compared to the other breeds, but with no differences between mixed and non-mixed animals, after that, differences were significant between mixed and non-mixed animal groups (8 h and 24 h). In RG breed, behavior of non-mixed animals followed a similar pattern than that already shown in **Figure 1** for this breed. In contrast, mixed animals behaved differently reaching the highest caspase activity at 8 h *post mortem*. This would be explained considering the protective action exerted by HSPs that may delay apoptosis process over *post mortem* time. It is recognized that transport is one of the most stressful events that influence bovine animals through the meat production chain due to loading/unloading, duration of transport, temperature, feed/water deprivation, noise, vibration, social regrouping and unfamiliar conditions [7,29]. Mixing of unfamiliar animals prior to slaughter can considerably upset group hierarchy, thus promoting the occurrence of behavioral stress [5] and leading to muscle glycogen depletion [30]. However, as already indicated, in the present study transport and lairage conditions did not affect meat pHu while breed did.

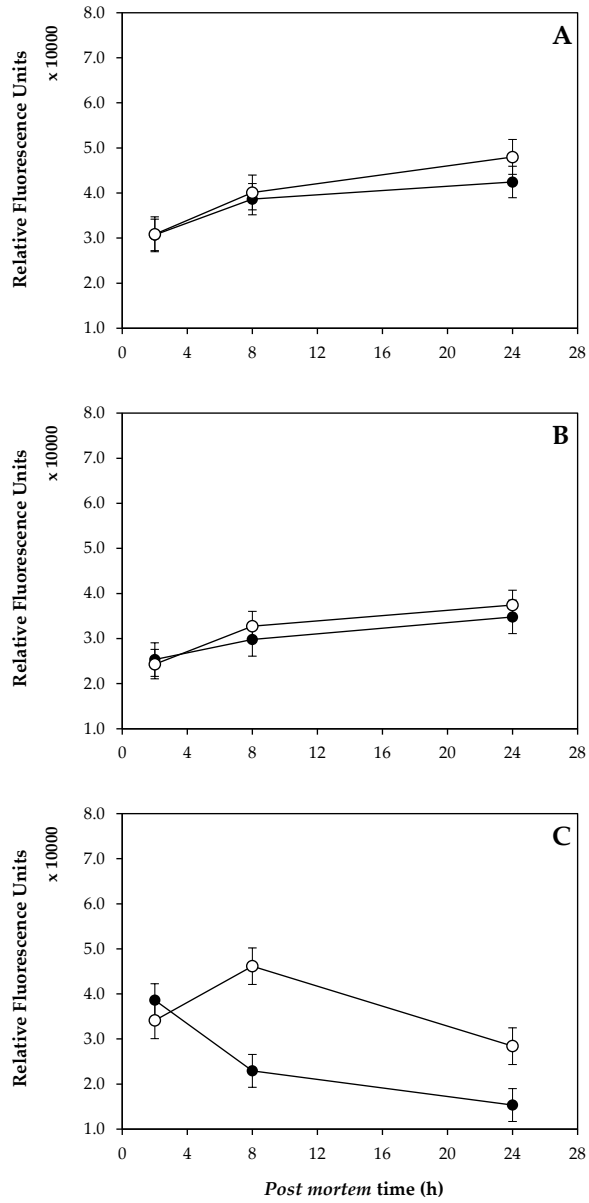

Figure 2. Evolution of caspase 9 activity over *post mortem* time as related to transport/lairage for mixed (○) and non-mixed (●) animal groups in each studied breed: A) Asturiana de los Valles; B) Retinta, and C) Rubia Gallega breed. Least square means and standard error of the means have been represented.

The activity of caspases 3/7 was significantly affected by transport ($P \leq 0.01$) and *post mortem* time ($P \leq 0.05$). However, the evolution of caspase 3/7 activity over time depended on breed ($P \leq 0.001$) and on the transport utilized within each production system and breed ($P \leq 0.01$; **Table 1**). The binary interaction between breed and *post mortem* time effects has been depicted in **Figure 3**. The different behavior of caspase 3/7 activity in RG compared to the other breeds (AV and RE) can be clearly observed. Caspase 3/7 activity values of RG breed were significantly different for all *post mortem* times (2, 8 and 24 h) compared to the other two breeds that were similar to each other. At 2 and 8 h *post mortem*, caspase 3/7 activity was the highest in RG breed, while at 24 h was the lowest. It is worthwhile mentioning the differences in animal age at slaughter, as RG were younger (10 months) than the rest of the animals (13 to 15 months). This fact could also explain, at least partially, the differences found in the *post mortem* muscle metabolism. In this line, Zhu et al. [31] reported that several apoptotic mechanisms, including caspase 3 activation, were more pronounced in younger rat brains as compared to more mature developmental states after induction of cerebral hypoxia-ischemia. When comparing both executioner caspase 3/7 and initiator caspase 9 activities, they showed a similar breed-dependant pattern although caspases 3/7 seemed to be slightly less sensitive, not being able to discriminate among breeds at 24 h *post mortem*. This could be explained considering that caspases 3/7 are involved in the last step of signalling caspase cascade, giving rise to a higher residual activity compared to caspase 9.

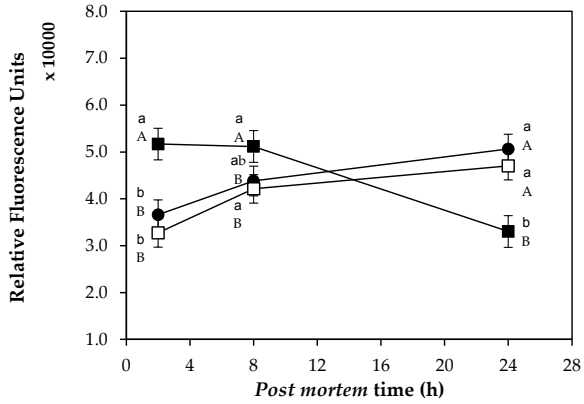

Figure 3. Interaction between breed and *post mortem* time factors for caspase 3/7 activity. Least square means and standard error of the means have been represented. Different capital letters indicate significant differences among breeds and different lower case letters indicate significant differences among *post mortem* times ($P \leq 0.05$). ●, Asturiana de los Valles; □, Retinta; ■, Rubia Gallega.

The interaction between transport/lairage and *post mortem* time for each breed is depicted in **Figure 4**. As for caspase 9, AV and RE breeds behaved with a similar increasing pattern over time, with no differences between mixed and non-mixed animals (**Figure 4A** and **4B**), whereas a very different pattern was observed for RG

breed (**Figure 4C**). At 2 h *post mortem*, caspase 3/7 activity values for RG breed were notably higher compared to the other breeds with no differences between mixed and non-mixed animals. However, at later *post mortem* times (8 and 24 h) differences were significant between mixed and non-mixed animal groups (**Figure 4C**), yielding again differences between breeds as observed for caspase 9.

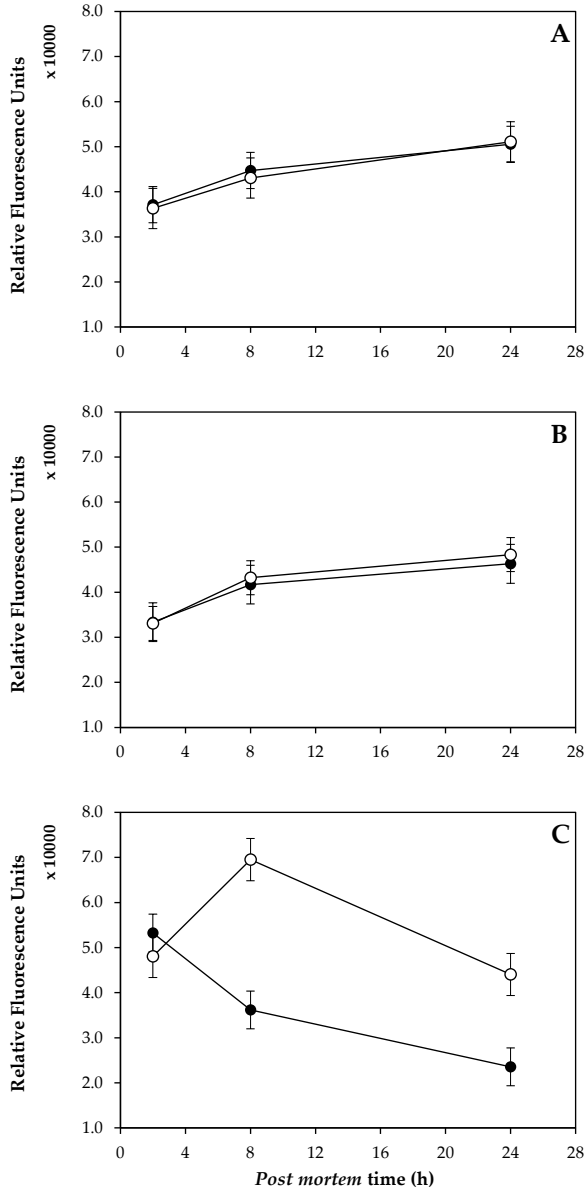

Figure 4. . Evolution of caspase 3/7 activity over *post mortem* time as related to transport/lairage for mixed (○) and non-mixed (●) animal groups in each studied breed: A) Asturiana de los Valles; B) Retinta, and C) Rubia Gallega breed. Least square means and standard error of the means have been represented.

Several studies have described the effect of handling practices on meat characteristics [24,32,33] and some of them have reported differences on caspase 3 expression patterns between normal and DFD samples, finding higher caspase 3 regulation levels in DFD samples at 24 h *post mortem* [14]. However, no studies have been previously reported concerning the relationship between caspase activity and stress-related factors. From our study we hypothesized that, depending on the external stimulus affecting animals before slaughter, different caspase activation responses could be triggered. In this regard, our work suggests that the intrinsic apoptotic pathway responding to stress and physiological damage may be the preferred mechanism to initiate the proteolytic cascade through the formation of the caspase 9 apoptosome complex.

6.4 Conclusions

Results obtained in the present work showed that levels of caspase activity over *post mortem* time greatly depends on breed and transport/lairage procedures, while production system played little effect. The behavior of Rubia Gallega breed over the studied *post mortem* period (2 to 24 h) was significantly different as compared to Asturiana de los Valles and Retinta breeds, being further research necessary to understand the evolution of both caspase activities in each breed. Caspase activity determinations were able to discriminate among breeds at both 2 and 24 h *post mortem*, especially between those breeds behaving differently (Rubia Gallega *vs* Asturiana de los Valles and Retinta). Moreover, enhanced sensitivity of caspase 9 activity at 24 h *post mortem* with respect to caspases 3/7 would indicate its preferable use as an indicator capable to detect breed and transport differences influencing animal meat characteristics. This innovative approach could be an interesting tool to improve assessment of meat quality in the industry. However, future research is necessary to fully address the prediction of stressful situations negatively influencing muscle metabolism and meat quality through assessment of either caspase 8 or 9 activity as the main contributors of the extrinsic and intrinsic apoptotic pathway, respectively.

Author Contributions

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

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 Sentandreu MA, Coulis G and Ouali A, Role of muscle endopeptidases and their inhibitors in meat tenderness. *Trends Food Sci Technol* **13**:400-421 (2002).

2 Herrera-Mendez CH, Becila S, Boudjellal A and Ouali A, Meat ageing: Reconsideration of the current concept. *Trends Food Sci Technol* **17**:394-405 (2006).

3 Kemp CM, Bardsley RG and Parr T, Changes in caspase activity during the postmortem conditioning period and its relationship to shear force in porcine longissimus muscle. *J Anim Sci* **84**:2841-2846 (2006).

4 Ouali A, Gagaoua M, Boudida Y, Becila S, Boudjellal A, Herrera-Mendez CH and Sentandreu MA, Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. *Meat Sci* **95**:854-870 (2013).

5 Tarrant PV, Animal behaviour and environment in the dark-cutting condition in beef-a review. *Ir J Food Sci Technol* **13**:1-21 (1989).

6 Gregory NG and Grandin T, *Animal welfare and meat science*. CABI Pub., (1998).

7 Ferguson DM and Warner RD, Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants? *Meat Sci* **80**:12-19 (2008).

8 Ijaz M, Li X, Zhang D, Hussain Z, Ren C, Bai Y and Zheng X, Association between meat color of DFD beef and other quality attributes. *Meat Sci* **161**:107954 (2020).

9 Grilo AL and Mantalaris A, Apoptosis: A mammalian cell bioprocessing perspective. *Biotechnol Adv* **37**:459-475 (2019).

10 Boatright KM and Salvesen GS, Mechanisms of caspase activation. *Curr Opin Cell Biol* **15**:725-731 (2003).

11 Taylor RC, Cullen SP and Martin SJ, Apoptosis: controlled demolition at the cellular level. *Nat rev Mol Cell Biol* **9**:231 (2008).

12 Towers AE, Oelschlager ML, Lorenz M, Gainey SJ, McCusker RH, Krauklis SA and Freund GG, Handling stress impairs learning through a mechanism involving caspase-1 activation and adenosine signaling. *Brain, Behav, Immun* **80**:763-776 (2019).

13 Alderman SL, Leishman EM, Fuzzen MLM and Bernier NJ, Corticotropin-releasing factor regulates caspase-3 and may protect developing zebrafish from stress-induced apoptosis. *Gen Comp Endocrinol* **265**:207-213 (2018).

14 Díaz-Luis A, Díaz F, Diñeiro Y, González-Blanco L, Arias E, Coto-Montes A, Oliván M and Sierra V, Nuevos indicadores de carnes (DFD): estrés oxidativo, autofagia y apoptosis. *Inf Tec Econ Agrar* **117**: 2-18 (2021).

15 García FIH and Cruz AMR, Nuevo tratamiento de induccion y sincronizacion ovulatoria que posibilita la concentracion de parideras y la utilizacion practica de la inseminacion artificial en vacuno retinto. *Inf Tec Econ Agrar* **26**: 416-218 (2005).

16 Huang F, Huang M, Zhang H, Guo B, Zhang D and Zhou G, Cleavage of the calpain inhibitor, calpastatin, during postmortem ageing of beef skeletal muscle. *Food Chem* **148**:1-6 (2014).

17 Huang F, Huang M, Zhang H, Zhang C, Zhang D and Zhou G, Changes in apoptotic factors and caspase activation pathways during the postmortem aging of beef muscle. *Food Chem* **190**:110-114 (2016).

18 Kemp C. M., King D. A., Shackelford S. D., Wheeler T. L., Koohmaraie M., The caspase proteolytic system in callipyge and normal lambs in longissimus, semimembranosus, and infraspinatus muscles during postmortem storage. *J Anim Sci* **87**:2943-2951 (2009).

19 Wang L, Han L, Ma X, Yu Q and Zhao S, Effect of mitochondrial apoptotic activation through the mitochondrial membrane permeability transition pore on yak meat tenderness during postmortem aging. *Food Chem* **234**:323-331 (2017).

20 Wang L, Ma G, Zhang Y, Shi X, Han L, Yu Q, Zhao S and Ma J, Effect of mitochondrial cytochrome c release and its redox state on the mitochondrial-dependent apoptotic cascade reaction and tenderization of yak meat during postmortem aging. *Food Res Int* **111**:488-497 (2018).

21 Wang L, Yu Q, Han L, Ma X, Song R, Zhao S and Zhang W, Study on the effect of reactive oxygen species-mediated oxidative stress on the activation of mitochondrial apoptosis and the tenderness of yak meat. *Food Chem* **244**:394-402 (2018).

22 Kultz D, Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *J Exp Biol* **206**:3119-3124 (2003).

23 Beere HM, "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. *J Cell Sci* **117**:2641-2651 (2004).

24 Ponnampalam EN, Hopkins DL, Bruce H, Li D, Baldi G and Bekhit AE, Causes and contributing factors to "dark cutting" meat: Current trends and future directions: A review. *Compr Rev Food Sci Food Saf* **16**:400-430 (2017).

25 Eusebi PG, Sevane N, Cortés O, Contreras E, Cañon J and Dunner S, Aggressive behavior in cattle is associated with a polymorphism in the MAOA gene promoter. *Anim Genet* **51**:14-21 (2020).

26 Ruiz Tena JL, Fomento, conservación y ordenación de las razas autóctonas españolas, in El Arca: Boletín de la Sociedad Española para los Recursos Genéticos Animales, Vol. 3, pp. 18–27 (Córdoba: Universidad de Córdoba; 1999).

27 Oliván M, Martínez A, Osoro K, Sañudo C, Panea B, Olleta JL, Campo MM, Oliver MÀ, Serra X, Gil M and Piedrafita J, Effect of muscular hypertrophy on physico-chemical, biochemical and texture traits of meat from yearling bulls. *Meat Sci* **68**:567-575 (2004).

28 Fiems LO, Double muscling in cattle: genes, husbandry, carcasses and meat. *Animals* **2**:472-506 (2012).

29 Swanson JC and Morrow-Tesch J, Cattle transport: Historical, research, and future perspectives. *J Anim Sci* **79**:E102-E109 (2001).

30 Warriss PD, Kestin SC, Brown SN and Wilkins LJ, The time required for recovery from mixing stress in young bulls and the prevention of dark cutting beef. *Meat Sci* **10**:53-68 (1984).

31 Zhu C, Wang X, Xu F, Bahr BA, Shibata M, Uchiyama Y, Hagberg H and Blomgren K, The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. *Cell Death Differ* **12**:162-176 (2005).

32 Gagaoua M, Monteils V, Couvreur S and Picard B, Identification of biomarkers associated with the rearing practices, carcass characteristics, and beef quality: An integrative approach. *J Agric Food Chem* **65**:8264-8278 (2017).

33 Warriss PD, The handling of cattle pre-slaughter and its effects on carcass and meat quality. *Appl Anim Behav Sci* **28**:171-186 (1990).

7 | APPENDIX VII MANUSCRIPT I

Caspase activity in *post mortem* muscle as a way to understand changes in ultimate meat pH[7]

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Abstract

BACKGROUND: Pre-slaughter stress can lead to the well-known 'dark cutting' defective meats characterized by a high ultimate pH (pHu) in cattle, causing high economical losses in the meat industry. Therefore, the study of biochemical mechanisms related to animal stress response is a fact that needs to be addressed. The aim of this work was to determine caspase 9 and 3/7 activities in high and normal pHu muscle samples at 24 h *post mortem* as a way to reveal stress situations in different Spanish local breeds and crossbred animals.

RESULTS: Either breed or pHu factors had an effect in both caspase activities, showing the high pHu group the highest caspase activity independently of the studied breed. In contrast, activity levels among breeds were slightly different for each caspase and between the high and normal pHu group in the case of caspase 3/7.

CONCLUSIONS: Overall, this study clearly demonstrated that caspase activity can be a good indicator to detect stress situations that might lead to defective high pHu meats.

Keywords

Caspases ● Cell Death ● Apoptosis ● *Bos taurus* ● Stress ● ultimate pH

7.1 INTRODUCTION

During the last decades, meat industry has moved towards the production of animals that are efficient feed converters, fast-growing and have high lean meat content with minimum production costs. This can negatively influence animal welfare and animals' stress status in relation to non-adequate animal handling practices, and consequently, increasing the appearance of meat quality defects such as *dry, firm and dark* (DFD) or 'dark-cutting' meat [1,2]. DFD meats are associated with any factor that lead to the depletion of muscle glycogen reserves prior to slaughter in reaction to acute stress. This causes the reduction of the substrate availability and modifies *post mortem* glycolytic metabolism, resulting in less lactic acid and high ultimate pH (pHu)[3,4]. In cattle, dark-cutting or high pHu meats cause consumers' rejection due to undesirable flavor, altered tenderness and short shelf-life [5-7]. This has a cost of around 55 and 20 million dollars per annum for Australian [8] and British [5] industry, respectively. Taking in mind its implications in terms of food quality and safety, but also in economic benefits for meat industry, an appropriate assessment of reliable indicators of pre-slaughter stress is currently necessary.

Measurement of pHu is the most common indicator for establishing the incidence of DFD meats since it is directly related to animals' pre-slaughter stress (PSS) [9,10]. However, usefulness of pHu assessment is compromised since high values do not necessarily certify the occurrence of true DFD meats [1]. New insights addressing the search of new stress indicators are needed to understand biochemical mechanisms underlying PSS condition and to overcome uncertainties from classic pHu determinations in meat [1]. In this line, Fuente-Garcia et al. (2020)[11] proposed, for the first time, the study of caspase activity over *post mortem* time as a way to understand the influence of pre-slaughter handling practices (feeding system and transport/ lairage conditions) on the stress status of several bovine breeds. Caspases constitute a family of cysteine dependent peptidases that are mainly synthesized as inactive zymogens (pro-caspases); however under internal (metabolic and/or hypoxic stress) and/or external stimuli they initiate a series of controlled reactions that ultimately lead to cell death [12-14]. They can be classified according to their point of entry into the cell death pathway as: a) initiator caspases (caspases 8, 9 10 and 12) and b) executioner caspases (caspases 3, 6 and 7). Previous studies have also associated the activation of these enzymes with stress situations reporting higher caspase 1 activity in stressed mice [15] and activation of caspase 3 in Zebrafish in response to heat stress [16]. In bovine cattle, Diaz et al. (2020) [17] found a higher expression of the caspase 3 large subunit in DFD beef samples at 24 h *post mortem*.

Our main goal was to study the activity of initiator caspase 9 and executioner caspases 3/7 from both high and normal pHu loin samples of different Spanish cattle breeds and crossbreeds animals at early *post mortem* times (24 h). This would be useful to understand the influence of pre-slaughter stress responsible of high

pHu meats and to be an early predictor of this condition in bovine cattle.

7.2 MATERIALS AND METHODS

7.2.1 Sample collection

In this work, a total of sixty-three (n = 63) beef samples were obtained from different Spanish local breeds: Asturiana de los Valles (AV, n = 18), Retinta (RE, n = 14) and Rubia Gallega (RG, n = 11). Additionally, another twenty samples (n = 20) were collected from crossbred (CB) animals (Asturiana de los Valles x Friesian). Male calves were handled in accordance with Directive 2010/63/EU (2010) and slaughtered at their 14-15 (AV, RE and CB) or 10 (RG) months of age in commercial abattoirs of each region following safety and welfare conditions according to European Union regulations (Council Regulation (EC) No 1099/2009).

At 24 h *post mortem*, approximately 10 g of *Longissimus thoracis et lumborum* (**LTL**) muscle sample were excised at 13th rib level from the left-half carcass of each animal, immediately frozen in liquid nitrogen and stored in Falcon tubes at -80 °C until further analysis. Muscle samples were sorted into two different groups according to their pHu values: 'high' pHu samples (9 AV, 10 CB, 7 RE and 5 RG) having pHu values higher than 5.9. The rest of samples were classified as 'normal' samples (9 AV, 10 CB, 7 RE and 6 RG) showing pH values below 5.9. The pHu measurements were done at the LTL muscle of the 6th rib level at 24 h *post mortem* using a penetration electrode (CRISON pH/mV-meter 506, CRISON Instruments SA, Spain).

7.2.2 Extraction of sarcoplasmic proteins

Protein extraction was carried out as described Fuente-Garcia et al. [18]. Briefly, half gram of each type of meat sample (high and normal pHu), was homogenized in 2 mL of extraction buffer (10 mM HEPES, pH 7.5, 0.03 % v/v Brij 35®, 10 % w/v sucrose, 1 mM EDTA, 1 mM PMSF) using an Ika ultra-Turrax device (Yellow Line Di 25 model, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The homogenate was centrifuged at 20,000 *g* for 20 minutes at 4 °C (Beckman Coulter Inc., Indianapolis, ID, USA), the supernatant filtered through 0.45 μm PVDF syringe filter and stored at -80 °C until further analyses.

7.2.3 Fluorogenic determination of caspase 9 and 3/7 activities

It was performed following the procedure proposed by Fuente-Garcia et al. [18]. Briefly, each sarcoplasmic protein extract (50 μL) was poured in each well of a 96-well microtiter plate followed by the addition of 20 μL of a 24 mM DTT solution. Microplate was pre-incubated for 30 min at 37 °C and the enzyme reaction was initiated by adding 50 μL of 0.1 mM of Ac-LEHD-AMC (caspase 9) or Ac-DEVD-AMC (caspases 3/7) substrate dissolved in HEPES-CHAPS buffer (10 % w/v sucrose, 100 mM NaCl, 50 mM HEPES pH.7.5, 0.1 % w/v CHAPS and 1 mM EDTA). Fluorescence intensity (expressed as Relative Fluorescence Units) was measured using a CLARIOstar

microplate fluorometer (BMG LABTECH GmbH, Ortenberg, Germany) for 30 min at 37 °C using excitation and emission wavelengths of 360 ± 15 and 480 ± 20 nm, respectively. Three technical replicates were done for each assay.

7.2.4 Statistical analysis

The General Linear Model of ANOVA was used to determine whether the presence or absence of significant differences ($P \leq 0.05$) in the caspase activity (9 and 3/7) at 24 h *post mortem*. Caspase activities were analysed using breed (Asturiana de los Valles, crossbreed, Retinta and Rubia Gallega) and pHu (high and normal pHu meat samples) as fixed factors. Normality and homoscedasticity of the variables were checked. Additionally, least square means (LSD) of dependent variables for the levels of breed and pHu fixed factors were compared using the LSD test and results were represented in box-plots. Significance level was declared at $P \leq 0.05$. Statistical analyses were performed using SPSS statistical software (version 25.0, New York, USA).

7.3 RESULTS AND DISCUSSION

Determination of meat pHu is critical for meat industry since authorities worldwide consider that values beyond 6.0 at 24 h *post mortem* are intimately associated to animals suffering PSS condition and occurrence of DFD meats [9,10,19]. Thus, other indicators such as caspase 9 and 3/7 activities were considered in order to relate them to changes in pHu values, and then, to evaluate their ability to explain stress situations. It is well-known that animals suffering higher stress levels would reach higher apoptosis levels compared to animals managed under less stressful conditions [15,16]. In our previous studies, we reported that caspase activity varied depending on *post mortem* time, being 24 h *post mortem* the most accurate period capable to discriminate among samples collected from several breeds managed under different feeding and transport/ lairage conditions[11].

Results of statistical significances of studied factors and binary interactions are reported in **Table 1**. In general, the activity of both caspases was affected by breed ($P \leq 0.001$) and pHu ($P \leq 0.001$), and the behaviour of both caspases was quite similar as observed in **Figures 1A.** and **2**. However, there was an interaction between breed and pHu for caspase 3/7 activity ($P \leq 0.001$).

Table 1. Statistical significances of studied factors and binary interactions.

Fixed effects and interactions	Caspase 9	Caspase 3/7
Breed	***	***
pHu	***	***
Breed x pHu	ns	***

Regarding the effect of breed on caspase 9 activity at 24 h *post mortem* (**Figure 1B.**), AV showed a significantly higher caspase activity compared to CB and RG ($P \leq 0.05$), while intermediate activities where observed for RE breed ($P > 0.05$). Observed activity data variability was also higher in AV breed, with highest maximum and lowest minimum activities, compared to other breeds. These results are partially in accordance to those reported by Fuente-García *et al*. (2021) [11], where AV and RE breed showed significantly higher caspase 9 activities at 24 h *post mortem* compared to RG breed. The higher caspase 9 activity in AV and RE at 24 h *post mortem* could be indicative of higher stress levels reached before slaughter. Some studies have reported the relationship between breed excitable temperament and stress susceptibility on these breeds [1]. While RE breed has widely described as tempered breed giving rise to high stress levels [20], AV and RG breed have been considered non-aggressive breeds [21,22]. It must noteworthy to mention that AV breed is known such as double-muscle or hypertrophied breed [23] and in this respect it has been considered more susceptible to stress due to their regularly limited mobility and reduced muscle capillary density as compared to normal cattle [24]. Concerning the effect of pHu, significantly higher caspase activity at 24 h *post mortem* was observed in high compared to normal pHu meat samples (**Figure 1C.**). Although there are not studies that analyse caspase 9 activity in relation to meat pHu, results would be comparable to those reported for caspase 3/7 activity. In this regard, some authors have revealed differences in caspase 3 regulation levels between normal and high pHu samples, reporting high expression of caspase 3 large subunit in high compared to normal pHu meat samples at 24 h *post mortem* [17]. Meanwhile, Fuente-García *et al*. (2019, 2021) [25,26] reported that some heat shock proteins (**HSPs**) corresponding to alpha-crystallin B and heat shock protein beta-1 and beta-6, were over abundant in high pHu meat samples. This finding seems to be in accordance with the fact that stress can induce the synthesis of HSPs that may delay the apoptosis process over *post mortem* time. Taking into account that caspase activation occurs immediately after animal exsanguinations and then decreasing over time, the idea of anti-apoptotic role exerted by heat shock proteins is becoming stronger.

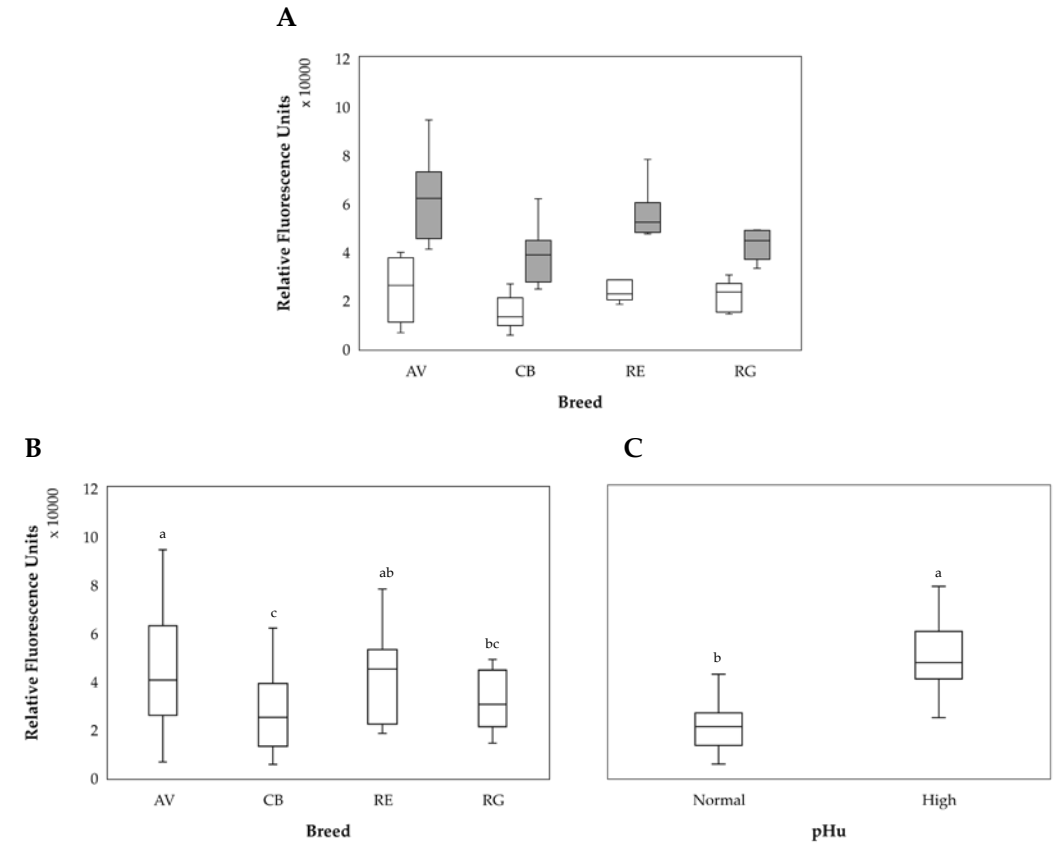

Figure 1. Box-plot with upper and lower whiskers representing the effect of (**A**) breed and meat pHu interaction (□ normal pHu meat samples; ■ high pHu meat samples) (**B**) breed and (**C**) meat pHu on caspase 9 activity at 24 h *post mortem*. Different letters indicate significant differences among breeds ($P \leq 0.05$).

As for caspase 9 activity, the activity of caspase 3/7 was significantly higher in meat samples with high compared to meat samples with normal pHu in all studied breeds ($P \leq 0.05$) (**Figure 2**). However, this pattern was quite different in RE breed with strong differences in caspase 3/7 activity between normal and high meat samples, reinforcing again the idea of the major role played by bovine breed in caspase activity. In this sense, as previously indicated, it has been described the aggressive behaviour and stress susceptibility of RE breed [20] compared to AV and RG breed that are similar to each other [21,22]. On the other hand, overall, crossbred animals showed the lowest caspase levels, in normal and high pHu meat samples, which could be related to the increased resilience to stress susceptibility of these animals. In this sense, some authors pointed out that crossbred animals yielded a better response to environmental changes and stressors compared to the pure breeds [27,28]. In terms of variability in activity, in general this was similar in both types of meat samples (normal and high pHu) for all studied breeds except for RG breed where activity variability was greater in high pHu meat samples (comparable to AV breed) compared to normal samples. Therefore, further research is necessary to

understand the influence of breed on caspase 3/7 activity.

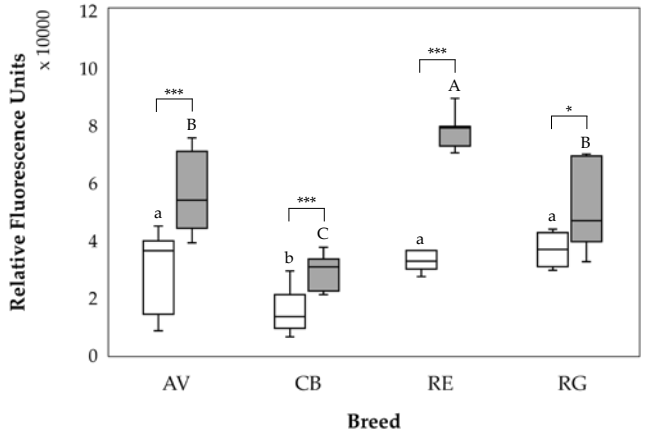

Figure 2. Box-plot with upper and lower whiskers representing the effect of breed and meat pHu on caspase 3/7 activity. Different lower case letters indicate significant differences among breeds in meat samples with normal pHu, and different capital letters indicate significant differences among breeds in meat samples with high pHu. Asterisks represent differences between the normal (□) and high (■) pHu meat samples for each studied breed. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

7.4 CONCLUSIONS

Overall, these results confirm that both caspase 9 and 3/7 activities were significantly higher in meat samples with high compared to normal pHu, independently of the studied breed and demonstrating that caspase activity measured at 24 h *post mortem* can be a good indicator of high pHu meat samples. However, differences among breeds vary in each studied caspase and are dependent on pHu in the case of caspase 3/7. Crossbred animals and Rubia Gallega breed showed lower and intermediate values, respectively, for both caspase activities and studied pHu groups as compared to other two breeds. However, Asturiana de los Valles and Retinta breeds showed slightly different activity levels for each caspase and between high and normal pHu group in the case of caspase 3/7. In this regard, further research is necessary to better understand how the behaviour of each breed can affect the activity of studied caspases.

AUTHOR CONTRIBUTIONS

Conceptualization, C. F.-G. and M.A.S.; methodology, C. F.-G., M.O., D.F., S.G.-T. and M.A.S.; investigation, C.F.-G.; data curation, N.A.; writing—original draft preparation, C.F.-G.; writing—review and editing, C.F.-G., N.A. and M.A.S.; supervision, N.A. and M.A.S.; project administration, M.A.S.; funding acquisition, M.A.S. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 Ponnampalam EN, Hopkins DL, Bruce H, Li D, Baldi G and Bekhit AE, Causes and contributing factors to "dark cutting" meat: Current trends and future directions: A review. *Compr Rev Food Sci Food Saf* **16**:400-430 (2017).

2 Ferguson DM and Warner RD, Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants? *Meat Sci* **80**:12-19 (2008).

3 McVeigh JM, Tarrant PV and Harrington MG, Behavioral stress and skeletal muscle glycogen metabolism in young bulls. *J Anim Sci* **54**:790-795 (1982).

4 Tarrant PV, Animal behaviour and environment in the dark-cutting condition in beef-a review. *Irish J Food Sci Tech* **13**:1-21 (1989).

5 Adzitey F and Nurul H, Pale soft exudative (PSE) and dark firm dry (DFD) meats: Causes and measures to reduce these incidences - a mini review. *Int Food Res J* **18**:11-20 (2011).

6 Newton KG and Gill CO, The microbiology of DFD fresh meats: A review. *Meat Sci* **5**:223-232 (1981).

7 Wulf DM, Emnett RS, Leheska JM and Moeller SJ, Relationships among glycolytic potential, dark cutting (dark, firm, and dry) beef, and cooked beef palatability. *J Anim Sci* **80**:1895-1903 (2002).

8 Jose CG, McGilchrist P, Perovic JL, Gardner GE and Pethick DW, The economic impact of dark cutting beef in Australia. (2015).

9 Loudon KMW, Lean IJ, Pethick DW, Gardner GE, Grubb LJ, Evans AC and McGilchrist P, On farm factors increasing dark cutting in pasture finished beef cattle. *Meat Sci* **144**:110-117 (2018).

10 Chambers PG, Grandin T, Heinz G and Srisuvan T, *Guidelines for humane handling, transport and slaughter of livestock.* FAO, Bangkok, (2001).

11 Fuente-Garcia C, Aldai N, Sentandreu E, Oliván M, Franco D, García-Torres S, Barron LJR and Sentandreu MÁ, Caspase activity in post mortem muscle and its relation to cattle handling practices. *J Sci Food Agric*, in press.

12 Grilo AL and Mantalaris A, Apoptosis: A mammalian cell bioprocessing perspective. *Biotechnol Adv* **37**:459-475 (2019).

13 Boatright KM and Salvesen GS, Mechanisms of caspase activation. *Curr Opin Cell Biol* **15**:725-731 (2003).

14 Taylor RC, Cullen SP and Martin SJ, Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* **9**:231 (2008).

15 Towers AE, Oelschlager ML, Lorenz M, Gainey SJ, McCusker RH, Krauklis SA and Freund GG, Handling stress impairs learning through a mechanism involving caspase-1 activation and adenosine signaling. *Brain, Behav, Immun* **80**:763-776 (2019).

16 Alderman SL, Leishman EM, Fuzzen MLM and Bernier NJ, Corticotropin-releasing factor regulates caspase-3 and may protect developing zebrafish from stress-induced

apoptosis. *Gen Comp Endocrinol* **265**:207-213 (2018).

17 Díaz-Luis A, Díaz F, Diñeiro Y, González-Blanco L, Arias E, Coto-Montes A, Oliván M and Sierra V, Nuevos indicadores de carnes (DFD): estrés oxidativo, autofagia y apoptosis. *Inf Tec Econ Agrar* **117**:3-18 (2021).

18 Fuente-Garcia C, Sentandreu E, Aldai N and Sentandreu MA, Optimization of a fluorogenic assay to determine caspase 3/7 activity in meat extracts. *Food Sci Technol Int*:1082013221993577 (2021).

19 Polati R, Menini M, Robotti E, Millioni R, Marengo E, Novelli E, Balzan S and Cecconi D, Proteomic changes involved in tenderization of bovine Longissimus dorsi muscle during prolonged ageing. *Food Chem* **135**:2052-2069 (2012).

20 García FIH and Cruz AMR, Nuevo tratamiento de induccion y sincronizacion ovulatoria que posibilita la concentracion de parideras y la utilizacion practica de la inseminacion artificial en vacuno retinto. *Inf Tec Econ Agrar* **26**:416-418 (2005).

21 Eusebi PG, Sevane N, Cortés O, Contreras E, Cañon J and Dunner S, Aggressive behavior in cattle is associated with a polymorphism in the MAOA gene promoter. *Anim Genet* **51**:14-21 (2020).

22 Ruiz Tena JL, Fomento, conservación y ordenación de las razas autóctonas españolas. in *El Arca: Boletín de la Sociedad Española para los Recursos Genéticos Animales,* Universidad de Córdoba, Servicio de Publicaciones, Córdoba, Vol. 3, pp. 18-27 (1999).

23 Oliván M, Martínez A, Osoro K, Sañudo C, Panea B, Olleta JL, Campo MM, Oliver MÀ, Serra X, Gil M and Piedrafita J, Effect of muscular hypertrophy on physico-chemical, biochemical and texture traits of meat from yearling bulls. *Meat Sci* **68**:567-575 (2004).

24 Fiems LO, Double muscling in cattle: genes, husbandry, carcasses and meat. *Animals* **2**:472-506 (2012).

25 Fuente-Garcia C, Aldai N, Sentandreu E, Oliván M, García-Torres S, Franco D, Zapata C and Sentandreu MA, Search for proteomic biomarkers related to bovine pre-slaughter stress using liquid isoelectric focusing (OFFGEL) and mass spectrometry. *J Proteomics* **198**:59-65 (2019).

26 Fuente-García C, Sentandreu MA, Aldai N, Oliván M and Sentandreu E, Proteomic pipeline for biomarker hunting of defective bovine meat assisted by liquid chromatography-mass spectrometry analysis and chemometrics. *J Proteomics* :104153 (2021).

27 Mäki-Tanila A, An overview on quantitative and genomic tools for utilising dominance genetic variation in improving animal production. *Agr Food Sci* **16**:188-198 (2007).

28 Peric T, Comin A, Corazzin M, Montillo M, Cappa A, Campanile G and Prandi A, Short communication: Hair cortisol concentrations in Holstein-Friesian and crossbreed F1 heifers. *J Dairy Sci* **96**:3023-3027 (2013).