UNIVERSIDAD DE CÓRDOBA

Programa de Doctorado Ingeniería Agraria, Alimentaria, Forestal y de Desarrollo Rural Sostenible por la Universidad de Córdoba y la Universidad de Sevilla

Del análisis at-line al control in situ en la industria del cerdo Ibérico utilizando sensores de Espectroscopía en el Infrarrojo Cercano

From at-line analysis to on-site control in the Iberian pig industry using Near Infrared Spectroscopy sensors

TESIS DOCTORAL

Juan Manuel Cáceres Nevado

Directores:

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Mayo 2021

TITULO: *From at-line analysis to on-site control in the Iberian pig industry using Near Infrared Spectroscopy sensors*

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Del análisis at-line al control in situ en la industria del cerdo Ibérico utilizando sensores de Espectroscopia en el Infrarrojo Cercano

TESIS DOCTORAL

para aspirar al grado de Doctor por la Universidad de Córdoba presentada por D. Juan Manuel Cáceres Nevado, Ingeniero Agrónomo y Máster en Proyectos y Gestión de Plantas Agroindustriales por la Universidad de Córdoba

El Doctorando

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Mayo 2021

Ana María Garrido Varo, Catedrática de Universidad del Departamento de Producción Animal de la Universidad de Córdoba y Emiliano Jesús De Pedro Sanz, Catedrático de Universidad del Departamento de Producción Animal de la Universidad de Córdoba

I N F O R M A N:

Que la Tesis titulada "**Del análisis *at-line* al control *in situ* en la industria del cerdo Ibérico utilizando sensores de Espectroscopia en el Infrarrojo Cercano**", de la que es autor D. Juan Manuel Cáceres Nevado, ha sido realizada bajo nuestra dirección durante los años 2018-2021; y cumple los requisitos académicos exigidos por la legislación vigente para optar al título de Doctor por la Universidad de Córdoba.

Y para que conste a los efectos oportunos firman el presente informe en Córdoba a 27 de mayo de 2021

Fdo.: Profª. Dra. Ana María Garrido Varo

Fdo.: Prof. Dr. Emiliano Jesús De Pedro Sanz

TÍTULO DE LA TESIS:

DEL ANÁLISIS *AT-LINE* AL CONTROL *IN SITU* EN LA INDUSTRIA DEL CERDO IBÉRICO UTILIZANDO SENSORES DE ESPECTROSCOPÍA EN EL INFRARROJO CERCANO

DOCTORANDO:

JUAN MANUEL CÁCERES NEVADO

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

La Tesis Doctoral cuyo título se menciona arriba se ha adaptado, desde sus inicios, a la metodología y al diseño programado, derivando todo ello en la obtención de resultados de indudable relevancia científica y tecnológica.

La investigación llevada a cabo en esta Tesis ha continuado una de las líneas emblemáticas del Grupo Ingeniería de Sistemas Agro-Ganaderos (AGR-128), relativa al potencial de la aplicación de la tecnología NIRS a los productos derivados del cerdo ibérico. Concretamente, la presente Tesis Doctoral se ha centrado en la puesta a punto de sensores NIRS para su uso en diferentes puntos de la cadena de producción y distribución de lomo de cerdo ibérico. La investigación básica y aplicada realizada confluye en la obtención de resultados de indudable relevancia científica y tecnológica.

En primer lugar, se abordó la evaluación con rigor científico, optimización y puesta a punto de un espectrofotómetro NIRS, de los denominados “transportables”, basado en tecnología de transformada de Fourier

(FT-NIR) para el control de calidad *on-line* de lomo ibérico, concretamente, la predicción del contenido de grasa intramuscular, proteína y contenido de agua en la industria transformadora. Para ello, se emplearon 227 muestras de lomo ibérico procedentes de animales sacrificados durante las campañas 2015-2016. Tal y como se recoge en el artículo publicado en *Meat Science (2019, 153: 86-93)*, las investigaciones llevadas a cabo mostraron que se obtuvo una mayor precisión y exactitud para los modelos quimiométricos desarrollados con muestras de lomo homogeneizadas frente a los obtenidos con muestras intactas debido a la heterogeneidad de las mismas, así como se comprobó que la optimización de la región espectral no suponía mejoras significativas de estos modelos en ambos modos de análisis.

En segundo lugar, se ha llevado a cabo la puesta a punto y optimización de un espectrofotómetro NIRS ultra compacto, portátil y low-cost (MicroNIR™ 1700) basado en tecnología de filtros de variables lineales (LVF), el cual tiene unas características ópticas más limitadas que el espectrofotómetro multicanal Matrix-F. El objetivo de esta investigación fue el desarrollo de modelos quimiométricos que permitiesen determinar *in situ* determinados parámetros químicos del lomo ibérico que son de gran importancia para la toma de decisiones llevadas a cabo por los genetistas en los programas de selección y mejora genética, tal y como es el contenido de grasa intramuscular. Para ello, se emplearon 524 muestras de lomo procedentes de animales sacrificados con un peso medio de 160 ± 11 kg y 14 meses de edad. Tal y como se recoge en el artículo publicado en *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy (2021, 258: 119865)*, se demuestra que los modelos quimiométricos desarrollados usando piezas intactas tienen una buena precisión para predecir el contenido intramuscular en tiempo real, evitando así la destrucción de la muestra, y permitiendo la clasificación individual de dichas piezas en función de su composición química, así como la toma de decisiones en los programas de selección y mejora genética. Por otro lado, se evaluaron algoritmos lineales y no lineales en el desarrollo de los modelos quimiométricos, comprobando que no había diferencias significativas entre ellos.

En tercer lugar, se ha evaluado el potencial de los dos instrumentos utilizados en los estudios anteriores (Matrix-F y MicroNIR™ 1700) con el objetivo de poder diferenciar espectralmente muestras de lomo frescas y muestras que han estado sometidas a congelación de forma rápida, *on-line* e *in situ*, ya que según el Reglamento (UE) 1169/2011 del Parlamento Europeo y del Consejo, de 25 de octubre de 2011, sobre la información alimentaria facilitada al consumidor, la venta de carne congelada es una práctica legal, pero el consumidor debe de ser informado de dicho tratamiento tecnológico a través de un correcto etiquetado. Para ello, se han evaluado 238 muestras de lomo ibérico pertenecientes a animales sacrificados durante las campañas 2018-2019 con un peso medio de 153 ± 11 kg. Tal y como se recoge en el artículo publicado en *Meat Science (2021, 175; 108440)*, se demuestra que los modelos cualitativos obtenidos con ambos instrumentos utilizando el algoritmo de clasificación PLS-DA muestran un "rendimiento" y precisiones similares, permitiendo una perfecta y clara distinción entre ambos tipos de muestras, lo cual es de enorme importancia para agentes de inspección de la industria agroalimentaria.

Estas investigaciones han dado lugar a tres publicaciones en revistas de alto impacto, por lo que se justifica plenamente que la forma más idónea de presentación de esta Tesis Doctoral sea el compendio de publicaciones científicas.

Asimismo, hay que destacar que el doctorando ha tenido la posibilidad de formarse en aspectos científicos-técnicos ligados a la tecnología NIRS realizando los siguientes cursos:

- Espectroscopia de Infrarrojo Cercano (NIRS). Aplicaciones en el Control de Calidad y Trazabilidad de Productos y Procesos. 17 de febrero de 2020 a 20 de marzo de 2020. Universidad de Córdoba (Córdoba, España).

- Online Course ‘Fundamentals and Applications of Near Infrared Spectroscopy’. 30 de mayo de 2020 a 30 de septiembre de 2020. Organizado por la Universidad de Córdoba y por el International Council for Near Infrared Spectroscopy (ICNIRS).

Los trabajos publicados en forma de artículos científicos relacionados con los resultados de la Tesis Doctoral son los siguientes:

1. Cáceres-Nevado, J.M., Garrido-Varo, A., De Pedro-Sanz, E., Pérez-Marín, D. 2019. Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of Iberian pork loins: Intact versus minced. Meat Science 153, 86-93. DOI: 10.1016/j.meatsci.2019.03.008. Journal Impact Factor: 3.644. Rank: 21/327, Q1, Food Science; Rank: 166/2284, Q1, Agricultural and Biological Sciences.

2. Cáceres-Nevado, J.M., Garrido-Varo, A., De Pedro-Sanz, E., Tejerina-Barrado, D., Pérez-Marín, D. 2021. Non-destructive Near Infrared Spectroscopy for the labelling of frozen Iberian pork loins. Meat Science 175, 108440. DOI: 10.1016/j.meatsci.2021.108440. Journal Impact Factor: 3.644. Rank: 21/327, Q1, Food Science; Rank: 166/2284, Q1, Agricultural and Biological Sciences.

3. Cáceres-Nevado, J.M., Garrido-Varo, A., De Pedro-Sanz, E., Pérez-Marín, D. 2021. NIR handheld miniature Spectrometer to increase the efficiency of Iberian pig selection schemes based on chemical traits. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 258, 119865, https://doi.org/10.1016/j.saa.2021.119865. Journal Impact Factor: 3.232. Rank: 7/42, Q1, Spectroscopy.

El doctorando ha participado en los siguientes congresos, simposios y conferencias:

- VII Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. Creando Redes - Investiga y Comunica. Córdoba. 6 y 7 de febrero de 2019. Presentación de la comunicación oral: "Espectrofotómetro de infrarrojo cercano por transformada de Fourier acoplado a un cabezal de fibra óptica para el control de calidad en lomo de cerdo Ibérico".

- XVIII Jornadas sobre Producción Animal, organizadas por la Asociación Interprofesional para el Desarrollo Agrario. Zaragoza, España. 7 y 8 de mayo de 2019. Presentación de la comunicación oral: "Tecnología FT-NIRS para control de calidad de lomo de cerdo Ibérico en la línea de procesado: intacto y homogeneizado".

- 19th International Council for NIR Spectroscopy Meting, NIR2019. Gold Coast, Australia. 15 a 30 de septiembre de 2019. Presentación de la comunicación escrita: "Predicting the composition of minced Iberian pig loin using a portable NIR spectrometer".

- X International Symposium of Mediterranean Pig. Florencia, Italia, 16 a 18 de octubre de 2019. Presentación de la comunicación oral: "Discrimination between fresh and frozen-thawed Iberian pig m. *Longissimus dorsi* by Near Infrared Reflectance Spectroscopy".

- Sólo Cerdo Ibérico, Octubre 2020, Nº43. Comunicación escrita: "Aplicación de la tecnología NIRS para diferenciar entre piezas frescas y congeladas de lomo de cerdo ibérico de bellota".

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, 27 de mayo de 2021

Fdo.: Profª. Dra. Ana María Garrido Varo

Fdo.: Prof. Dr. Emiliano Jesús De Pedro Sanz

A mi familia

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En primer lugar, deseo expresar mi agradecimiento, gratitud y reconocimiento a los directores de esta tesis, Dra. Ana María Garrido Varo y Dr. Emiliano Jesús de Pedro Sanz, catedrática y catedrático de Universidad del Departamento de Producción Animal de la Universidad de Córdoba, por la dedicación y apoyo que han brindado en la dirección y elaboración de este trabajo, y por su rigor y profesionalidad durante este largo, difícil y apasionante periodo de aprendizaje.

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Al Grupo de Investigación AGR-128 "Ingeniería de Sistemas de Producción Agroganaderos", del Departamento de Producción Animal de la Universidad de Córdoba; en particular, al profesor José Emilio Guerrero Ginel.

Pero un trabajo de investigación es también fruto del reconocimiento y apoyo vital que nos ofrecen las personas que nos estiman, sin el cual no tendríamos la fuerza y energía que nos anima a crecer como personas y como profesionales.

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Table of Contents

Resumen

RESUMEN

En las últimas décadas, numerosas publicaciones científicas han mostrado como un riguroso y continuo trabajo científico en las áreas de nutrición, genética, procesamiento tecnológico y, más recientemente, en el control de calidad, trazabilidad y autenticidad de las piezas cárnicas procedentes del cerdo Ibérico, han permitido ofrecer al consumidor productos de excelentes propiedades nutricionales, organolépticas y saludables. No obstante, para conseguir todos estos logros, miles de laboratorios a nivel mundial han necesitado y aún necesitan llevar a cabo análisis fisicoquímicos y sensoriales para poder confirmar que los parámetros de calidad de los productos cárnicos derivados de tales ensayos están dentro de las expectativas. Este tipo de análisis tiene dos limitaciones principales: en primer lugar, los análisis sensoriales y fisicoquímicos tradicionales son caros, destructivos y requieren de laboratorios bien equipados y altamente especializados; en segundo lugar, dichos análisis generan residuos tóxicos y requieren un elevado tiempo (normalmente días) para generar resultados.

Por tal razón, productores, especialistas en genética, nutrición y reproducción, industriales, inspectores de calidad, y como último eslabón, el propio consumidor, demandan técnicas y tecnologías que generen información fiable, rigurosa, contrastada y en tiempo real, fácil de usar y que evite la destrucción de la muestra.

La Espectroscopía de Reflectancia en el Infrarrojo Cercano (en inglés, Near Infrared Reflectance Spectroscopy, NIRS) ha sido definida por diferentes autores como una tecnología no invasiva, no contaminante, rápida, de bajo coste, versátil, que permite realizar análisis *at-line*, *on-line*, *in-line* e *in situ*, con facilidad y precisión de la medida. Estos atributos de la tecnología NIRS permiten su implementación en diferentes puntos del sector cárnico, tanto en campo, como matadero, en las salas de despiece, salas de procesado y puntos

finales de venta y consumo, permitiendo la obtención de información de parámetros relativos a la calidad, seguridad y vida útil de productos cárnicos.

Como otras tecnologías basadas en la fotónica, la instrumentación NIRS ha experimentado una fuerte evolución desde los años sesenta hasta la actualidad, existiendo hoy en día una amplia gama de instrumentos que difieren entre sí en sus características ópticas, tamaño, peso, rango espectral, coste, adecuación a diferentes puntos de implantación en la industria, operatividad y precisión, permitiendo la adaptabilidad de dicha tecnología a las necesidades reales y actuales de las industrias agroalimentarias. Sin embargo, la mayor parte de trabajos científicos se han realizado con sensores *at-line.* Por ello, son numerosos los retos científicos y tecnológicos a abordar, particularmente en el caso de productos heterogéneos y con un alto contenido de agua, como es el caso de las piezas cárnicas intactas, objeto de estudio en esta Tesis Doctoral. Entre ellos, podemos destacar: la optimización de la medida para el análisis de producto intacto en función del punto de implementación a lo largo de la cadena de producción, así como la evaluación de algoritmos lineales y no lineales y la cuantificación de la pérdida de precisión y exactitud de los modelos predictivos NIRS obtenidos con diferentes instrumentos portátiles de reciente desarrollo.

La presente Tesis Doctoral intenta contribuir al desarrollo del conocimiento sobre el potencial de sensores NIRS para el análisis *on-line e in situ* de la calidad de productos cárnicos derivados del cerdo Ibérico, en particular, en el músculo *Longissimus dorsi.* Para ello, se ha evaluado la capacidad predictiva de dos espectrofotómetros NIRS, uno basado en la Transformada de Fourier (en inglés, FT-NIR) que permite realizar mediciones *on-line*, y otro basado en la tecnología de filtros variables lineales (en inglés, LVF), diseñado para análisis *in situ*. Se han desarrollado modelos quimiométricos cuantitativos y cualitativos con colectivos muestrales constituidos por más de 300 muestras de lomo ibérico, evaluando algoritmos lineales y no lineales, así como diferentes formas de presentación de la muestra. Dichas muestras fueron suministradas por las empresas participantes en el proyecto INIA titulado "*Estudio de calidad y*

valoración económica de productos del cerdo Ibérico bajo diferentes condiciones de producción y procesado tecnológico", así como por la empresa Sánchez Romero Carvajal, Jabugo S.A.

Los resultados obtenidos en los distintos trabajos de investigación que forman parte de esta Tesis Doctoral han puesto de manifiesto el potencial de la tecnología NIRS para su incorporación *in situ* y *on-line* en el sector del cerdo Ibérico como sensor y herramienta de apoyo a la decisión, de utilidad tanto a nivel de investigación (programas de selección y mejora genética, nutrición, etc.), como para su uso por la industria para el control de productores y la toma de decisiones sobre el destino de cada pieza individual de lomo obtenida de las partidas de animales sacrificados en cada campaña. La información generada también puede ser útil para responsables de inspección en puntos de distribución y venta.

Summary

SUMMARY

In recent decades, numerous scientific papers have shown rigorous and continuous scientific work in the areas of nutrition, genetics, technological processing and, more recently, in the quality control, traceability and authenticity of the meat cuts from the Iberian pig, which have allowed to offer to consumers meat cuts of excellent nutritional, organoleptic and health properties. However, to obtain all these achievements, thousands of laboratories in the world have needed -and still need- to carry out physicochemical and sensory analysis to be able to confirm the quality parameters of these meat products. This type of analysis has two main limitations: first, traditional sensory and physicochemical analyses are expensive, destructive, and require well-equipped and highly specialized laboratories; second, these analytical procedures generate toxic waste and require a long time (usually days) to provide results.

For this reason, producers, specialist in genetics, nutrition and reproduction, industrialists, quality inspectors, and, finally, the consumer himself, demand techniques and technologies that generate reliable, rigorous, contrasted and real-time information, easy to use, avoiding sample destruction.

Near infrared Reflectance Spectroscopy (NIRS) has been defined by different authors as a non-invasive, non-polluting, fast, precise, low-cost, versatile technology that allows *at-line, on-line, in-line and on-site* analysis. These attributes of NIR technology allow its implementation in different points of the meat sector, i.e., in the field, in the slaughterhouse, in cutting and processing rooms and at final points of sale and consumption, enabling to obtain information about the parameters related with the quality, safety and shelf life of the meat products.

Like other photonics-based technologies, NIRS instrumentation has shown a strong evolution from the sixties to the present. Today there is a wide range of instruments that differ from each other in their optical characteristics,

size, weight, spectral range, cost, adaptation to different points of implementation in the industry, operability and precision, allowing an adaptability of NIR technology to the real and current needs of the different agri-food industries. However, most scientific works have been done with *at-line* sensors. For this reason, there are still numerous scientific and technological challenges to be addressed, particularly in the case of heterogeneous products with high moisture content, such as intact meat pieces, the object of study of this Doctoral Thesis. Among them, it can be highlighted: the optimization of the measurement for the analysis of intact product, taking into account the point of implementation along the production chain or the evaluation of linear and non-linear algorithms and the quantification of the loss of accuracy of NIR predictive models obtained with the recently developed portable NIR instruments.

This Doctoral Thesis tries to contribute to the development of knowledge about the potential of NIRS sensors for the *on-line* and *on-site* analysis of the quality of Iberian pig meat products, in particular, in the *Longissimus* dorsi muscle. To do this, the predictive capacity of two NIR spectrophotometers, one based on Fourier Transform (FT-NIR), which allows *on-line* measurements, and other based on variable filter technology (LVF), designed for *on*-site analysis, have been evaluated. Quantitative and qualitative chemometric models, with samples of more than 300 Iberian loin samples, were developed evaluating linear and non-linear algorithms, as well as different ways of spectra collecting (mode of analysis). The samples were supplied by Sánchez Romero Carvajal, Jabugo S.A. and by other companies participating in the INIA project entitled "*Study of quality and economic evaluation of Iberian pig products under different conditions of production and technological processing*".

The results obtained in the different research works that are part of this Doctoral Thesis have revealed the potential of NIRS technology for its *on-line* and *on-site* implementation in the Iberian pig sector, as a sensor and decision support tool, useful, both at the research level (genetic selection programs, nutrition, etc.), as well as for use by the industry to control producers and make

decisions about the destination of each individual piece of loin obtained in each campaign. The information generated can also be useful for inspection managers at distribution and sales points.

CHAPTER I:

INTRODUCTION

Chapter I. INTRODUCTION

The pig come from the archaic Mediterranean pig (*Sus mediterraneous* or *Sus scrofa*) is called Iberian pig, which is found mainly in the central and southern geographical areas of the Iberian Peninsula, that is, Extremadura, Andalusia and the Portuguese Alentejo. Throughout this geography, it can be found different varieties of the Iberian pig, among which it must be highlighted the following: Lampiño, Entrepelado, Mamellado, Dorado, Gaditano, Torbiscal, Retinto and Manchado de Jabugo (Dieguez, 2000). Currently, the most widespread is the Extremaduran Retinto with its Silvela, Villalón and Valdesequera lines, and the Entrepelado (Dieguez, 2001).

This breed is characterized by the presence of animals with high rusticity, a consequence of their adaptation to the pasture ecosystem (dehesa), low or no susceptibility to stress, a tendency to accumulate fat and a high level of veining of the muscles (Zuzuarregui, 1976).

Analysing the evolution of the sector, the number of heads of Iberian pigs has suffered strong fluctuations throughout history. Around the 60s, the Iberian sector suffered a sharp decline as consequence of the entry into the market of white pig reared in intensive farms, reducing the profitability of Iberian products, the introduction of new crosses, rural depopulation, and the abandonment of the field, as well as a consequence derived from the African Swine Fever. According to the official MAPA censuses, pure Iberian pig females in those years represented around of 40% of the pig herd, barely reaching 4% at the beginning of the 1980s. However, from the 1980s on, the sector boomed as a result of a trend that promoted the quality of Iberian products, which led to an increase in profitability and consumption and, consequently, in the profitability of its production.

During the last decade, from 2010 to 2019, in the Spanish geography there has been an increase of 33% of heads of Iberian pigs, going from 2,536,564 animals in 2010 to 3,375,281 in 2019. In this last year, they were registered 1,870,909 pigs of 50 of more kg of live weight, and a total of 347,664 pure Iberian pig females, of which 52% were covered. Currently, the Iberian pig herd represents around 10% of the national pig herd (MAPA, 2019).

In terms of consumption, the Iberian pig went from being a supplier of fresh meat and cured products until 1960s, to being relegated to the production of high-quality cured products. Changes in the purchasing power of the consumer, and the enhancement of the nutritional quality of their meats, have brought with an increase in the consumption of fresh Iberian pork meat in recent years, highlighting among these meat cuts the secret, the prey, the sirloin, and the loin (Ortiz et al., 2020, 2021).

The improvement of the production was based on genetics and nutrition, fundamental parameters on the quality of Iberian products (Fernández et al, 1999), which caused confusion about the product quality concept. This situation led to the elaboration and establishment of legal guidelines that would regulate this production and its commercialization, as well as the industrial processes of the product elaboration, the third most important parameter of quality (Fernández et al., 1999), giving rise to the Standard of Quality for the meat, ham, shoulder, and cane of Iberian loin (Real Decreto 4/2014).

This standard was initially published in 2001 and, after several modifications, the one published on January 10, 2014, is currently in force, and has two main objectives. First, to preserve and maintain the Iberian breed and, secondly, to ensure the correct and fair competition in the market, as well as to defend the rights of consumers. For this reason, respect to the previous standard, it highlights the need to improve the racial purity of the animals, for which establishes that reproducers must be registered in the Genealogical Book of the

Iberian Pig Breed, intensifies the management conditions and feeding the animals and reduces the authorized livestock load, requires the registration of the "dehesa" in the Geographic Information System of Agricultural Parcels (SIGPAC) in the "acorn" animals and has improved the traceability and control system with the implantation of the coloured labels with the aim of helping to the consumer to differentiate the different products categories. Besides, the monitoring measures of compliance with quality standard have been strengthened, stricter and clearer requirements have been established in relation to the labelling of products such as the compulsory mention of the genetic percentage of the Iberian pig breed (Real Decreto 1083/2001; 144/2003; 1781/2001; 1079/2008; 4/2014).

Regarding the sale of products, it is mandatory to highlight three designations: designation by product type, whether they are made or sold as fresh product; designation by feeding and production system: "bellota", "cebo de campo" or "cebo"; and racial designation: 100% Iberian, or Iberian, which can be 75% or 50%, depending on the genetics of the male. It will be 75% if it comes from a 100% Iberian female registered in the herd book and male from 100% Iberian mother and a 100% Duroc father, both registered in the herd book. On the other hand, it will be 50% Iberian when they come from 100% Iberian females and 100% Duroc males, both registered in the herd book (Real Decreto 4/2014).

The sensory quality of Iberian pig products is mainly influenced by the presence of intramuscular fat in these products, i.e., the adipogenic character of this breed plays a fundamental role on this parameter (López-Bote, 1998; Ventanas et al., 2005). Due to this, it is of great importance to know the intramuscular fat content of the different meat cuts of the Iberian pig, as well as the changes in its content and composition throughout the processing of these products, since it has a fundamental role in the different stages of technological

processing of these products and, therefore, in their sensory and nutritional characteristics (Ventanas, 2001; Ruíz & López Bote, 2002).

From the point of view of technological quality, the intramuscular fat content is of great importance since it determines the degree of penetration into the muscle of the salt, additives and/or spices using during processing, as well as the level of product desiccation and, as a consequence, its maturation time (Palumbo et al., 1977). The content and composition of intramuscular lipids are related to sensory attributes such as juiciness (Wood et al., 1986), aroma and flavour (Mottram & Edwards, 1983; Cameron et al., 1990; Cameron & Enser, 1991), which are precisely the most important attributes in the Iberian products (Ruiz et al., 2000; Ruiz et al., 2002). On the other hand, this parameter is of great importance in order to develop and implement breeding selection schemes focused on the Iberian purebred products, labelled as "Bellota" (Muñoz et al., 2020).

One of the sensory attributes related to the appearance of products derived from Iberian pigs is the brightness of the cut surface, a direct consequence of the intramuscular fat content and its fluidity. Another of the most characteristics sensory attributes of these products is the high degree of marbling (Ruiz et al., 2000). These authors also highlight another sensory attribute influenced by the intramuscular fat content, such as juiciness, which has a greater influence on the acceptability of Iberian pork products by consumers.

For several years, producers, industrials and consumers have demanded more information about products that reach the market, which have very high prices. However, official methods such as gas chromatography are slow, destructive and very expensive. For this reason, it is necessary to implement and consider other methods for the innovation in the quality control and authentication systems to substitute or complement the gas chromatography (Order PRE/3844/2004), currently considered an official technique.

These methods must contemplate reliable, objective, fast, low-cost and, fundamentally, non-destructive analysis techniques, providing confidence to consumers. In addition, another of the intended objectives with these new techniques is to identify individual quality, which would allow to pay per registered animal and not based on the average results of the batches of animals. These new systems must allow to control the quality of the raw materials that are going to be used by the processing industry, in order to be able to make real time and efficiently decisions, certifying the quality and generating added value that ensure consumer confidence (Pérez-Marín et al., 2007, 2009, 2021; Solís et al., 2001; Zamora-Rojas et al., 2011, 2012, 2013).

There are several studies and research works that support the viability of Near Infrared Reflectance Spectroscopy (NIRS) for the quality control needed in the Iberian pig sector. However, most of these studies have been carried out with laboratory instrumentations, that is, outside the processing line (De Pedro et al., 1992; García-Olmo, Garrido & De Pedro, 1998; Garrido & De Pedro, 2007; González-Martín et al., 2002; Hervás et al., 1994; Martínez et al., 1998; Ortiz-Somovilla et al., 2007; Pérez-Marín et al., 2007, 2008; Solís et al., 2001; Zamora-Rojas et al., 2011). For this reason, and due to the demand of the sector and the evolution and advances of NIRS instrumentation, it is possible to think about implementing this technology in the processing line, allowing to control the products *on-line* and *in situ* with adapted equipment to the industry and with small portable devices. Currently, there are already some studies carried out with Iberian pigs products using portable instruments based on the analysis of adipose tissue using MEMS (micro-electromechanical-system), LVF (linear variable filter) and post-dispersive diode array technology (Pérez-Marín et al., 2009, 2021; Zamora-Rojas et al., 2012, 2013), but there are no studies about the implementation of NIRS technology for *on-line* and *on-site* quality control of Iberian loin through the use of portable equipment using LVF technology, also called "transportable instruments" (Crocombe, 2018) or small version of laboratory analysis, but with ability suited to be used for *on-site* analysis. To that

second group belong the FT-NIRS multiplexed instruments based on Fourier Transform.

Given the wide diversity in terms of characteristics and benefits of these new NIRS instruments, it is necessary and advisable to evaluate them in order to determine which of them could be more suitable for a specific application. In addition, the on-line incorporation of NIR sensors requires a detailed methodology of analysis, which includes all aspects related to sampling (taking spectra), as well as the selection of the optimal spectral region, integration time, etc.

The Department of Animal Production from the Faculty of Agriculture and Forestry Engineering (University of Cordoba, Spain), and more specifically, the Research Group "Agro-Livestock Production Systems Engineering" (AGR-128, ISPA), has been carrying out, since the 90s, an important and outstanding research activity in the quality control of agri-food products and processes, an in particular research on the high quality products derived from the Iberian pig.

Scientific research carried out with Iberian pig products has focused primarily on demonstrating the potential of NIRS technology for the determination of chemical parameters such as the percentage of intramuscular fat, water, and protein in minced meat samples, as well as the determination of the fatty acid profile in subcutaneous adipose tissue using bench top devices, which have high scientific-technical performance, but they are very expensive and are not adapted to *on-line* or *in situ* analysis.

As a consequence, this thesis addresses the development and evaluation of strategies and methodologies that allow to develop, evaluate, and implement NIRS calibrations, based on extensive databases, for the quality control of Iberian loin using an on-line NIRS device, Matrix-F (Bruker Optics GmbH, Ettlingen, Germany), and an ultra-light-compact portable microspectrometer,

MicroNIRTM 1700 (VIAVI Solutions, Inc., San Jose, California, USA), which allows the *in situ* analysis.

Due to the fact that the main objective of this PhD dissertation is the generation of new scientific knowledge for the Iberian pig sector and to enable them to make decision in real time for guaranteeing the control of certain quality parameters and offering a secure certification system, and with the object of facilitating its dissemination, the results here obtained are shown as a compendium of research articles published in indexed scientific journals.

In order to facilitate the reading, this PhD dissertation has been divided in different chapters:

- In Chapter I, a general introduction to the different research papers of this PhD dissertation is written.
- In Chapter II, the objectives of this PhD dissertation are clarified and exposed.
- In Chapter III, instrumental comparison to predict, on-line and *in-situ*, the quality parameters in Iberian pork loins using a new generation of NIRS sensors is studied.
- In Chapter IV, instrumental comparison for the labelling of frozen Iberian pork loins is carried out.
- In Chapter V, the conclusions of this PhD dissertation are written.
- In Chapter VI, recommendations for further research are exposed.
- In Chapter VII, references used are included.

CHAPTER II:

OBJECTIVES

Chapter II. OBJECTIVES

2.1. General objective

The general objective of this PhD dissertation is to design, develop, evaluate, and optimize methodologies for quality control products and process on-line and *in-situ* in the Iberian pig processing industry through the use of sensors based on NIRS technology.

2.2. Specific objectives

The specific objectives of this PhD dissertation are:

1. Development and validation of methodologies to obtain robust NIRS predictive models from extensive databases of pork meat products obtained over several years using handheld portable and on-line NIRS sensors for real-time decision-making and on-vine monitoring. [*This objective was reached in the research articles: 'Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of IBERIAN pork loins: Intact and minced'. Meat Science 153, 86–93 (2019); 'NIR handheld miniature spectrometer to increase the efficiency of Iberian pig selection schemes based on chemical traits'. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 258, 119865 (2021)*].

2. Evaluation and optimization of portable and handheld NIRS spectrometers for its implementation on-line and *in-situ* in the quality control and authentication of Iberian pig products. [*This objective was reached in the following research article*: *'Non-destructive Near Infrared'. Meat Science 175, 108440 (2021)*].

CHAPTER III:

INSTRUMENTAL COMPARISON TO PREDICT, ON-LINE AND *IN-SITU*, THE QUALITY TRAITS IN IBERIAN PORK LOINS USING A NEW GENERATION OF NIRS SENSORS

3.1. Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control IBERIAN pork loins: Intact versus minced. Meat Science 153, 86–93 (2019)

Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of Iberian pork loins: Intact versus minced

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Abstract

Conventional chemical analyses of meat products are time-consuming, expensive, and destructive. The advantages of NIR spectroscopy are its speed, portability, suitability for both at-line and on-line analysis, low cost, and the possibility of simultaneously measuring many different parameters in a large number of samples. The purpose of this study was to develop and validate calibrations for the prediction of moisture, protein and fat in Iberian pig pork loins using an FT-NIR instrument coupled to a 5-metre fibre optic sensor head. The best equations obtained for intact loin in both modes of analysis (full and optimal spectral range) displayed Standard Error of Cross-Validation (RMSECV) of 1.06% and 1.09% and Determination Coefficient of Cross-Validation (RCV2) of 0.69 and 0.77 for fat: RMSECV of 0.87% and 0.77% and RCV2 of 0.67 and 0.73 for moisture; while for protein, the RMSECV values were 0.51% and 0.49% and the RCV2 values were 0.66 and 0.70.

Keywords: Iberian pig loin; FT-NIR spectroscopy; fibre optic probe; intact and minced meat analysis; chemical composition.

1. Introduction

Iberian pig (IP) products elicit a considerable degree of rate consumer demand, leading to high prices in the market. Consumers assess the quality of these products based on their exceptional organoleptic, health and sensory characteristics, but guarantying the quality meat products is a complex task that requires control procedures. Fresh meat is a very heterogeneous product and the determination of the major chemical constituents, such as fat, moisture and protein, is important for labelling purposes and for preparing good mixtures to produce dry-cured and processed products (Garrido & De Pedro, 2007; Prieto et al., 2009).

Traditional wet chemistry to determine these parameters is time-consuming, tedious, costly and destructive (Zamora-Rojas et al., 2011).

Near-Infrared Spectroscopy (NIRS) has demonstrated its ability to determine the chemical composition of raw meat and meat products. Numerous studies have reported the successful use of NIRS for the at-line prediction of the fat, moisture or/and protein content (Prevolnik, Candek-Potokar & Skorjanc, 2004; Prieto, et al., 2009; Tejerina, López-Parra & García-Torres, 2009) of meat products. A number of studies have found that NIRS technology is specifically applicable to this type of analysis in Iberian pork products (De Pedro et al., 1992; Hervás et al., 1994; García-Olmo, J., Garrido, A., & De Pedro, E. 1998; Martínez et al., 1998; Solís et al., 2001; González-Martín et al., 2002; Garrido & De Pedro, 2007; Ortiz et al., 2004; Pérez-Aparicio et al., 2004; Ortiz-Somovilla et al., 2007; Pérez-Marín et al., 2007, 2008, 2009; Zamora-Rojas et al., 2012, 2013a, 2013b). However, most of the research has been conducted on adipose tissue or using minced cuts of meat (e.g., loins). IP pork loins are relatively expensive pieces of meat, and the meat industry consequently requires technologies that are able to analysis of intact pieces, which later will be processed into cured loin.

To obtain clear conclusions about the viability of using NIR for the analysis of intact meat, avoiding recourse to mincing of meat samples, it is necessary to develop robust models with a large number of samples exhibiting considerable

variability. This however is an expensive research undertaking, because the project needs to assume the cost of a high number of these expensive IP loin pieces. Previous publications related to the development of NIRS equations for the analysis of intact pork loins have therefore used a low number of samples and have not been properly validated (Barlocco et al., 2006; Fan et al., 2018; Solís et al., 2001). Other research used very thick slice samples (Hu et al., 2008), or fibre optic equipment with small windows and limited length, making its on-line application difficult (Fan et al., 2018; Pérez-Marín et al., 2008).

NIRS instrumentation has evolved enormously in recent years (Ropodi, Panagou & Nychas, 2016). These days it is possible to extend its on-line use to process control, using instruments adapted to the critical conditions prevailing in the industrial environment and capable of taking measurements at various points of the process. There is, however, no rigorous scientific regarding the potential for using instruments that have recently appeared on the market for the on-line control of intact pork loin. Such instruments include equipment based on FT-NIRS (Fourier Transform-Near-Infrared Reflectance Spectroscopy) technology. Some of these new devices provide multiplexed option i.e., they enable to take measurements in several points at the same time with different accessories, which can be connected via fibre optic probes (up to 100 m length).

The development and enhancement of NIRS sensors for the quality control of intact loins is therefore still in need of research investigation to evaluate and demonstrate the added value of the various equipment appearing on the market, as well as to provide the advice to those industries wishing to acquire this type of instrument.

The aim of the present study was to evaluate an FT-NIRS instrument coupled to a 5-metre fibre optic sensor head for determining the chemical composition of intact pieces of Iberian pig loin, which was analysed both intact and minced. A secondary goal of the study was to evaluate the influence of the optimum spectral range on the predictive ability of the equations developed.

2. Material and methods

2.1. Sample set

A total of 277 samples of Iberian pig loin belonging to animals reared during 2015 and 2016 provided by Sanchez Romero Carvajal Jabugo S.A. were used in this study. The animals were slaughtered at the age of 14 months with an average weight of 160 kg.

The samples were refrigerated and stored at -20 ºC. Prior to analysis they were defrosted and tempered until they reached 19-20 ºC.

2.2. Reference analysis

Reference values for fat, moisture and protein content were determined on ground samples in accordance with the official meat analysis methods. Fat content was determined following the Soxhlet procedure (ISO-R-1443). Total protein was determined by the Kjeldahl method (ISO-R-937) and moisture content was measured by lyophilisation, using FTS SYSTEMS equipment, at -50 ºC for 48 hours.

2.3. NIRS measurements

Spectra were collected using a FT-NIR spectrometer (MATRIX-F, Bruker Optics, MA, USA), working in reflectance mode in the spectral range 834.2-2502.6 nm, with a constant interval of 1.074312 nm.

This instrument was interfaced to a fibre optic NIR illumination and detection head (Q412) containing tungsten sources for illuminating the sample. The head requires environmental conditions of 5-40ºC. In this study, the instrument was in a room with a controlled temperature of 21ºC. The detector has a diameter of 10 mm. The scattered light is collected and guided via a fibre optic cable (5 m length; 0.6mm core diameter) to the spectrometer. This fibre optic cable has two SMA connectors, low OH quartz in gel-filled, gas-tight, and electrically conductive PE protective hose.

The sample is located 10 cm away from the head, which allows measuring a sample area of around 38.46 cm^2. The samples had approximately 2 cm

thickness. Two sample presentation modes were evaluated for the analysis of loin samples: intact and minced loin. In intact mode, two spectra per sample were collected, one for each side of the loin. These samples were subsequently minced using a homogeniser "Heidolph Diax 900" and two spectra were collected from each sample.

Spectral data were recorded using OPUS 7.0.122 software (Bruker Optics GmbH, Ettlingen, Germany), selecting 32 scans per spectra at a speed of 10 kHz with a resolution of 16 cm^{-1}. These parameters were previously stablished in other studies carried out using the same instrument (Garrido et al., 2018).

2.4. Data pre-treatment

Data processing was performed using two software packages: WinISI package ver. 1.5 (Infrasoft International, Port Matilda, PA, USA) for performing spectral repeatability analysis, principal component analysis and selecting calibration and validation sets; and OPUS 7.0 (Bruker Optik GmbH, Ettlingen, Germany) for calibration development.

2.4.1.Principal component analysis and detection of spectral outliers

Prior to the development of NIR calibrations, the structure and spectral variability of the sample population were determined following the method recommended by Shenk & Westerhaus (1991), using the CENTER algorithm included in the WinISI II software package, version 1.50 (Infrasoft International, Port Matilda, PA, USA). This algorithm performs a principal components analysis (PCA), reducing the original spectral information to a small number of linearity-independent variables, which facilitate the calculation of spectral distances. This spectral information allowed to calculate the centre of sample population and the distance of each sample from that centre (Mahalanobis 'GH' distance). Samples with a GH value greater than 3 were considered spectral outliers (Shenk & Westerhaus, 1996).

The spectra were pre-treated using Standard Normal Variate (SNV) and Detrending (DT) (Barnes, Dhanoa, & Lister, 1989) algorithms to remove the multiplicative interferences of scatter. In addition, four derivate mathematical

treatments were performed: (1,5,5,1), (1,10,5,1), (2,5,5,1) and (2,10,10,1) where the first digit is the order of the derivate, the second is the gap over which the derivate is calculated, the third is the number of data points in a running average or smoothing, and the four is the second smoothing (ISI, 2000).

2.4.2.Definition of calibration and validation sets

Samples for the calibration and validation sets were selected in accordance with Shenk & Westerhaus (1991), using the methodology described above. Thus, the samples were ordered based on the Mahalanobis distance to the centre of the population using the intact loin set, since this set had a larger spectral variability than the minced set. One out of every three samples were then selected to be part of the validation set (S^1), and the remaining samples were reserved for calibration (S^0). For both analysis modes (intact and minced), the calibration (N = 185) and validation (N = 92) sets were finally made up of the same samples.

2.5. Calibration development and validation

Calibration was carried out using the OPUS 7.0 software (Bruker Optik GmbH, Ettlingen, Germany). Prediction models were developed for determining fat, moisture and protein content in IP loins.

OPUS 7.0 enables calibrations to be developed using the full spectral range or an optimum spectral range for each application.

The optimisation (using the QUANT algorithm include in OPUS software) was run automatically by trying different combinations of predefined wavelength regions and data pre-processing methods. The result of the optimisation process shows the rank (number of PLS vectors) and RMSECV (Root Mean Square Errors of Cross Validation) value for each combination of predefined wavelength regions and data pre-processing methods. The combination of RMSECV and number of latent variables (LVs) was the criteria chosen to develop the final calibration model. In this paper, both options, the full range and the selected spectral range, are compared.

In addition, various signal pre-treatments were evaluated: no scatter correction, Multiplicative Scatter Correction (MSC) and Standard Normal

Variate (SNV), together with first and second derivatives (1, 5), (1, 9), (2, 9), where the first digit is the order of the derivate, the second is the number of the smoothing points.

To recognise spectral outliers, the squared spectral residual is compared with the mean value of all others by calculating the *FValue.* Spectra poorly represented by the PLS vectors have a high *FValue.*

$$FValue_i = \frac{(M-1)(SpecRes_i)^2}{\sum_{j\neq 1}\left(SpecRes_j\right)^2} \quad (1)$$

where the spectral residual "*SpecRes"* is calculated by a summation over all selected frequency points of the difference spectrum:

$$SpecRes = \sqrt{\sum (x_i - s_i)^2} \quad (2)$$

"Chemical outliers" can be detected automatically as those samples for which the difference between the true concentration determined by the reference method and the predicted concentration is large and statistically significant. *FProb* indicates the probability that a sample is a chemical outlier, considering outliers samples with Fprob values around 1 (OPUS, 2004). In this case an *FValue is calculated:*

$$FValue_i = \frac{(M-1)(Differ_i)^2}{\sum_{j\neq 1}\left(Differ_j\right)^2} \quad (3)$$

$$FProb_i = \frac{\int_0^{FValue} f(FValue)d(FValue)}{\int_0^{\infty} f(FValue)d(FValue)} \quad (4)$$

The following statistics were used to select the best equations: Root Mean Square Error of Estimation (RMSEE), Root Mean Square Error of Cross Validation (RMSECV), and Determination Coefficient of Cross Validation (R^2_{CV}). The other statistic used was the Residual Predictive Deviation (RPD_{CV}) (Williams & Sobering, 1996), calculated as the ratio between the standard deviation of the reference data for the calibration set and the Root Mean Square Error of Cross Validation (RMSECV).

The RMSEP(C) were compared using the method proposed by Roggo et al., (2002). This method is based on a Fisher test and defines a confidence interval for errors with non-significant differences with minimum error obtained ($Error_{min}$):

$$\left(Error_{min}, Error_{min}\sqrt{F_{critical(1-\propto,n-1,n-1)}}\right) \quad (5)$$

where α is the significance level (5% in this study) and (n-1) the degrees of freedom.

According to Roggo et al., (2002), a model that has a RMSEP(C) (RMSEP bias corrected) value between $RMSEP(C)_{min}$ (the smallest RMSEP) and the RMSEP confidence limit $(SEP(C)_{min} \cdot F_{critical})$ can be considered as not significantly different.

The best models were subjected to evaluation using samples not involved in the calibration procedure. A validation set (S^1) composed of 92 samples, not used previously for the model development, was evaluated.

For the validation of the calibration models, the protocol proposed by Windham, Mertens, & Barton, (1989) was followed. The procedure consists essentially in determining the possible existence of a significant known error, or bias, and an unexplained error, termed the Root Mean Square Error of Prediction Corrected for bias or RMSEP(C). Both errors contribute to the RMSEP value. For calibration sets comprising 100 or more samples and validation sets composed of nine or more samples, Shenk, Workham & Westerhaus (2001) assume the following limit control: RMSEP should not exceed 1.30 times the RMSEE and Bias should not exceed 0.6 times the RMSEE. The Determination Coefficient of Prediction (R_P^2) should be higher than 0.6, and the slope should be between 0.9-1.1. These statistics and RPD_P values calculated from the RMSEP were used to evaluate the predictive ability of the models generated. The best model was selected according to the highest R_P^2 and RPD values and the lowest RMSEP.

$$RMSECV = \sqrt{\frac{1}{M} \cdot \sum_{i=1}^{M} (Differ_i)^2} \quad (6)$$

$$RMSEE = RMSEC = \sqrt{\frac{1}{M-R-1} \cdot \sum_{i=1}^{M} (Res_i)^2} \quad (7)$$

$$\mathrm{RMSEP} = \sqrt{\frac{1}{\mathrm{M}} \cdot \sum (\mathrm{Differ_i})^2} \quad (8)$$

$$\mathrm{Bias} = \frac{\sum_{\mathrm{i}} \mathrm{Differ_i}}{\mathrm{M}} \quad (9)$$

$$\mathrm{SEP} = \sqrt{\frac{\sum_{\mathrm{i}} (\mathrm{Differ_i} - \mathrm{Bias})^2}{\mathrm{M} - 1}} \quad (10)$$

In these equations, R is the number of PLS factors of the model and M is the number of samples.

3. Results and discussions

3.1. Spectrum interpretation

Figure 1 shows the mean spectra, as log (1/R), for both modes of analysis. It is evident that the absorbance of intact samples is higher than that of minced samples. This fact can be explained by the disruption of the meat structure when the sample is minced, which interferes with the light absorbance (Cozzolino et al., 2000; Fan et al., 2018). Meat NIR spectra present broader peaks, making band assignment more difficult than for other agri-food products. However, it is clear that the spectra are dominated by strong absorptions due to fat (1190 nm), protein (1550 nm), water (1900 nm) and protein and fat (2220 – 2350 nm) (Murray, 1986; Cozzolino et al., 2002). To overcome this issue, Figure 2 shows the pre-treated spectra of intact and minced meat to remove the scatter effects, enhancing differences due to chemical composition. As can be observed, clear absorption valleys and peaks are now present.

3.2. Population structure and detection of spectra outliers

After studying the population structure of the initial set made up of 277 samples using PCA using the CENTER algorithm, no samples were found with a Mahalanobis distance higher than 3. Thus, no outliers were detected.

3.3. Calibration development and validation

After the application of the CENTER algorithm, the calibration and validation sets were selected.

The selection of the most representative samples based on the Mahalanobis distance as described in section 2.4.2, yielded a calibration set (S^0 = 185 samples) and a validation set (S^1 = 92 samples) with similar means and standard deviations for all the chemical parameters studied (Table 1). This indicates that spectral data *per se* are very useful for selecting well-structured training and test sets.

Once the calibration and validation sets were established, the prediction models were developed for both analysis modes (intact and minced loin), employing two approaches to optimise the results: the full spectral range of the FT-NIRS spectrometer and using the recommended spectral range by running the OPUS QUANT algorithm included in the OPUS software.

Firstly, a comparison of signal pre-treatments with the same number of calibration sample (without removing outliers) was done. After that, the best pre-treatment was optimized removing chemical outliers. Tables 2, 3, 4 and 5 show the statistics of cross validation for the best equation developed for each parameter and analysis mode. While pre-treatments seem to be of importance in calibration development, the statistical significance of the improvements obtained in the prediction ability of NIRS equations, is often not established (Fernández-Cabanás et al., 2007). Moreover, most published works conclude with the use of a specific pre-treatment based on the minimum values obtained for the root mean square error of cross validation (RMSECV) or root mean square error of prediction (RMSEP). This way of obtaining conclusions about the optimum pre-treatment of the spectral data involves a risk, mainly when other factors affecting the final RMSECV or RMSEP values may have influenced the

results (e.g., outliers samples removed from the calibration set). Several authors (Fearn, 2001; Roggo et al., 2002*;* Fernandez Cabanas et al., 2007; Pérez-Marín, et al., 2008) have suggested the use of statistical tests to compare RMSECVs and RMSEPs.

Fernández-Cabanás et al., (2007) concluded that the method proposed by Fearn (2001) and Roggo et al., (2002) produces similar results. In this paper, the Roggo et al., (2002) procedure was applied to ascertain whether there were statistical differences between equations developed with several math pre-treatments.

The results of the Fisher's F-test (Table 6), comparing mathematical pre-treatments, both in intact and minced loin and for both calibration approaches (full and optimal range), reveal that F_{values} are inferior to $F_{critic\text{-}values}$ ($p > 0.05$) for the three parameters studied. These results provide grounds to conclude that the different mathematical pre-treatments applied provide similar results.

The same test was also applied to ascertain whether there were differences between the two calibration strategies evaluated, i.e., the one using the full range (831-2502 nm) and the one using spectral regions as recommended when using the QUANT algorithm. The results of the Fisher's F-test provided lower F_{values} than $F_{critic\text{-}values}$ ($p > 0.05$) for range approaches for intact and minced loins. This finding demonstrates that there are no statistically significant differences between the two calibration approaches. This result is of enormous practical importance, since QUANT is designed to calibrate by optimising the spectral regions. However, this calibration option involves a processing time to obtain calibrations that is much higher than the one proposed in this paper, using a single region. By carefully looking at the most frequently selected regions for each analytical parameter and each mode of analysis, it is not possible to conclude that there are reduced regions that lead one to think about the possibility of using instruments of lower spectral range, since they cover practically the full spectral range. However, it is noted that, in general, the water region is omitted when this selection is done.

In accordance with the above results and considering the RPD values, the best equations for intact and minced loins are shown in Tables 2, 3, 4 and 5.

The coefficients of determination of cross-validation (R^2_{CV}) of the best equations (see Table 4 and Table 5) were excellent for minced samples (above 0.9) and around 0.7 for all parameters in intact loin (Table 2 and Table 3), which would only allow adequate discrimination between samples of high, medium and low content in the tested parameter (Shenk & Westerhaus, 1991).

As expected, and as mentioned by other authors (Solís et al., 2001; Barlocco et al., 2006; Hu et al., 2008; Fan et al., 2018;), the calibration equations for intact loin have a lower predictive ability than those obtained with minced loins.

In fact, for intact loins, the best values of the RPD_{CV} for all the analytical parameters studied are around 2. These values are lower than that recommended (RPD > 3) by Williams & Sobering (1996). For minced loin however, regardless of the chemical parameters evaluated, the RPD_{CV} values are much higher than the minimum value established by Williams & Sobering (1996). Nevertheless, as indicated by Fearn (2014), the RPD statistic is highly correlated to the R^2 since its calculation can be done by using the formula $1/(1-R^2)$, both being highly dependent on the range of the data in the calibration. For this reason, Fearn (2014) argues that it is probably not the best statistic to employ when comparing calibrations for the same analyte across different experiments. This view is borne out by the results obtained here (Tables 2, 3, 4 and 5), which indicate a close match between the highest and lowest RPD values in the two analysis modes and the highest and lowest R^2 values for the respective equations.

The RPD statistic is widely used in NIRS research for assessing the efficiency of NIRS predictions. Williams (2001) suggests that RPD values of between 3 and 5 indicate acceptable efficiency. Other research however, (Vega, 2013), suggest that this criterion cannot be generalised to all types of product or all NIRS instruments; it was obtained using equations for NIRS analysis of finely-ground samples of grains (mainly wheat), using a high-performance laboratory monochromator. Esbensen, H.K., Geladi, P., & Larsen, A. (2014) have recently argued that RPD values depend on the kind of sample, on its prior

preparation and on the way, it is presented to the instrument. According to this approach, the RPD values obtained for intact samples are smaller than for minced samples. Intact muscles have different levels of organization (muscle fibres) and characteristics (chemical and physical) due to a diverse range of factors (e.g., myofibrillar birefringence, myoglobin and protein precipitation in the sarcoplasm, macroscopic surface reflectance properties of cut meat, sarcomere length, pH, intramuscular fat, protein coagulation surface, and moisture content) (Cozzolino et al., 2000; Cozzolino & Murray, 2002).

The results obtained for minced samples in this work are quite similar to those obtained by Solís et al., (2001), Barlocco et al., (2006) and González-Martín et al., (2002).

As mentioned above, a certain amount of work has been done using intact loin samples. Hu et al., (2008) obtained RMSEE and RPD values of 0.4% and 2.61 for intramuscular fat; RMSEE of 0.51% and RPD of 1.95 for moisture; while for protein, the RMSEE and RPD values were 0.25% and 3.38 respectively. The slightly better results obtained by Hu et al., (2008) compared to those obtained in the present paper could be attributable to the characteristics of the instrument they used, a monochromator, suitable for lab conditions but not for on-line analysis. Fan et al., (2018), using a small set of intact meat (calibration n = 70 and validation n = 24) obtained RMSEE and RMSEP values of 0.124 and 0.528 for intramuscular fact. It is clear that the equations were over fitted, causing the RMSEP value to be four times the RMSEE. Barlocco et al., (2006), in a study of intact muscle samples, obtained poorer PLS calibration models for IMF (Intramuscular fat) and moisture ($R^2 < 0.70$).

The last step in the calibration development process is to validate the equations with an external set of samples. Evaluation of the best models for each parameter was performed using the validation set (S^1) and is shown in Fig. 3-4. The validation statistics for intact loins fulfil the limits established by Windham et al., (1989) and Shenk et al., (2001), in the case of the bias and the RMSEP(C), but not for the R_P^2 or slope values (Figure 3). The values of R_P^2 for fat and protein range between 0.5 and 0.6. These statistics show that the equation for these

parameters can only be used to discriminate samples with different fat and protein content, not to provide an accurate prediction of the percentage of fat and protein. In the case of moisture, the R_P^2 value is lower than 0.5. This low R_P^2 value and the dispersion of samples for moisture content (Figure 3.B) shows a low correlation between the values predicted by NIRS and the reference values. This may be attributable to the analysis procedure, i.e., from the time the sample is analysed in intact mode to the analysis of the sample by the reference method and minced there is a period of time, in which the sample is processed (minced, stored, etc.), and it is clear that there are changes or losses in the moisture content that affect the precision of the prediction models developed. This loss of moisture is reflected in the values of the statistics mentioned above.

In fact, this problem does not appear in the case of minced loins, for which all the statistics comply with the established limits (Figure 4).

4. Conclusions

The calibration equations for intact loin have shown a lower predictive ability than those obtained with minced loins. However, the equations have potential use for classifying intact pieces of loin into high, low and medium values according to their composition in terms of moisture, fat and protein. This would allow the industrial sector to make real-time decisions about the subsequent use of these highly-prized pieces of meat.

It has been observed not only with the instrument used in the present paper but also with other NIRS instruments that the existing commercial instrumentation has difficulties analysing intact loins, because they are very heterogeneous samples, and as consequence, the proportion of incident light that interacts with the sample, but it is not reflected, and therefore it does not reach the detectors of the spectrometer, is higher than those when samples are homogenized. The main challenge facing the on-line analysis of intact loins is therefore to refine the instrumentation and fibre optics in order to increase the amount of reflected light. In the opinion of the present authors, a second and important challenge is the crucial contribution of industrial enterprises in

providing a high number of samples at no or affordable cost for this type of research project.

Finally, the optimisation of the spectral range does not noticeably improve the equations that are developed, and the time taken to develop the equations is higher than when the full spectral range is used. Such considerations make it advisable to calibrate with the full spectral range.

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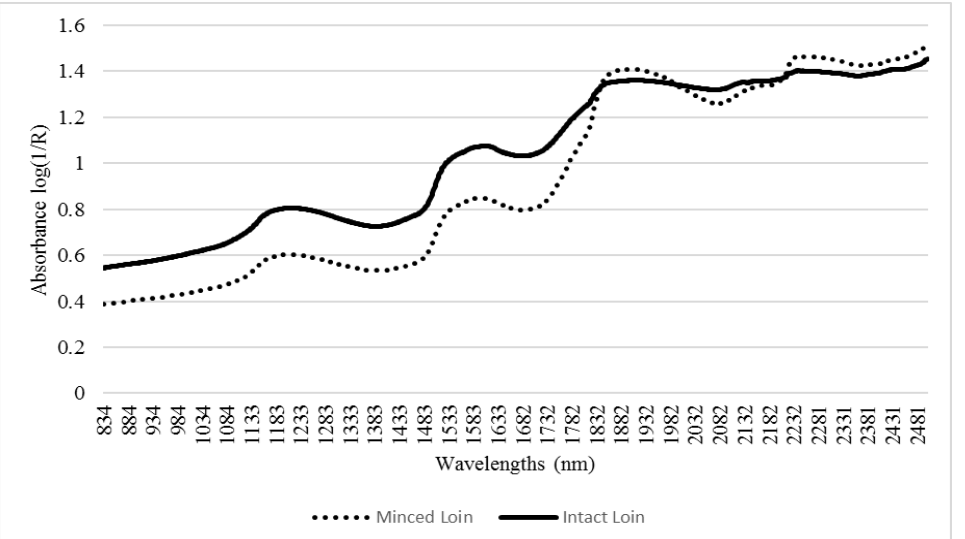

Figure 1. Mean spectra of minced and intact loins.

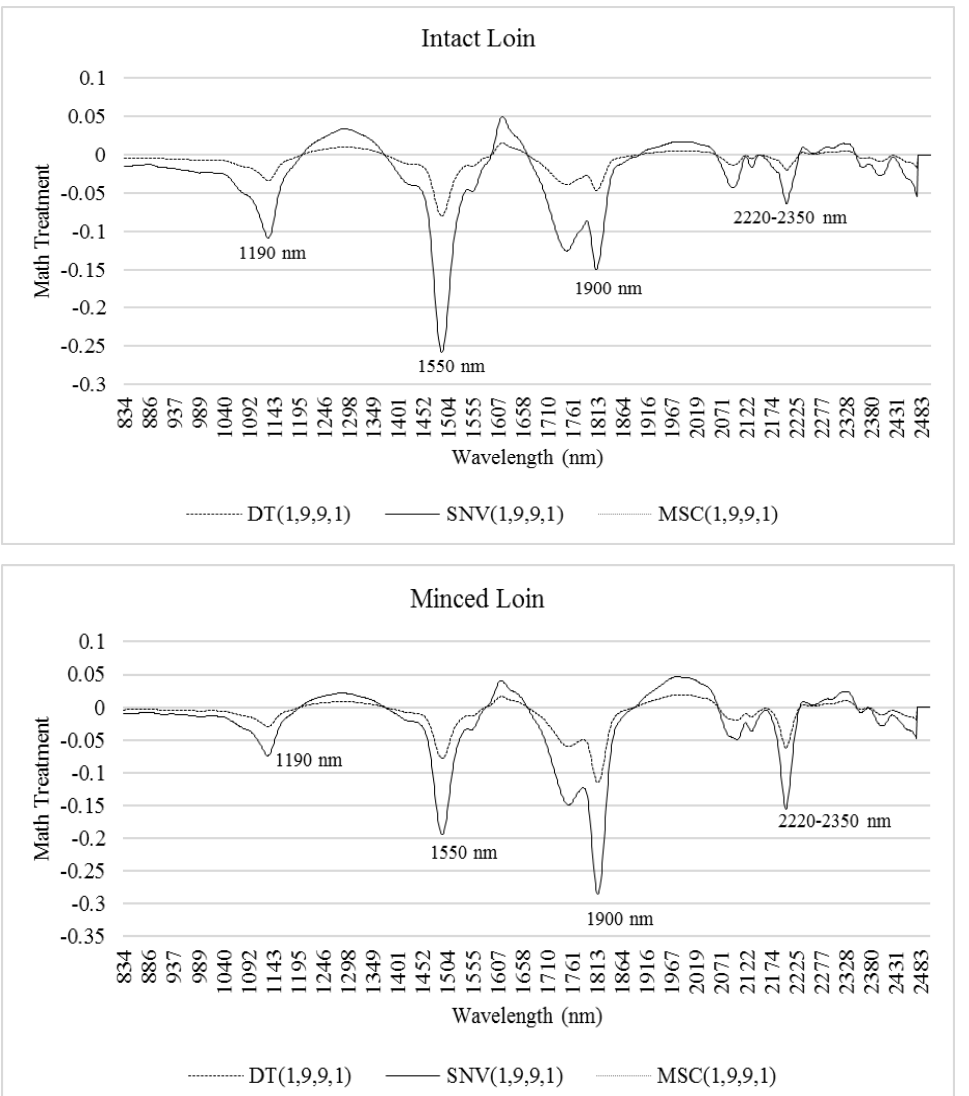

Figure 2. Math pretreatment spectra for intact and minced loins.

Graphics of math pre-treatments DT (1, 9, 9, 1) and MSC (1, 9, 9, 1) coincide in both graphs.

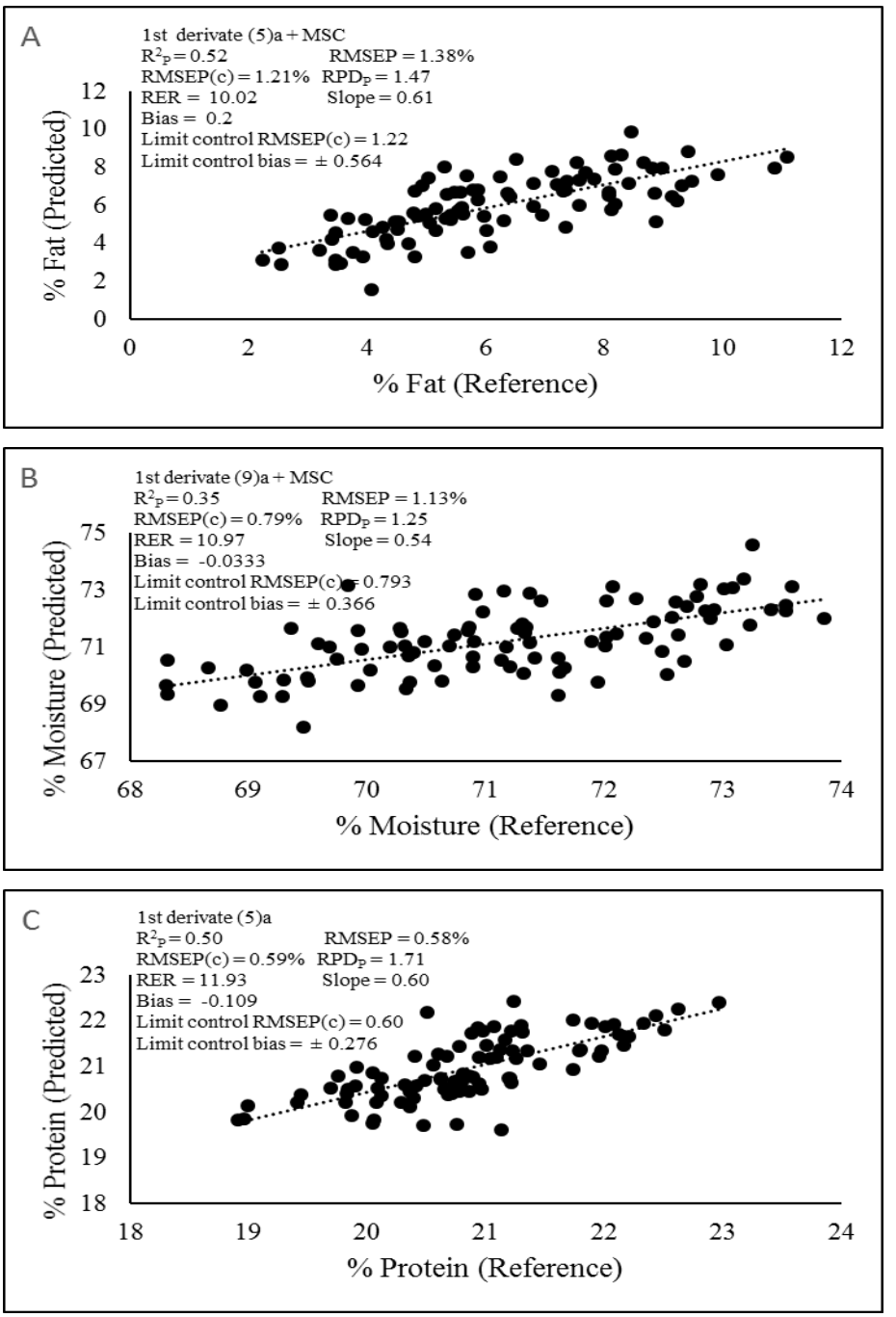

Figure 3. Predicted versus reference values based on the validation set for intact loin using the full spectral range. RMSEP: root mean square error of prediction; RMSEP(c): RMSEP corrected by bias; RPD_P: residual predictive deviation of prediction; RER: range error ratio (range/standard deviation); ($\boldsymbol{R_P^2}$) Determination Coefficient of Prediction.

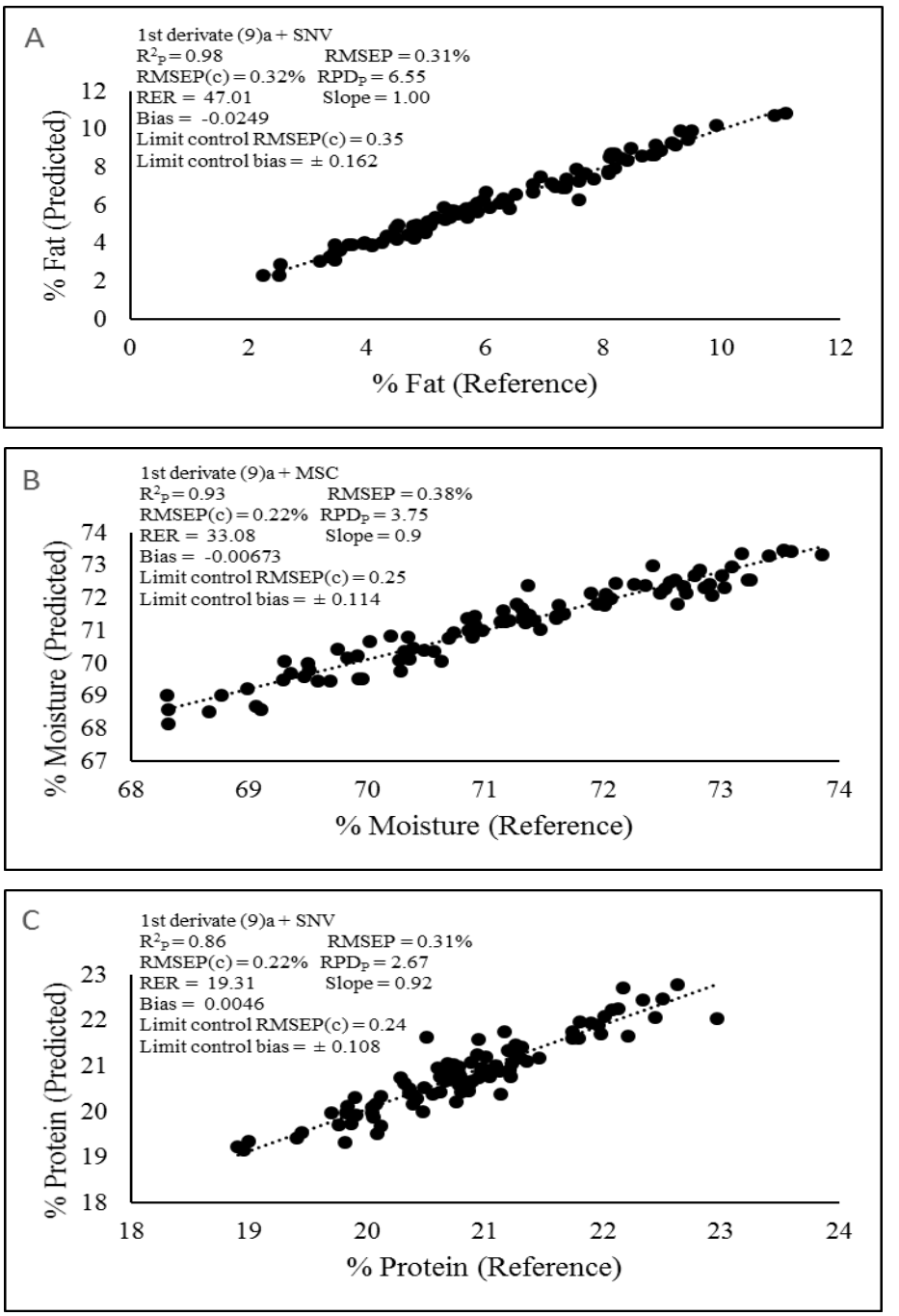

Figure 4. Predicted versus reference values based on the validation set for minced loin using the full spectral range. RMSEP: root mean square error of prediction; RMSEP(c): RMSEP corrected by bias; RPD_P: residual predictive deviation of prediction; RER: range error ratio (range/standard deviation); ($\boldsymbol{R^2_P}$) Determination Coefficient of Prediction.

Table 1. Descriptive statistics for training and calibration test (values expressed as % of wet weigh).

Parameter	**Calibration set (185 samples)**				**Validation set (92 samples)**			
	Min	Max	Mean	SD	Min	Max	Mean	SD
Fat (%)	1.66	15.2	5.65	2.37	2.24	11.09	6.18	2.01
Moisture (%)	64.89	74.45	71.55	1.55	68.31	73.86	71.2	1.41
Protein (%)	17.8	23.87	21.03	0.94	18.91	22.97	20.85	0.82

(Min: Minimum; Max: Maximum; SD: Standard Deviation).

Table 2. Parameters of PLS multivariate calibration models of fat, moisture and protein for intact loin using full spectral range.

Constituent	**Fat**	**Moisture**	**Protein**
Latent Variables	3	8	5
N	168	178	178
Wavelength Range (nm)	834.2-2502.6	834.2-2502.6	834.2-2502.6
Spectra pre-processing	1st (5)[a] + MSC	1st (9)[a] + MSC	1st (5)[a]
Mean (%)	5.34	71.54	21.08
SD (%)	1.91	1.51	0.87
RMSEE	0.94	0.61	0.46
RMSECV	1.06	0.87	0.51
R^2_C	0.76	0.85	0.75
R^2_{CV}	0.69	0.67	0.66
RPD_{CV}	1.8	1.73	1.71

RPD_{cv} residual predictive deviation of cross validation.

RMSEE root mean square error of estimation.

RMSECV root mean square error of cross validation.

R^2_{CV} coefficient of determination of cross validation.

R^2_C coefficient of determination of estimation.

SD standard deviation.

a: The number of smoothing points.

Table 3. Parameters of PLS multivariate calibration models of fat, moisture and protein for intact loin using optimal spectral range by OPUS QUANT.

Constituent	Fat	Moisture	Protein
Latent Variables	4	3	4
N	180	177	177
Wavelength Range (nm)	834.2-1334.8 1501.4-1668.9 2002-2336.1	834.2-1168.3 1501.4-1668.9 2168.5-2336.1	834.2-1334.8 1834.4-2336.1
Spectra pre-processing	1st (9)[a] + MSC	1st (5)[a] + MSC	1st (9)[a]
Mean (%)	5.58	71.57	21.09
SD (%)	2.27	1.48	0.89
RMSEE	0.97	0.61	0.42
RMSECV	1.09	0.77	0.49
R^2_C	0.82	0.84	0.79
R^2_{CV}	0.77	0.73	0.7
RPD_{CV}	2.08	1.92	1.82

RPD_{cv} residual predictive deviation of cross validation.

RMSEE root mean square error of estimation.

RMSECV root mean square error of cross validation.

R^2_{CV} coefficient of determination of cross validation.

R^2_C coefficient of determination of estimation.

SD standard deviation.

a: The number of smoothing points.

Table 4. Parameters of PLS multivariate calibration models of fat, moisture and protein for minced loin using full spectral range.

Constituent	**Fat**	**Moisture**	**Protein**
Latent Variables	3	10	9
N	173	173	181
Wavelength Range (nm)	834.2-2502.6	834.2-2502.6	834.2-2502.6
Spectra pre-processing	1st (9)[a] + SNV	1st (9)[a] + MSC	1st (9)[a] + SNV
Mean (%)	5.5	71.63	21.03
SD (%)	2.22	1.47	0.92
RMSEE	0.27	0.19	0.18
RMSECV	0.29	0.31	0.26
R^2_C	0.99	0.98	0.96
R^2_{CV}	0.98	0.96	0.92
RPD_{CV}	7.66	4.74	3.54

RPD_{cv} residual predictive deviation of cross validation.

RMSEE root mean square error of estimation.

RMSECV root mean square error of cross validation.

R^2_{CV} coefficient of determination of cross validation.

R^2_C coefficient of determination of estimation.

SD standard deviation.

a: The number of smoothing points.

Table 5. Parameters of PLS multivariate calibration models of fat, moisture and protein for minced loin using optimal spectral range by OPUS QUANT.

Constituent	**Fat**	**Moisture**	**Protein**
Latent Variables	3	7	6
N	175	177	181
Wavelength Range (nm)	834.2-1835.5 2002-2336.1	1000.7-1334.8 1501.4-1835.5	1167.2-1334.8 1667.9-1835.5
Spectra pre-processing	1st (5)[a] + MSC	1st (9)[a]	1st (5)[a]
Mean (%)	5.53	71.59	21.03
SD (%)	2.2	1.5	0.93
RMSEE	0.26	0.22	0.19
RMSECV	0.28	0.27	0.21
R^2_C	0.99	0.98	0.96
R^2_{CV}	0.98	0.97	0.95
RPD_{CV}	7.86	5.56	4.43

RPD_{cv} residual predictive deviation of cross validation.

RMSEE root mean square error of estimation.

RMSECV root mean square error of cross validation.

R^2_{CV} coefficient of determination of cross validation.

R^2_C coefficient of determination of estimation.

SD standard deviation.

a: The number of smoothing points.

Table 6. Fisher test results.

	Fat			**Moisture**			**Protein**		
	F_{value}	$F_{critic\ value}$	P_{value}	F_{value}	$F_{critic\ value}$	P_{value}	F_{value}	$F_{critic\ value}$	P_{value}
Intact loin-Full range (inter math pre-treatments)	0.264	2.106	0.934	0.058	2.106	0.999	0.031	2.106	0.999
Intact loin-Optimal range (inter math pre-treatments)	0.077	2.106	0.998	0.027	2.106	0.999	0.115	2.106	0.995
Intact loin Full Range *vs* Intact loin Optimal Range	0.383	1.724	0.975	0.046	1.724	0.999	0.081	1.724	0.999
Minced loin-Full range (inter math pre-treatments)	0.126	2.106	0.993	0.05	2.106	0.999	0.012	2.106	0.999
Minced loin-Optimal range (inter math pre-treatments)	0.078	2.106	0.998	0.085	2.106	0.998	0.042	2.106	0.999
Minced loin Full Range *vs* Minced loin Optimal Range	0.095	1.724	0.999	0.066	1.724	0.999	0.025	1.724	0.999

3.2. NIR handheld miniature spectrometer to increase the efficiency of Iberian pig selection schemes based on chemical traits. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 258, 119865 (2021)

NIR handheld miniature spectrometer to increase the efficiency of Iberian pig selection schemes based on chemical traits

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Abstract

Five hundred twenty-four Iberian Pig loin samples were used to develop near-infrared (NIR) calibrations for the non-destructive and real time predicting the percentage of intramuscular fat, together with the protein and moisture contents, as chemical traits in selection schemes and breeding programs. Spectra were collected at 6.2 nm increments using a MicroNIR™ 1700 LVF (Linear Variable Filters) spectrophotometer. For the development of NIRS prediction models, two chemometric approaches were compared: MPLS (modified partial least square), a linear regression algorithm traditionally used for developing NIRS applications, and a local regression, LOCAL patented algorithm. For all predicted parameters, LOCAL calibrations were shown to slightly correct non-linearity problems, which is reflected in the best slope values, improving them between 5% and 10% with respect to those obtained when using MPLS regression equations. However, the use of LOCAL did not show to improve the standard error of prediction (SEP), bias values, and determination coefficient values (R^2_P) compared with GLOBAL calibrations.

Keywords: Near-Infrared spectroscopy; Iberian loins; Miniature spectrometer; Linear and Non-Linear Regressions; LOCAL algorithm; Iberian pig selection schemes.

1. Introduction

The scientific literature of the last decades has shown, as a rigorous and continuous scientific work in the areas of nutrition, genetics, technological processing and, more recently, in the quality control, traceability and authenticity of the meat cuts derived from research projects undertaken with numerous local breeds and their crosses, has allowed to offer consumers meat cuts of excellent nutritional, organoleptic and health properties. To achieve that goal, thousands of laboratories in the world have needed -and still need- to perform analyses of the quality of the meat cuts to confirm if they were in accordance with what was expected. There are two important limitations in this type of research: firstly, the classical physico-chemical and sensory analysis are destructive, expensive and requires laboratories well equipped with both personnel and scientific equipment; secondly, in many cases, they use highly toxic reagents and produces a large amount of toxic waste. Other limitations are the high economic cost and time required to obtain such information, which makes the number of samples analysed rather limited.

Near Infrared Spectroscopy is a non-destructive, digital and rapid analytical technology. Besides, it is cheaper than the traditional wet chemistry and does not use reagents or generate toxic waste. It allows to significantly increase the number of samples analysed daily. As a consequence, NIR spectroscopy is being increasingly adopted in high-throughput analysis in the agri-food industries [1].

It is difficult to find studies involving a large number of meat samples coming research projects as the mentioned before. This drawback is very important for selection and breeding programs supported - at national level - by the respective governments, DOs (Denominations of Origin), Interprofessional Associations or by the own industrials. In fact, for decades, pig breeding programs have focused mainly on reducing the production costs of pork. Selection has been aimed to increases the litter size and the percentage of lean meat, in addition to weight gain and improving feed conversion. However, currently, to meet consumer expectations, breeding targets are shifting their focus to meat quality traits due to its high economic value [2].

Specifically, in the case of the Iberian pig (IP), many research projects have been carried out in Spain making possible the industrialization of an activity that in its beginnings was familiar. The extraordinary transformation is the result of many scientific efforts and governmental support for selection and breeding programs.

However, in order to develop and implement breeding selection schemes focused on the Iberian purebred products, labelled as "Bellota", some parameters as intramuscular fat percentage (IFM) have been measured and studied [3]. The selection programs have enabled a significant improvement of the Iberian products quality and, therefore, their acceptance associated to their health and organoleptic quality traits, allowing the flourishing of new markets, both in the EU and in Third Countries. Furthermore, it has been demonstrated that IFM has a high hereditability component, which makes it suitable to be included in selection and breeding programs aimed at improving the yield of premium cuts and the quality of the meat of Iberian pigs and theirs crosses with Duroc [4, 5].

Since the 90's, the research group of the authors of this paper has a dedicated and specific research line to demonstrate the role that NIRS technology could play to support both, breeding programs and quality control schemes, at an industry level and for inspection bodies. However, most of these studies have been developed with adipose tissue from Iberian pigs using at-line and portable instruments [6, 7, 8, 9, 10, 11, 12, 13].

The more expensive pieces of the Iberian pigs are the hams and shoulders, so the analysis or part of the loins (M. *Longissimus*) is preferred in research programmes, where there is a need to evaluate the final meat product quality. Iberian loins are meat pieces whose length range between 60-70 cm and their weight from 1.5 to 2.5 kg, approximately. Thus, it is possible to take a small portion of this piece for wet chemical analysis or NIR, and the rest of this piece could be commercialized as cured or raw without any problem.

Zamora et al. [13, 14] demonstrated the excellent ability of NIR technology for the determination of meat quality using minced Iberian Pigs loin samples, in order to determine chemical composition (IMF, protein and moisture).

Later on, Cáceres-Nevado et al. [15] attempted to develop calibrations for predicting the same parameters but using intact Iberian Pig loins. This research showed the lower accuracy of the calibrations developed using intact samples in relation to those equations obtained with minced samples, as demonstrated by other authors in other types of meat [16, 17, 18, 19]. However, the models obtained by Cáceres-Nevado et al. [15] for the prediction of IFM explained around 52% of the variability encountered in that important quality meat trait, which it may be of the help for Iberian pig selection and breeding programs. This study was developed using a multiplexed FT-NIR instrument, Matrix-F, which it can be implemented on-line in an industrial plant [20]. This instrument was employed for this study because it enables to measure until in six different points of the slaughterhouse, reducing the cost of developing different applications and the use of different instruments in the meat industry.

Although this instrument could be of interest to large industrial companies, it is important to point out that technological evolution that has taken place in recent years has allowed the appearance of portable spectrometers based on MEMS (Micro-electro Mechanical Systems) and LVF (Linear Variable Filters) technologies, which have provided substantially reduction in size and weight, while presenting better tolerance to more extreme conditions, such as temperature, humidity, etc. allowing on-line and on-site measurements. These devices are offering new opportunities for its applicability to the agri-food industries due to their excellent portability and low-cost [1, 21, 22].

Therefore, it is desirable to obtain more accuracy models for intact loin than those obtained by Cáceres-Nevado et al. [15], so it is necessary to continue the evaluation of other strategies to obtain spectroscopy data and their treatment. In addition, the high heterogeneity of loin samples reduces the repeatability of the NIR measurement. This makes difficult to meet the linearity requirements for the application of traditional regression techniques (e.g., PLS) for NIR model development. Different authors had shown that the non-lineal LOCAL algorithm allows better accuracy models for heterogeneous products or for the predictions of analytical parameters of a non-chemical nature [10, 23, 24, 25, 26].

The main goal of the present research was to evaluate a handheld miniature NIR instrument based on LVF technology for its applicability to the prediction of quality traits in intact Iberian pig loins, and its comparison with minced samples analysis, using linear and non-linear algorithms.

2. Material and methods

2.1. Sample set

Samples of *Longissimus dorsi* muscles (MLD) were collected from 524 carcasses of Iberian pigs from "Sánchez Romero Carvajal Jabugo S.A.". The animals were slaughtered at a live weight of about 160 ± 11 kg and at the age of 14 months. These samples were cut transversally from the zone between the thirteenth and fourteenth dorsal vertebrae of the animals to provide a slice measuring 2 cm x 6 cm x 10 cm (thickness, width, and length).

Each sample was immediately packed in plastic bag, vacuum-sealed and transported to the laboratory of the Animal Production Department (University of Cordoba) at a temperature of 4-6ºC, where they were stored at minus 20ºC and then, prior to NIR analysis, the samples were gently thawed at 4ºC for 24 hours and were tempered until they reached 20 ± 2ºC.

2.2. Reference analysis

Reference chemical analysis were performed with homogenised samples in accordance with the official meat analysis methods [27]. Intramuscular fat content was determined following the Soxhlet procedure as indicated in the ISO-R-1443; total protein was determined by the Kjeldahl method following the ISO-R-937; and moisture content was measured by lyophilisation, using FTS SYSTEMS equipment, at -50 ºC for 48 hours.

In the analysis for both, intramuscular fat and protein, two determinations were made, taking the mean after eliminating the values in which there was an error higher than 2%. When a value is eliminated, the process was repeated. The chemical composition is presented in percentage by weight.

2.3. NIR spectrum acquisition

NIR spectra of loin samples were collected using the MicroNIR™ 1700 spectrophotometer (VIAVI Solutions, Inc., San Jose, California, USA), a portable instrument designed for *in-situ* analysis, working in reflectance mode. This miniature instrument is a fully integrated NIR spectrophotometer and extremely light (250 g of weight), which make it very suitable to be used in different points of the meat supply chain [28]. This instrument uses Linear Variable Filter (LVF) technology as dispersing element, without moving parts and a single InGaAs photodetector array [22, 29]. The spectrometer scans at 6.2 nm intervals across a range of wavelengths (908.1-1676.2 nm). Its optical window size is around 227 mm^2. Spectral data were recorded using the MicroNIR Pro v2.2 software (VIAVI Solutions). The instrument's performance was checked every 10 min: a white reference measurement was obtaining using a NIR reflectance standard (Spectralon™) with 99% of diffuse reflectance, while a dark reference was obtained from a fixed point in the room [30].

Two sample presentation modes were evaluated for the analysis of the loin samples: intact and minced loin (for comparison purposes). Minced samples were homogenised using a homogeniser "Heidolph Diax 900". Four spectral measurements were taken with this instrument in intact loin (two per face) and two spectral measurements in minced loin with a measurement time of 4 seconds. In both modes of analysis, all spectra for each sample were averaged to provide a mean spectrum for each loin's sample.

2.4. Data pre-treatment

Data pre-processing and chemometric treatments were performed using the WinISI II software package version 1.50 (Infrasoft International LLC, Port Matilda, PA, USA) [31].

2.4.1. Detection of spectral outliers

Prior to the development of NIR calibrations, the structure and spectral variability of the sample population were determined following the procedure

recommended by Shenk et al. [32], using the CENTER algorithm included in the WinISI software (Infrasoft International, Port Matilda, PA, USA). The CENTER algorithm performs a principal component analysis (PCA), reducing the original spectra information to a small number of linearity-independent variables, which facilitate the calculation of the spectral distances. Once each sample in the global set was ordered by its spectral distance (from shorter to longer) to centre of the global population, those samples with GH > 3 were considered as spectral outliers [33].

2.5. Calibration and validation sets

In order to develop quantitative models, the entire set was divided into calibration and validation sets using the methodology described by Shenk et al. [32]. Thus, throughout the spectra of intact loin (since this set had a larger spectral variability than the minced set) ordered according to their Mahalanobis distance to the centre of the population, one out of every three samples were selected to be part of the validation set (N = 152 samples) and the remaining samples were used for calibration (N = 322 samples) (Table 1). For both analysis modes (intact and minced), the calibration and validation sets were finally made up of the same samples to facilitate the comparison of the results.

2.6. Developing and validation of prediction models using linear and non-linear regression strategies

Modified Partial Least Square (MPLS) regression [34] and the LOCAL algorithm [26] were used to obtain NIR calibration models for the prediction of quality parameters (intramuscular fat, protein, and moisture) in Iberian pig loins.

For the development of global MPLS models, spectral data were pre-treated testing four derivatives treatments: 1,5,5,1; 1,10,10,1; 2,5,5,1 and 2,10,10,1, with the first digit representing the order of the derivative, the second digit representing the derivation segment length in data points, and the third and fourth digits representing the number of data points used for smoothing [31, 35]. In addition, they were combined with Standard Normal Variate (SNV) and

Detrending (DT) methods, used for scatter correction [36]. Non scatter correction was also tested.

Cross Validation was applied to select the optimal number of factors and avoid overfitting [35]. Thus, the calibration set was divided into 4 cross validation groups, using alternatively all the samples for calibration or validation. Thus, the prediction errors were combined in order to obtain the standard error of cross validation (SECV), which is one of the best estimators of the uncertainty of prediction for samples not included in the calibration set [33].

The statistics used to select the best global MPLS equations were the coefficient of determination for calibration (R_C^2), the standard error of calibration (SEC), the coefficient of determination for cross validation (R_{CV}^2) and the standard error of cross validation (SECV). Additionally, the Residual Predictive Deviation (RDP_{CV}) for cross validation was calculated as the ratio of the standard deviation (SD) of the reference data to the SECV [30]. The RPD enables SECV to be standardized, facilitating the comparison of results obtained with sets of different means [37].

The best model for each parameter analysed (intramuscular fat, protein and moisture) were selected by statistical criteria, and prior to external validation, tests were run for looking for significant differences between the predictive capacities' models developed for each parameter. The SECV values for the best equations obtained for both analysis modes were compared using Fisher's test [38, 39]. Values for F were calculated as:

$$F = \frac{(SECV_2)^2}{(SECV_1)^2} \quad (1)$$

were $SECV_1$ and $SECV_2$ are the standard error of cross calibration of two different models and $SECV_1 < SECV_2$. F is compared to $F_{critical\ (1\text{-}P,\ n1\text{-}1,\ n2\text{-}1)}$, as read from the table, with P = 0.05 and n_1 is the number of times measurement is repeated using the optimal spectral range, while n_2 is the number of times the measurement is repeated using the full spectral range when we compare different spectral ranges using the same analysis mode. On the other hand, when we compared the different analysis modes (intact vs minced), n_1 is the number of

times measurement is repeated with intact samples, and while n_2 is the number of times the measurement is repeated with minced samples. If F is higher than $F_{critical}$, the two SECV values are significantly different.

Finally, the best models selected by statistical criteria [33, 37] were subjected to external validation using a group of samples not involved in the calibration development, following the validation protocol outlined by Windham et al. [40].

The LOCAL algorithm [26, 41] is a procedure designed to locate and select, within a large spectral database (several thousand of samples is recommended), those samples that resemble the unknown sample to be predicted. Selection is based on the coefficient of correlation between the spectrum of the unknown sample and the spectra forming the whole database. The selected samples are then used as a calibration set to develop a specific equation for predicting the unknown sample. For the prediction of a second sample, the algorithm will select a different calibration set and perform a new specific calibration. LOCAL uses PLS regression to develop the specific equations with the selected samples.

To optimize the LOCAL procedure, working with a given spectral library, a number of parameters need to be evaluated, although the three basic factors to be defined [10, 24, 25] are: the number of samples to select from the spectral library (*k*); the maximum number of PLS terms to be used (*l*); and the number of predicted values generated with the first PLS terms to be excluded from the calculation of final predicted values, since the accuracy of the predictions obtained with LOCAL improves when the predicted values generated with the first PLS terms are excluded from the calculation of mean predicted values [26]. At the same time, as in MPLS calibrations, other factors need to be optimized, as the signal pretreatment (derivatives and light scatter correction).

In this study, all the above-mentioned factors were pre-set, except the number of samples selected for calibration (*k*), for which six values (60, 80, 100, 120, 150, and 160) were tested. In all cases, the maximum number of PLS terms to be included (*l*) was set at 16, eliminating predicted values from the first three PLS terms (*m*) from the final calculation. SNV and detrend treatments [36] were

used for scatter correction, in combination with the derivation treatments 1,5,5,1 and 2,5,5,1 [35].

3. Results and discussion

3.1. Spectrum interpretation

Fig.1 shows the raw spectra, as a log (1/R) being R the reflectance, for both modes of analysis (intact *vs* minced). It is evident that the absorbance of intact samples is higher than that of the minced samples. This could be explained by the disruption of meat structure when samples are minced, which interferes with the light absorbance [15, 17, 42]. Both spectra show broader peaks, usual in high moisture products, making band assignment more difficult than for other agri-food products. However, it is clear that the spectra are dominated by strong absorptions due to IFM (1200 nm). Another important band is observed around 1450 nm which is related to combinations of the bond C-H (CH_2 at 1440 nm and aromatic structure at 1446 nm), OH stretch first overtone (1450 nm) and NH stretch first overtone (urea at 1460 nm and $CONH_2$ at 1463 nm). In meat products, this band has been linked with water and protein due to the OH and NH bonds [15, 18, 43, 44, 45, 46].

3.2. Population characterization and detection of spectral outliers

Using the whole population (N = 524), the Mahalanobis distance between each sample and the centre of the population was calculated in order to detect potential spectral outliers, as described in section 2.5. GH values greater than 3 were found for 50 samples (less than the 10% of the population, as it is recommended). After the examination of their reference data, it was found that these samples were located in the extreme of the variation range, mainly in terms of percentage of intramuscular fat (greater than 9% or below 2%). Consequently, these samples were removed from the original set.

After removing spectral outliers, the structured selection of the calibration (N = 322 samples) and validation (N = 152 samples), as described in section 2.5, yielded two sets with similar means and standard deviations for all chemical

parameters studied (Table 1), which indicates that spectral data *per se* are very useful for selecting well-structure training and test sets.

Similarly, Table 1 shows that the parameter with the greatest variability is the intramuscular fat content, with a CV of 42.25% and 42.23% for the training and test sets, respectively. This variability was due to the wide range and standard deviation obtained for this parameter because the samples were taken from animals with a great genetic variability, and IFM content directly depend on this factor. However, for moisture (CV_c = 2.10%, CV_v = 2.24%) and protein (CV_c = 4.15%, CV_v = 4.17%) the groups show less variability, which could be explained by the fact that all animals tested were reared under the same feeding conditions.

3.3. Prediction of quality parameters using Global MPLS

Once the calibration and validation sets were established, calibration equations were developed for both intact and minced loin samples.

Table 2 shows the SECV (standard error of cross-validation) and R_{cv}^2 (coefficient of determination of cross-validation) values obtained for the best Global MPLS equations developed to predict the percentage of the three chemical parameters (IFM, protein, and moisture) evaluated in this research paper.

Generally speaking, according to the criteria established by Shenk et al. [32], the results suggest that the equations obtained afford an excellent degree of precision and accuracy for minced samples (around 0.9) for all parameters tested. For intact samples, the accuracy obtained was good for IFM and protein, enabling an adequate discrimination between samples of high, medium and low content in the tested parameter. However, in intact samples, the equation developed to predict moisture content showed a low degree of precision (less than 0.6), so it could not be used to predict and quantify this parameter.

As can be seen in Table 2, there were notable statistical differences ($P < 0.05$, $F > F_{critical}$) between the analysis modes (intact *vs* minced). Thus, as it was

expected, the lowest SECV values were obtained in the minced mode analysis for all the parameters.

The higher SECV values shown by the models developed using intact samples could be associated to the irregular shape and surface that present these samples, which increases the amount of scattered radiation and, consequently, the amount of light that does not reach the detectors. Furthermore, other factors that could be influence on this would be the complex molecular structure that intact meat presents [17, 18] and its heterogeneity (zones with more or less presence of intramuscular fat or connective tissue, for example). Nevertheless, the analysis of intact loins enables to have an instantaneous response without destroying the product. This is a very important advantage for taking decisions in the industry that we do not have with the minced analysis.

For comparing the efficiency of the models, some authors have used the RPD statistic. Most of the authors refer to the recommended values for the RPD [47]. However, this criterion cannot be generalised to all type of products or all NIRS instruments [48], because it was set for applications developed with finely-ground samples of grains (mainly wheat) and using high-performance laboratory monochromators. Furthermore, Esbensen et al. [49] have reported that RPD values depend on the kind of sample, on its prior preparation and on the way, it is presented to the instrument.

As it shown in Table 2, the RPD_{cv} values for intact samples are lower than those achieved in the case of calibration models developed with minced samples. In both cases, the highest RDP_{cv} values were obtained for IFM parameter, which could be attributed to its higher standard deviations compared to those of the moisture and protein parameters.

Several authors have carried out their research using intact meat samples [15, 50, 51, 52, 53, 54]. All of them, except [15, 51], used a much smaller set of samples than that used in the present study, and furthermore, did not to carry out external validation. Thus, González-Martín et al. [50], analysing 56 samples of Iberian pork loins with a monochromator instrument (Foss NIRSystems 5000) adapted to a fiber optic probe (with a 5cm x 5cm windows surface), obtained

R^2_C, SEC and SECV values of 0.94%, 0.80% and 1.26 for intramuscular fat; while for protein were 0.88%, 0.56% and 0.83%, respectively. Although the R^2_C values obtained by these authors are higher than our results, these models show higher errors of calibration and cross validation, presenting therefore lower accuracy. Besides, Chan et al. [51] obtained SECV and RPD_{CV} values of 0.62% and 2.26 for intramuscular fat; 0.43% and 1.88 for protein; 0.58% and 2.38 for moisture. The better results obtained by these authors compared to those obtained in the present research could be attributable to the use of high-performance instruments suitable for laboratory conditions but no for on-line analysis. In our work, a portable NIRS instrument has been evaluated, which is a low-cost instrument, with a lower spectral range and a more reduced optical window (227 m^2).

On the other hand, Barlocco et al. [53], using a FOSS NIRSystem device, obtained similar PLS calibration models for IMF and moisture ($R^2 < 0.70$); Cáceres-Nevado et al. [15], using a Bruker spectrophotometer (Matrix-F), obtained very similar statistics values for all parameters; and Hoving-Bolink et al. [52], using Zeiss MCS 511/522 device with a measuring area of 1 mm^2 and MLR (Multiple Linear Regression) models, obtained a lower accuracy.

Regression coefficient plots were also used to check the influence of different wavelengths (variables) in the determination of the IMF, protein and moisture contents (Fig. 2). Large absolute values indicate the importance and significance of the most relevant wavelengths in the models. It can be appreciated in Fig. 2 that the weight of the coefficients was almost double for intact than for minced samples. That it is good agreement with the highest absorbance values (log 1/R) obtained for intact samples (Fig. 1).

Fig. 2 shows that the peaks with the highest weights were located at 980, 1270 and 1630 nm for IFM in intact samples and around 1200 and 1250 nm for minced samples. The region around 980 nm is related to the third overtones of C-H stretching modes while regions around 1200-1270 and 1630 nm could be associated with C-H second overtones. Analysing the plot for protein (Fig. 2), the most important wavelengths are located around 1200, 1300 and 1500 nm

using intact samples, and around 1150-1250 using minced samples. These regions are related to the third and second overtones of N-H stretching modes with the absorption pf the peptide bonds in protein. On the other hand, the peaks with the highest weight for moisture models using intact samples were around 960 and 1550. However, with minced samples were located around 960 and 1490 nm. Regions around 960, 1400 and 1550 nm could be associated with O-H bonds, mainly related to the moisture content in the muscle tissue [15, 18, 43, 44, 45, 46].

The differences found between the weights for similar wavelength in both analysis modes could be due to the complex nature of the muscle before and after its homogenisation, since during this process the fibre and IFM undergo several important modifications and, mainly, the water content is slightly reduced.

Evaluation of the best models for each parameter was performed using the validation set (N = 152). The results are shown in Fig. 3 and Fig.4.

The validation statistics for intact loins (Table 5), fulfil the limits stablished by [40, 44], in the case of the bias and the SEP(c). On the other hand, models constructed for predicting IFM and protein attained the recommended value of 0.6 for R_p^2, and 0.9 for slope, which indicate that these models could provide a good prediction of the percentage of these parameters when they were used in routine analysis. However, moisture models did not attain the recommended minimum value of 0.6 for R_p^2, and 0.9 for slope. As a consequence of these results, these equations could only be used to discriminate samples with different water content (high, medium or low), not to provide an accurate prediction of the percentage of moisture.

This low R_P^2 value and the dispersion of samples for moisture content (Fig. 3) shows a low correlation between the values predicted by NIRS and the reference values. This may be attributable to the analysis procedure, i.e., from the time the sample is analysed in intact mode to the analysis of the sample is analysed by the reference method and minced there is a period of time, in which the sample is processed (minced, stored, etc.), and it is clear that there are changes or losses in the moisture content that affect the precision of the

prediction models developed. This loss of moisture is reflected in the values of the statistics mentioned above.

In fact, in the case of minced loin samples (Table 6), all parameters analysis met the validation requirements in terms of the coefficient of determination for prediction, R_p^2 ($R_p^2 > 0.6$) and slope (minimum slope 0.9), and both the standard error of prediction corrected for bias (SEP(c)) and bias were within the confident limits: the equations thus ensure accurate prediction and can be applied routinely.

3.4. Prediction of quality parameters using LOCAL regressions

LOCAL calibrations (using the LOCAL algorithm) were also developed for the prediction of the 3 parameters studied.

The results obtained applying the LOCAL algorithm for predicting the chemical composition of Iberian pig loins, using a spectral library of 474 samples, and selecting 152 samples as the validation set, are shown in Tables 3 and 4 for intact and minced samples, respectively. These tables show the prediction results obtained with each one of the values for the parameter *k* that were tested, i.e., the maximum number of samples used for calibration, for each of the 152 samples making up the validation set.

As stated in the Materials and Methods section, this is one of the three major parameters to be fixed when developing LOCAL equations. Six different *k* values (60, 80, 100, 120, 150 and 160) were tested for each parameter combined with first and second derivative and scatter correction treatments. It can be appreciated in Tables 3 and 4 show that for all parameters the best results were obtained when *k* was set at 150. This means that instead of calibrating with 322 samples, calibration only uses 150 samples; these 150 samples, of course, are not random samples, but spectrally selected as being the most representative set, and the best spectrally fitted to the sample to be predicted.

For the parameters studied here, the accuracy and precision of the predictions obtained using LOCAL algorithm, evaluated by means of the statistical SEP, bias, and R_p^2, were similar than those of the predictions obtained using Global MPLS calibrations (Tables 5 and 6), but yielded a slope values

much closed to one (Fig. 3 and Fig.4), which indicates that this algorithm has slightly corrected the non-linearity.

As well as employing the MPLS algorithm, with the LOCAL regression the validation statistics for intact and minced loins (Tables 5 and 6) fulfil the limits stablished by [40, 44], in the case of the bias and the SEP(c).

However, although the number of samples employed in this research paper are high, it is not large enough for LOCAL to achieve lower SEP values and better accuracy of the predictive models.

It is important to highlight that this aspect will be due to deepen in future research.

As can been seen in Table 7, the best models developed using MPLS and LOCAL strategies do not show significant differences between them ($F < F_{critical}$), both in intact and minced analysis. In fact, Pérez Marín et al. [23], using 7423 compound feed samples, showed that LOCAL calibrations resulted in a significant improvement in both standard error of prediction (SEP), increasing it more than 20%, and bias values compared with GLOBAL calibrations. On the other hand, Berzaghi et al. [24], using a multi-product database composed by 6599 samples, also obtained lower values of SEP for LOCAL calibrations than those obtained of generic global calibrations. It is possible that the number of samples used in the present paper for developing LOCAL calibrations is not enough to observe important improvements in predictive ability.

Most of the papers related to the evaluation of NIRS for the prediction of qualitative traits in intact meat pieces used PLS regressions. To our knowledge, there is only one paper which evaluates non-linear regression algorithms for the prediction of IFM in intact samples of *Longissimus dorsi* muscles [42]. The best model obtained for the prediction of IFM by using LS-SVM (Least-Square Support Vector Machine) showed a high precision and accuracy (R^2_{cal} of 0.94, R^2_{val} of 0.92, SEC of 0.233, SEP of 0.462, and RPD of 2.29). However, given that the values of the SECV and SEPs differ by more than 20%, and that the number of samples used for calibration development was low (N = 92), that could be indicative of overfitting of the model, and therefore the value of the SEP could

be considered as optimistic. In our case, even though the SEP value was higher, the similarity of the SECV and SEP indicate that the model could be more robust

It is important to highlight that the instrument used in the present paper has previously demonstrated its potential to combine with IoT to be used for the prediction in real time of the fatty acids' composition of IP carcasses at the speed of slaughtering line [28]. In a similar way, the equations obtained here may be implemented for the analysis of all the piece of loins produced at the Iberian pig industry or in breeding programmes and the information may be send to any server in fractions of seconds.

4. Conclusion

The results of this paper show that models obtained using minced samples has a high accuracy, but the most important advance of this research is that equations developed using intact samples enable to predict with a good accuracy the IFM content in real time without having to destroy the samples. This is a very important finding since the equation it would allow to classify individual pieces of loins, and therefore individual carcasses, on the basis of their intramuscular fat content, enabling to take important decisions in selection schemes and breeding programmes. Besides, the industrial sector would make real-time decisions about the subsequent use of these highly prized pieces of meat.

The results showed that Global MPLS is enough for predicting the composition of Iberian loin, both in intact or minced analysis mode, and in this case the use of a non-linear algorithm does not give specific advantages for this application, since the results of both methods could be considered similar. Nevertheless, it is important to stress that the maintenance of LOCAL algorithms is easier since in this case only needs to enlarge the spectral library. This could be a point of interest for the future, when large spectral libraries are available, and maybe in this case the effect of LOCAL algorithm could be more, appreciated.

In general, this paper demonstrates that the equations obtained have an accuracy enough to be used with a handheld NIR device to analyse every intact

pork loin at the industrial plant, which may be of tremendous help and impact in Iberian pig selection schemes based on IMF.

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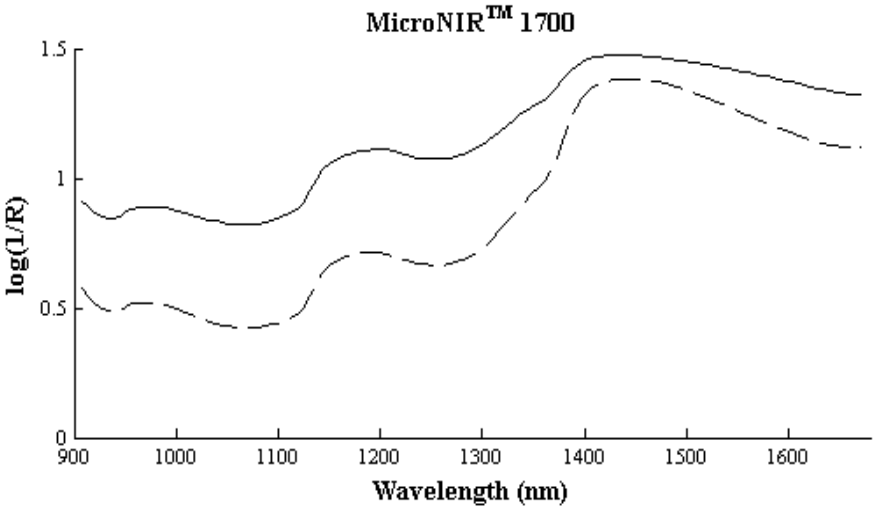

Figure 1. Raw spectra of intact (continuous line) and homogenised (dashed line) loins.

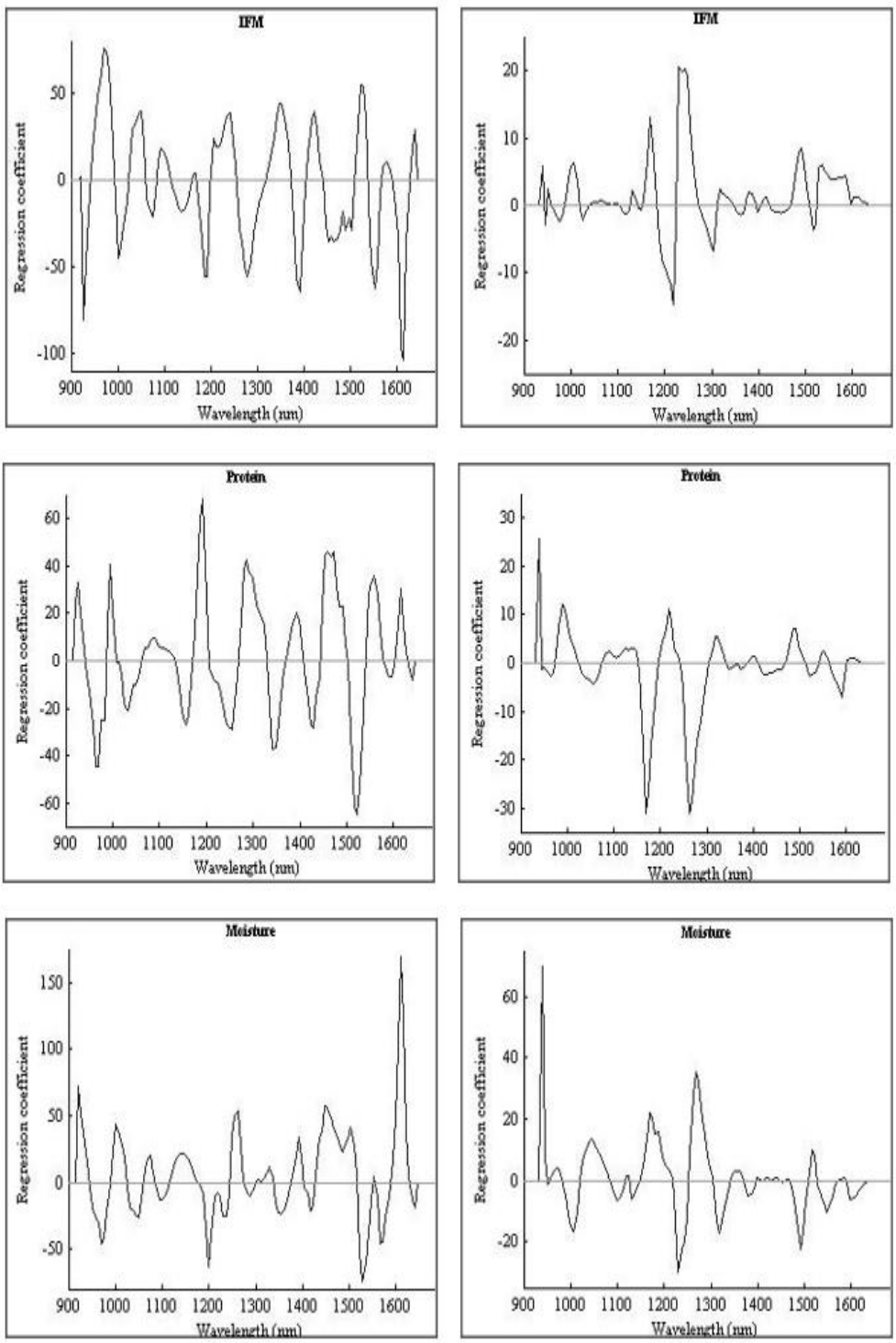

Figure 2. Regression coefficients of the best calibration MPLS models for IFM, protein and moisture using intact (left part) and minced (right part) loin samples.

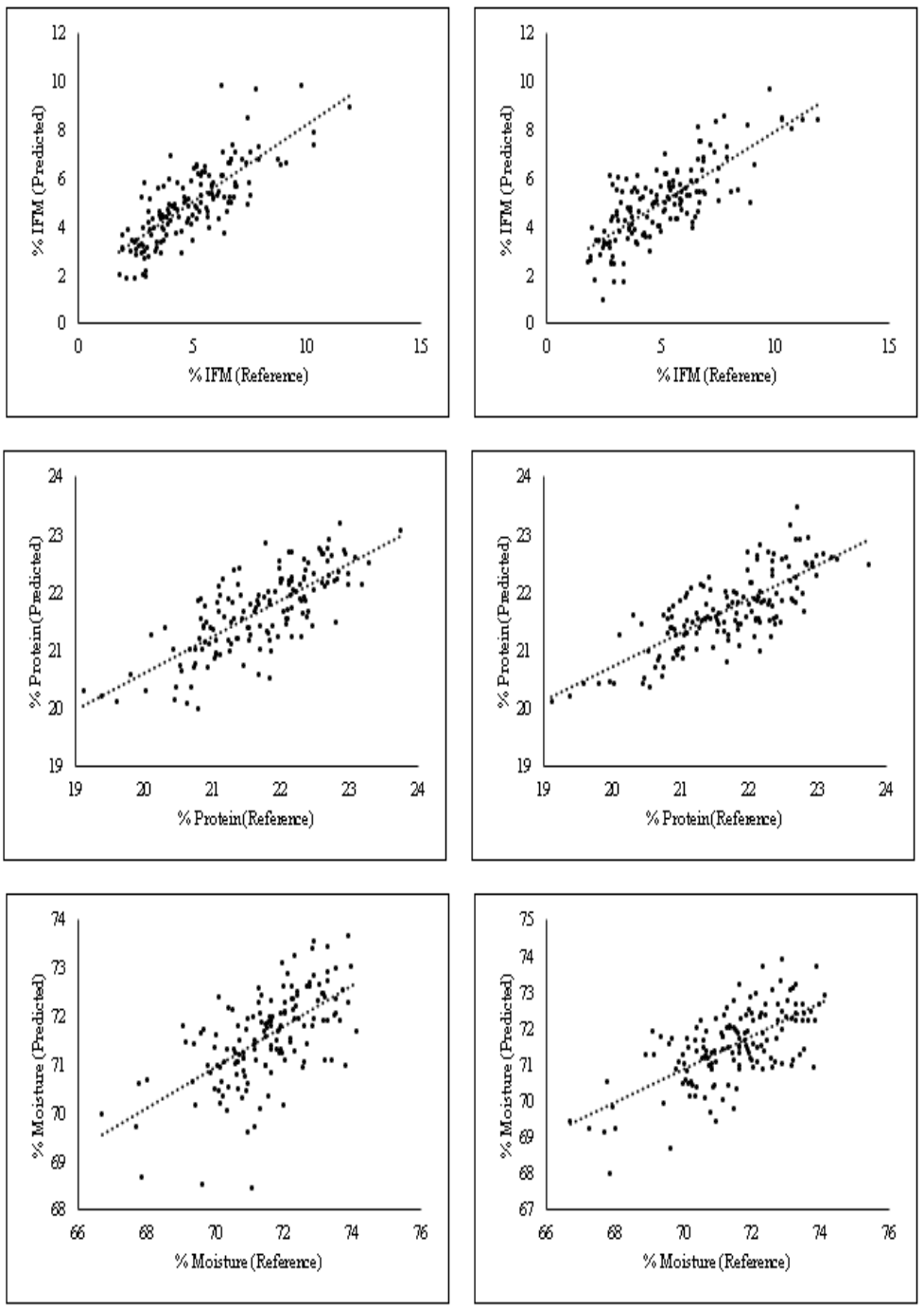

Figure 3. Reference versus NIR-predicted data for the validation intact loin samples set (N = 152) using MPLS (left part) and LOCAL (right part) algorithm.

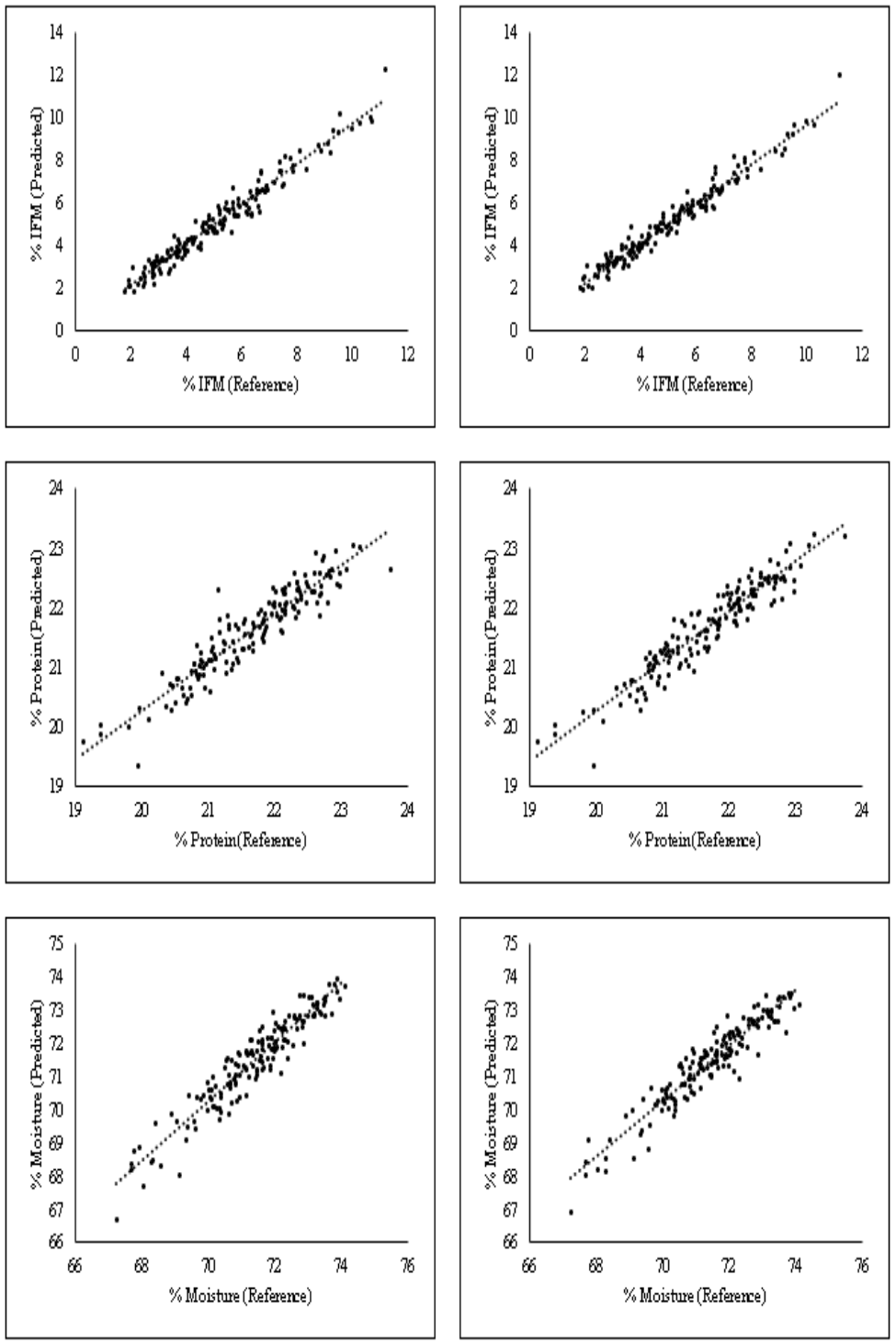

Figure 4. Reference versus NIR-predicted data for the validation homogenised loin samples set (N = 152) using MPLS (left part) and LOCAL (right part) algorithm.

Table 2. Descriptive statistics of the calibration and validation sets, for each parameter.

Parameter	Calibration set (N=322)					Validation set (N=152)				
	Min	Max	Mean	SD	CV (%)	Min	Max	Mean	SD	CV (%)
IFM (%)	1.16	12.90	4.97	2.10	42.25	1.80	11.90	5.21	2.20	42.23
Moisture (%)	66.20	74.70	71.50	1.50	2.10	66.70	74.10	71.3	1.60	2.24
Protein (%)	19.06	24.00	21.70	0.90	4.15	19.10	23.70	21.6	0.90	4.17

(Min: Minimum; Max: Maximum; SD: Standard Deviation).

Table 2. Statistics corresponding to the best calibration equations (N = 322 samples), for two analysis modes, for predicting intramuscular fat, protein, and moisture in Iberian pig loin samples using the MPLS algorithm.

Parameter	Analysis Mode	Math treatment	[a] N	[b] LVs	Range	[c] Mean	[d] SD	[e] SEC	[f] SECV	[g] R_{cv}^2	[h] RPD_{CV}	F	$F_{critical}$
IFM (%)	Intact	1,5,5,1	291	9	1.16-11.73	4.81	1.97	0.94	1.07	0.71	1.84	7.53	1.21
	Minced	2,5,5,1 + [i] SNV + [j] DT	302	5	1.16-11.12	4.79	1.93	0.36	0.39	0.96	4.95		
Protein (%)	Intact	1,5,5,1	303	10	19.06-24.00	21.71	0.84	0.44	0.50	0.64	1.68	3.19	1.21
	Minced	2,5,5,1 + [i] SNV + [j] DT	311	8	19.06-24.00	21.73	0.84	0.25	0.29	0.89	3.00		
Moisture (%)	Intact	1,5,5,1	305	9	66.32-74.68	71.59	1.41	0.88	0.97	0.54	1.45	3.62	1.21
	Minced	2,5,5,1 + [i] SNV + [j] DT	307	8	66.20-74.68	71.58	1.50	0.46	0.51	0.88	2.94		

[a] Number of samples

[b] Latent Variables

[c] Mean of the calibration set

[d] Standard deviation of the calibration set

[e] Standard error of calibration

[f] Standard error of cross validation

[g] Coefficient of determination of cross validation

[h] Residual predictive deviation for cross validation

[i] Standard normal variation

[j] Detrending.

Table 3. Validation statistics for predicting intramuscular fat, protein and moisture in IP intact loins applying the LOCAL algorithm.

Number of samples to select from the spectral library (k)	**1,5,5,1 + [a] SNV + [b] DT**						**2,5,5,1 + [a] SNV + [b] DT**					
	IMF		**Protein**		**Moisture**		**IMF**		**Protein**		**Moisture**	
	[c] SEP	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**
60	1.494	0.501	0.645	0.457	1.269	0.319	1.469	0.521	0.634	0.474	1.250	0.346
80	1.415	0.540	0.592	0.527	1.215	0.349	1.364	0.568	0.584	0.533	1.172	0.387
100	1.376	0.556	0.578	0.537	1.162	0.382	1.317	0.589	0.559	0.561	1.133	0.408
120	1.336	0.577	0.550	0.575	1.135	0.400	1.320	0.587	0.560	0.570	1.112	0.422
150	**1.110**	**0.600**	**0.547**	**0.600**	**1.110**	**0.400**	1.325	0.584	0.560	0.570	1.117	0.415
160	1.367	0.559	0.560	0.589	1.180	0.368	1.335	0.577	0.560	0.570	1.154	0.384

[a] Standard normal variation

[b] Detrending

[c] Standard error of prediction

[d] Coefficient of determination of prediction.

Table 4. Validation statistics for predicting intramuscular fat, protein and moisture in IP minced loins applying the LOCAL algorithm.

Number of samples to select from the spectral library (k)	**1,5,5,1 + [a] SNV + [b] DT**				**2,5,5,1 + [a] SNV + [b] DT**							
	IMF		**Protein**		**IMF**		**Protein**		**IMF**		**Protein**	
	[c] SEP	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**	**[c] SEP**	**[d] R_p^2**
60	0.454	0.944	0.373	0.830	0.513	0.874	0.449	0.942	0.303	0.810	0.516	0.871
80	0.450	0.950	0.306	0.849	0.502	0.880	0.448	0.950	0.288	0.864	0.507	0.876
100	0.420	0.956	0.297	0.857	0.500	0.880	0.427	0.955	0.270	0.882	0.500	0.880
120	0.414	0.957	0.289	0.865	0.489	0.886	0.411	0.958	0.268	0.883	0.483	0.890
150	0.408	0.959	0.287	0.869	0.487	0.888	**0.396**	**1.000**	**0.267**	**0.900**	**0.475**	**0.900**
160	0.401	0.960	0.285	0.870	0.482	0.890	0.408	0.959	0.268	0.886	0.481	0.891

[a] Standard normal variation

[b] Detrending

[c] Standard error of prediction

[d] Coefficient of determination of prediction.

Table 5. Validation statistics for the best models for the prediction of IFM, protein and moisture in intact Iberian loin samples using MPLS and LOCAL algorithms.

Parameter	Regression method	Math treatment	[c] SEP	[d] SEP(c)	Bias	[e] R_p^2	Slope	[f] RPD_P
IFM (%)	MPLS	1,5,5,1	1.012	1.013	-0.024	0.690	0.940	1.560
	LOCAL (150, 16, 3)	1,5,5,1 + [a] SNV + [b] DT	1.110	1.114	0.055	0.600	1.000	1.383
Protein (%)	MPLS	1,5,5,1	0.490	0.491	0.032	0.630	0.910	1.410
	LOCAL (150, 16, 3)	1,5,5,1 + [a] SNV + [b] DT	0.547	0.548	-0.024	0.600	1.000	1.129
Moisture (%)	MPLS	1,5,5,1	0.980	0.983	-0.006	0.470	0.820	0.960
	LOCAL (150, 16, 3)	1,5,5,1 + [a] SNV + [b] DT	1.110	1.110	-0.084	0.400	0.935	0.891

[a] Standard normal variation

[b] Detrending

[c] Standard error of prediction

[d] Standard error of prediction corrected by bias

[e] Coefficient of determination of prediction

[f] Residual predictive deviation for prediction.

Table 6. Validation statistics for the best models for the prediction of IFM, protein and moisture in minced Iberian loin samples using MPLS and LOCAL algorithms.

Parameter	Regression method	Math treatment	[c] SEP	[d] SEP(c)	Bias	[e] R_p^2	Slope	[f] RPD_P
IFM (%)	MPLS	2,5,5,1 + [a] SNV + [b] DT	0.372	0.373	-0.018	0.970	0.970	5.430
	LOCAL (150, 16, 3)	2,5,5,1 + [a] SNV + [b] DT	0.396	0.397	-0.061	1.000	1.021	4.696
Protein (%)	MPLS	2,5,5,1 + [a] SNV + [b] DT	0.284	0.285	-0.007	0.890	0.900	2.610
	LOCAL (150, 16, 3)	2,5,5,1 + [a] SNV + [b] DT	0.267	0.267	0.023	0.900	1.050	2.600
Moisture (%)	MPLS	2,5,5,1 + [a] SNV + [b] DT	0.436	0.438	-0.001	0.900	0.910	3.170
	LOCAL (150, 16, 3)	2,5,5,1 + [a] SNV + [b] DT	0.475	0.478	0.034	0.900	1.015	2.677

[a] Standard normal variation

[b] Detrending

[c] Standard error of prediction

[d] Standard error of prediction corrected by bias

[e] Coefficient of determination of prediction

[f] Residual predictive deviation for prediction.

Table 7. Comparison between MPLS and LOCAL algorithms.

Parameter	Analysis Mode	SEP of the best GLOBAL equation	SEP of the best LOCAL equation	F	Fcritical	F *vs* Fcritical	
IFM (%)	Intact	1.012	1.110	1.203	1.308	F < Fcritical	
	Minced	0.372	0.396	1.133	1.308	F < Fcritical	
Protein (%)	Intact	0.490	0.547	1.246	1.308	F < Fcritical	No statistical differences
	Minced	0.284	0.267	1.131	1.308	F < Fcritical	
Moisture (%)	Intact	0.980	1.110	1.283	1.308	F < Fcritical	
	Minced	0.436	0.475	1.189	1.308	F < Fcritical	

CHAPTER IV:

INSTRUMENTAL COMPARISON FOR THE LABELLING FROZEN IBERIAN PORK LOINS

4.1. Non-destructive Near Infrared Spectroscopy for the labelling of frozen Iberian pork loins. Meat Science 175, 108440 (2021)

Non-destructive Near Infrared Spectroscopy for the labelling of frozen Iberian pork loins

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Abstract

Conventional chemical analyses of meat products are time-consuming, expensive, and destructive. The advantages of NIR spectroscopy are its speed, portability, suitability for both at-line and on-line analysis, low cost, and the possibility of simultaneously measuring many different parameters in a large number of samples. The purpose of this study was to develop and validate calibrations for the prediction of moisture, protein and fat in Iberian pig pork loins using an FT-NIR instrument coupled to a 5-metre fibre optic sensor head. The best equations obtained for intact loin in both modes of analysis (full and optimal spectral range) displayed Standard Error of Cross-Validation (RMSECV) of 1.06% and 1.09% and Determination Coefficient of Cross-Validation (RCV2) of 0.69 and 0.77 for fat: RMSECV of 0.87% and 0.77% and RCV2 of 0.67 and 0.73 for moisture; while for protein, the RMSECV values were 0.51% and 0.49% and the RCV2 values were 0.66 and 0.70.

Keywords: Iberian pig loin; FT-NIR spectroscopy; fibre optic probe; intact and minced meat analysis; chemical composition.

1. Introduction

Currently, there are more than ten different cuts of meat from acorns-fed Iberian pigs (AFIP), but the loin is one of the most expensive pieces. As stablished in article 6.3 in the Quality Standard for Iberian pork products, Iberian pigs fed on acorns and pasture grass are slaughtered from December 15 to March 31. Therefore, AFIP loin can be considered seasonal food, which only can be found fresh, at the marketplace, during a limited period of the year (BOE, 2014). This makes necessary that industrials pack and deep-freezing meat of the season. In fact, there are two main reasons to explain why industrials freeze the fresh meat. Firstly, because sometimes they do not have enough capacity to process or sell — as fresh meat — all the production in such short time. Secondly, as a marketing strategy aimed to offer their seasonal meat all year round.

Consumers demand foods of high "integrity", which is a comprehensive term for nutritive, healthy, taste, safe, authentic, traceable, as well ethically, environment-friendly and sustainably food product (Elliot, 2014). It must be highlighted that selling frozen-thawed meat is a legal practice, but consumers must be informed with a correct labelling about what they purchase. Thus, the label of a meat product that has been previously frozen and is sold defrosted, shall contain the designation "defrosted" (EU, 2011). However, one of the most frequent meat frauds is to declare as "fresh meat", meat previously frozen-thawed. In this case, the attractiveness for these fraudulent practices is obvious since fresh meat is more valuable than frozen meat. Furthermore, fresh and frozen-thawed pig meat samples are visually indistinguishable (Ballin & Lametsch, 2008).

Until now, many studies have been conducted to investigate the differences between fresh and frozen-thawed meats by using various approaches including enzymatic (Ellerbroek, Lichtenberg, & Weise, 1995; Gottesmann & Hamm, 1983), physiological (Park et al., 2000), physical (Carballo, Cofrades, Solas, & Jiménez-Colmenero, 2000; Evans, Nott, Kshirsagar, & Hall, 1998) and microbiological (Kim et al., 2013) methods. However, most of these currently

available testing methods are laborious, time consuming, invasive, expensive and require sophisticated laboratory procedures with tedious sample preparation steps (Bae et al., 2014; Mamani-Linares, Gallo, & Alomar, 2012).

During the past five years, Near Infrared Spectroscopy (NIRS) combined with specific data processing techniques has been considered as one of the most challenging non-targeted methods for the authentication and confirmation of the integrity of food products (Pérez-Marín, 2019). This has been possible thanks to its high scientific reputation and, specifically, its growing acceptance and uptake by food industrials.

Moreover, in the last decade, it has appeared in the market a range of miniaturized NIR sensors and robust instruments coupled to long fibre-optic probes, which make possible to implement NIRS sensors in the processing plant for real-time *in situ* measurements and decision making. However, most of these new instruments were designed for the pharmaceutical and chemical industries rather than for food applications, where the complex nature of the food matrix is a particular challenge (Garrido-Varo, Riccioli, Fearn, De Pedro, & Pérez-Marín, 2018a; Crocombe, 2018). More recently, scientific papers are appearing for implementing NIR for *in situ* analysis in real food industry situations, where specific developments and adaptations are needed for each food product and application (Garrido et al., 2018a; Garrido-Varo, Sánchez-Bonilla, Maroto-Molina, Riccioli, & Pérez-Marín, 2018b).

Some previous works (Downey & Beauchêne, 1997; Chen, Cai, Wan, & Zhao, g2011; Grunert, Stephan, Ehling-Schulz, & Johler, 2016; Huang, Li, Wu, Dong, & Wang, 2016; Attanassova, Stoyanchev, Yorgov, & Nachev, 2018) have investigated the usefulness of NIR for discriminating fresh *vs* frozen-thawed meat cuts of different species (beef, poultry and pork). Some of them were developed over minced meat and others over intact meat. Nevertheless, they should be considered as feasibility studies, because the number of samples used is rather low (between 16 and 120) to obtain robust predictive models.

One important and highly demanded research in that field is the rigorous scientific evaluation of novel portable and on-line NIR instrumentation

appearing in the market, useful to help industrials and inspection bodies to take decisions about the uptake of NIR technology to fight against food frauds. Another important challenge is the correct validation of the so-called "non-targeted" methodologies for food authentication issues, being NIRS one of the most promising (Baeten et al., 2016; Pérez-Marín, 2019).

Considering this, the present study has been developed with two main goals. Firstly, to compare the performance of two different near infrared spectroscopy instruments — based on Fourier Transform and Linear Variable Filter technologies — for the *in-situ* discrimination of fresh and frozen-thawed acorns-fed Iberian pig loins using Partial Least Squares Discriminant Analysis. Secondly, the evaluation of the performance of these binary models using scalars and graphical methods.

2. Material and methods

2.1. Samples

This study was carried out with AFIP loins (*Longissimus dorsi)* belonging to animals slaughtered during the first three months of 2018 and 2019. The animals were reared in the region of Badajoz (Spain), ending their growing - the last fattening phase - in "Montanera" (i.e. in free range and fed with acorn and grass in the Dehesa agro-forestry system). They were slaughtered with an average weight of 153 ± 11 kg.

A total of 238 samples were evaluated in this study: 143 fresh samples (F) and 95 frozen-thawed samples (FT). Freezing was carried out in freezing tunnels at -40ºC with recirculating air systems and a freezing speed of 1-5 cm/h until the centre of the sample reached the temperature desired (-20ºC), at which time they were stored in conventional freezing chambers by air recirculation at -20ºC until their analysis, three months (N=47) or six months (N=48). Freezing process was developed using the whole loins. The loin steaks samples were obtained when the whole loins were thawed (FT samples) or previously to freezing (F samples). Both types' samples, fresh and frozen-thawed were taken from adjacent areas on the loin.

All samples were pieces of 2.5-3 cm thick and 60-70 g weight, which were cut 10 cm from de caudal zone. The fresh meat samples (N=143) were immediately packed in plastic bags, vacuum-sealed and transported to the University of Cordoba, refrigerated at 4ºC temperature. The samples were kept refrigerated at 4ºC, until the next day when they were analysed by NIRS. The defrosting process was carried out in conventional refrigeration chambers by recirculation of air at 0-2ºC for 48 hours until whole loins were tempered (0ºC). After this, FT samples were taken and were refrigerated at 4ºC during the day before the analysis, and then tempered to room temperature (20 ± 2 ºC) for NIR analysis.

2.2. NIR Spectral Measurements

The NIR spectra of the loin samples were collected using two instruments with very different optical characteristics, size, weight and price.

A MicroNIR™ 1700 microspectrophotometer (VIAVI Solutions, Inc., San Jose, California, USA) was selected by its high portability (250 g weight), which make it very suitable to be used in different points of the meat supply chain (Garrido et al., 2018a). The instrument uses Linear Variable Filters (LVF) as dispersing element, without moving parts and a single InGaAs photodetector array (O'Brien et al., 2012; Alcalá et al., 2013). Spectra were obtained in reflectance mode in the spectral range from 908.1 to 1676.2 nm, with a bandwidth of 6.2 nm. The data were recorded using the MicroNir Pro v2.2 software (VIAVI Solutions). The instrument's performance was checked every 10 minutes. A white reference measurement was obtained using a NIR reflectance standard (Spectralon™) with a 99% diffuse reflectance, while a dark reference was obtained from a fixed point in the room (Torres, Sánchez, Entrenas, Garrido-Varo, & Pérez-Marín, 2019).

A MATRIX-F Fourier Transform NIR spectrophotometer (Bruker Optics GmbH, Ettlingen, Germany) was selected for this study, since joins high technical optical features and a number of attributes that makes it very suitable for *in situ*/on-line/in-line analysis in the agri-food industry (Garrido et al.,

2018b). The spectra were recorded in reflectance mode in the spectral range 834.2-2502.6 nm, with a bandwidth of 1.1 nm. The MATRIX-F was interfaced to a 100 m length fibre optic NIR illumination and detection head, which contains tungsten sources to illuminate the sample.

The sample was located 10 cm away from the head, which allowed measuring of a sample area of around 38.46 cm^2 (Cáceres-Nevado, Garrido-Varo, De Pedro-Sanz, & Pérez-Marín, 2019). Spectral data were recorded using OPUS 7.0.122 software (Bruker Optics GmbH, Ettlingen, Germany), selecting 32 scans per spectra at a speed of 10 kHz with 16 cm^{-1} resolutions. An internal white reference was also collected every thirty minutes. These parameters were previously established in other studies carried out using the same instrument (Garrido et al., 2018b).

Two spectra per sample were collected with Matrix-F and four with MicroNIR™ 1700, one and two for each side of the loin samples, respectively, to minimize any possible effects of structural variation in the samples. The average spectra were used for further data treatment.

2.3. Multivariate data analysis

The spectral data, expressed as (log1/R) being R the reflectance, were exported from the MicroNIR Pro v2.2 and OPUS 7.0.122 software into the PLS Toolbox (Eigenvector Research Inc., Wenatchee, WA), working under Matlab R2013b (The Mathworks, Inc., Natick, MA), for the data processing.

2.3.1. Principal component analysis (PCA)

PCA reduces the co-linearity in the data and is the most used unsupervised chemometric tool for data exploration and pattern recognition. PCA transforms the original spectral variables into a smaller number of linearly independent new variables, called principal components (PCs) (Cowe & McNicol, 1985; Mark, 2001). PCA was performed using the raw data and the data pretreated, using a second derivative signal pretreatment to reduce baseline variation and enhance the spectral features (Naes, Isaksson, Fearn, & Davies, 2002).

2.3.2. Discriminant Partial Least Squares (PLS-DA) model.

PLS-DA is one of the most powerful classification methods and it is, normally, the reference method in supervised classification studies within food adulteration, authenticity and traceability (Khakimov, Gurdenizc & Engelsen, 2015). In the present study, PLS-DA models were developed to identify fresh or frozen-thawed intact Iberian pig meat cuts, using the two evaluated NIR instruments, different pre-processing treatments, and spectral ranges. The MicroNIR™ 1700 has a much largest bandwidth (6.2 nm) than the one of the Matrix-F (1.1 nm). Therefore, in order to better understand how much this important technical specification of the instrument could affect to the classification, PLS–DA models were also developed for the Matrix-F using the spectral range and the bandwidth of the MicroNIR™ 1700.

In order to minimize additive and multiplicative scatter effects and baseline shifts, several spectral pre-treatments (standard normal variate, detrend, mean centre and Savitzky-Golay first and second derivatives) and their combination were evaluated for the development of the PLS-DA models. The derivative pre-treatments tried were 1,5,5; 1,5,11; 2,5,5 and 2,5,11, where the first digit indicates the derivative order, the second digit the polynomial order and the third digit is the filter width (Savitzky & Golay,1964; Barnes, Dhanoa & Lister, 1989; Naes et al., 2002).

Partial Least Square-Discriminant Analysis (PLS-DA) models were developed to classify the samples in two classes. Class 1 was referred to fresh (F) samples and class 2 was referred to frozen-thawed (FT) samples, including in this last one both samples frozen during three months and those frozen during six months.

The models seek to correlate spectral variations (X) with defined classes (fresh or frozen-thawed), attempting to maximize the covariance between the two types of variables. In this type of approach, the Y variables used were not continuous, as they are in quantitative analysis, but rather categorical “dummy” variables (Naes et al., 2002; Kramer, Workman & Reeves III, 2004; Pérez-Marín, Garrido-Varo, & Guerrero-Ginel, 2006). In the present work, the dummy

variable assigned for the class 1 "fresh meat" was 1 and for the class 2 "frozen-thawed meat" was 0.

The full data set (N=143 F and N=95 FT samples) was divided into training and validation sets (80% and 20% of total samples respectively) using the Kennard-Stone algorithm, which enables to select samples with a uniform distribution over the predictor space (Kennard & Stone, 1969). The training set consisted of 192 samples (115 F and 77 FT) and the validation set of 46 samples (28 F and 18 FT). The optimal number of latent variables (LV) for the PLS-DA models was determined using tenfold cross validation (CV) with venetian blinds method. The number of LV yielding the lowest percentage of misclassification (error rate) was chosen as the optimal model.

For the MicroNIR™ 1700, the PLS-DA models were developed comparing two spectral ranges: 908.1-1676.2 and 908.1-1403.6 nm, this last one eliminating areas of low signal to noise ratio. For the Matrix-F instrument, the spectral ranges evaluated were 834.2-2502.6 and 908.1-1676.2 nm, in this last case reducing also the bandwidth to that of the MicroNIR™ 1700 (6.2 nm).

2.3.3. Evaluation and interpretation of PLS-DA models.

Firstly, the best PLS-DA models developed with the training set were selected using the percentage of samples correctly classified, when the validation samples were predicted. For the evaluation and interpretation of the predictive capacity of each of the best PLS-DA models, the following statistics were used:

- ✓ Sensitivity, specificity and accuracy. These three statistics measure the performance of the test, being the most commonly associated with a binary classification test (Yerushalmy, 1947; Feldsine, Abeyta & Andrews, 2002; Tharwat, 2018).

$$Sensitivity = \frac{TP}{TP + FN} \quad (1)$$

$$Specificity = \frac{TN}{TN + FP} \quad (2)$$

$$Accuracy = \frac{TN + TP}{TN + TP + FN + FP} \quad (3)$$

where TP = True Positive; TN = True Negative; FP = False Positive; FN = False Negative.

Here, the sensitivity, specificity and accuracy are expressed in percentages.

- ✓ Matthews Correlation Coefficient (MCC) (Matthews, 1975).

$$MCC = \frac{(TP \times TN - TP \times FN)}{\sqrt{(TP + FN)(TP + FP)(TN + FN)(TN + FP)}} \quad (4)$$

A value of 1 for the MCC indicates a perfect prediction; -1 represents a total disagreement between prediction and true values; and zero means "no better than random prediction" (Boughorbel, Jarray & El-Anbari, 2017; Tharwat, 2018).

- ✓ Receiver Operating Characteristics (ROC) and the Area Under the ROC Curve (AUC).

The ROC curve is a two-dimensional graph in which the sensitivity or true positive rate is plotted in the y-axis and the false positive rate (i.e. "1-specificity") is plotted in the x-axis The ROC curve is generated by changing the threshold on the confidence score; hence, each threshold generates only one point in the ROC curve. To compare classification models, ROC curves can be reduced to a single scalar value representing expected performance. A common method is to calculate the area under the ROC curve (AUC). Since the AUC is a portion of the area of the unit square, its value will always be between 0 and 1.0. A perfect test has an area of 1.0, whilst a non-discriminating test has an area of 0.5 (Moscetti et al. 2013; Oliveri & Downey, 2012). Further details about the use and interpretation of ROC graphs may be found elsewhere in the literature (Tharwat, 2018; Fawcett, 2016: Streiner, Norman & Cairney, 2015; Broadhurst & Kell, 2006).

PLS loadings and Variable Importance in Projection (VIP) were used for model's interpretation. The most relevant wavelengths in the PLS-DA models were identified using the loading weights and the VIP. The loading weights show how much each wavelength contributes to explain the response variation along

each latent variable of the model. The VIP measure is defined as expressed in Eq.5.

$$VIP_j = \sqrt{p \sum_{a=1}^{A} \left[SS_a \left(\frac{W_{aj}}{\|W_a\|} \right)^2 \right] / \sum_{a=1}^{A} SS_a} \quad (5)$$

where SS_a is the sum of squares explained by the a^{th} latent variable. The VIP_j weight is a measurement of the contribution of each *j* variable according to the variance explained by each latent variable, where $\left(\frac{W_{aj}}{\|W_a\|}\right)^2$ represents the importance of the j^{th} variable (Wold, Johansson & Cochi, 1993; Mehmood, Liland, Snipen, & Saebo, 2012).

Wavelengths with higher VIP scores are considered more relevant in the classification (Bjarnestad & Dahlman, 2002). According to Eriksson et al., (2001), the wavelengths could be classified according to their relevance in explaining Y: VIP > 1.0 (highly influential), 0.8 < VIP < 1.0 (moderately influential) and VIP < 0.8 (less influential).

3. Results and discussion

3.1. Spectra information.

The average spectra of the raw data (log 1/R) from fresh and frozen-thawed samples, analysed in the two instruments studied, are shown in Fig.1. The NIR spectra have similar shapes and show the same main peaks and valleys throughout the NIR range, and there are not noticeable absorbance differences at different wavelengths depending on the conservation process (fresh and frozen-thawed). As expected, there is a lack of clear visual differences between the fresh and frozen-thawed spectra, apart from a slightly less absorbance of the frozen-thawed meat samples. The observed additive baseline drifts presumably arise from alterations in the physical structure of at least in the surface layer of meat (Downey & Beauchêne, 1997).

Furthermore, it can be observed that above 1400-1600 nm not enough signal reaches the detector in both instruments. Downey & Beauchêne (1997) using a FOSS NIRSystems 6500 monochromator -coupled to an interactance fibre optic

probe- and scanning intact beef loins, observed similar problem but at a lower wavelength (1100 nm). This is a demonstration of the advantages in optical designs that have occurred in the last ten years.

By using only, the raw spectral data it seems risky to make any interpretation of relevant bands for the discrimination. Later on, attempts will be done with that purpose using the PLS loadings and VIP graphs.

3.2. Principal Component Analysis

PCA analysis was applied to show intuitive differences in the loin cuts that have been undergone to different conservation processes (F and FT).

Fig. 2 shows the scores on PC1 and PC3 of the all samples, with the F and FT classes distinguished by plotting symbol and colour. PC1 and PC3 explained together the 93.22% and 96.03% of the variance in the spectral data for the loin samples analysed in the MicroNIR™ 1700 and Matrix-F, respectively. Although there is overlap between the classes, there is enough separation for the MATRIX-F plot, to suggest that classification may be possible using these spectra. In the case of MicroNIR™ 1700, the overlapping is much more visible, making difficult to distinctly discriminate between fresh and frozen-thawed loin steaks, but it is expected that the use of more latent variables for the development of the PLS models could help in this discrimination.

There are three samples located at the up-right side of the Fig. 2b which could be considered spectral outliers. However, given that only happens for the MATRIX-F, it was decided to keep them for a more rigorous comparison of the performance of both instruments.

3.3. *Development and validation of PLS-DA models for discriminating fresh and frozen-thawed Iberian pig loin samples.*

The best PLS-DA models obtained with the spectral data collected in the MicroNIR™ 1700 and the Matrix-F devices, for each one of spectral ranges studied, are shown in Tables 1 and 2, respectively. The PLS-DA models obtained with both instruments offered very high discriminant ability, showing consistent values for sensitivity, specificity and accuracy. These values were greater than

90%, both in cross validation and prediction. One exception was the model developed with the Matrix-F but using the same range and the bandwidth of the MicroNIR™ 1700, for which the prediction results got worse considerably.

Sensitivity, in the context of the paper, is the ability of the model to correctly classify the authentic fresh loin samples, while specificity is referred to the ratio of samples classified as frozen-thawed to the total number of frozen-thawed samples. In other words, specificity is the capacity of the model to correctly identify the samples of non-authentic fresh loin samples. The accuracy is expected to measure how well the models predict both classes.

Accuracy is a common and generalized statistic to measure the performance of classification models, however many authors reported that it can be a misleading metric evaluator when the data are imbalanced. Therefore, several other metrics have been proposed, such as the Matthews Correlation Coefficient (MCC), which represents the correlation between the observed and predicted classifications. The MCC produces a high score only if the prediction obtains good results in all the categories (i.e., true positives and true negatives). That means MCC score is high only if the model is performing well on both the negative and the positive elements. This fact can be observed in Table 2, looking carefully at the descriptive statistics for the model developed with the Matrix-F but using the range and the bandwidth of the MicroNIR™ 1700. In this case, at prediction level, the model seems to recognize the 100% of the frozen-thawed samples and the accuracy is relatively high (almost 77%). However, its ability to recognize the authentic fresh IP loin samples is only 53.57%. Therefore, the MCC score is only 0.56, because - as it was explained previously - MCC only will produce high values in the case that the model is working well for true positives (fresh) and true negatives (frozen-thawed).

According to all the performance statistics and, in particular, to the scores for MCC, the best PLS-DA model for the MicroNIR™ 1700 was obtained by using as signal pre-treatment, the combination of first derivative and smoothing followed by mean centering, detrending and SNV (Table 1). For the Matrix-F, the best model was obtained using a first derivative and smoothing followed by

detrending and mean centering (Table 2). Curiously, the best model obtained with the spectra collected in the Matrix-F is the one using the same spectral region of the MicroNIR™ 1700, but with its own resolution (1.1 nm instead of 6.2 nm).

This information is useful to complete what it has been previously appreciated in Fig. 1, where it can be observed that not enough signal reaches the detector from 1400 nm. However, it can now be confirmed that, for the type of application considered in this paper (intact meat cuts), the useful wavelength range can be extended up to 1676 nm.

Fig. 3 and 4 show the classification results for the training and test sets for the best PLS-DA models developed on both instruments. The dashed line indicates the threshold value, which is the cut-off for whether a sample belongs to fresh or frozen-thawed classes. In this paper, the threshold value considered was 0.5. Thus, spectra with prediction values greater than the threshold are classified as fresh samples and if the prediction values are lower than the threshold are classified as frozen-thawed samples. As can been observed in Fig. 3, all samples belonging to the training sets are correctly classified by the Matrix-F, while for the MicroNIR™ 1700 there is one misclassified sample, corresponding to one of the samples frozen for 6 months. Furthermore, for the Matrix-F it could be also appreciated that the distance between classes is higher, i.e., the discrimination is clearer. Finally, for the test sets (Fig. 4), both instruments correctly classified all samples.

The evaluation of the discriminant results was completed with the ROC curves. ROC curves have been used in clinical studies to choose the most appropriate cut-off for a diagnostics test, for many years now. They are widely considered one the best means by which to describe the utility of a variable in binary classification models (Andersen & Bro, 2010). However, the use of ROC curves for the evaluation of classification models in food authentication issues is more limited (Riccioli, Pérez-Marín & Garrido-Varo, 2018). In the present study, the ROC curves have been used to add statistical information that can be of help to the EU normative bodies for a more practical understanding of the contribution

of NIR technology for the detection of fresh and frozen-thawed meat cuts. They can be also useful to compare classification performance using NIR or others more expensive and destructive methods.

ROC curves, for the validation of the best discriminant model selected with each instrument, are shown in Fig. 5. Here, the specificity (the number of samples predicted as not belonging to a class divided by the number of samples that are really not in the class) is plotted against sensitivity (the number of samples predicted as belonging to a class divided by the number of samples that are really in the class), for each of the two classes. Fixing the threshold for both classes at 0.5, the model sensitivity (True Positive Rate) and model specificity (True Negative Rate or One minus the False Positive Rate) was 1.0 for both instruments (see red circle in Fig. 5, the umbral position), leading to an overall accuracy of 100% (see Table 1 and 2), respectively. On the other hand, the AUC values indicate a perfect test because its value is 1.0 using MicroNIR™ and Matrix-F.

In addition, this methodology, that could be apply also to training set, could be very useful to set new thresholds, in particular, when the misbalancing of the data commits the results or in order to guaranty to the consumers or inspection bodies the authenticity of one of the classes. In the present case of study, considering the excellent results, where the discrimination is perfect for the Matrix-F device or almost perfect for the MicroNIR™ 1700, this consideration was not evaluated.

To the best of our knowledge, only three papers have been published concerning the use of NIR to differentiate intact cuts of fresh *vs* frozen-thawed or fresh *vs* freeze meat samples (Downey & Beauchêne, 1997; Chen et al., 2011; Atanassova et al., 2018). However, the two first papers provided results using high-performance spectrophotometers adapted to laboratory work conditions, as FOSS NIRSystems 6500 monochromator and Antaris II FT-NIR, Thermo Electron Co., USA (1000-2500 nm), and only the last one reported information using a portable instrument, the NIR Quest 512, Ocean Optics, Inc. (900-1700 nm).

A general comment to the three papers is that they should be considered what is called a "viability study", because they used very low number of samples, such as 32 beef samples (Downey & Beauchêne, 1997), 67 white pork samples (Chen et al., 2011) and 24 poultry samples (Attanassova, et al., 2018). Furthermore, it is impossible to draw conclusions from the robustness of the models, due not only to the limited number of samples used in validation, but also because they only provide information on the percentage of correctly classified samples, without any other information that provides, for example, information on the number of false positives (fresh) or negatives (frozen-thawed).

The models obtained in the present work analysing intact IP loins, both with a portable device and an instrument adapted to on-line measurements, have a better performance than other obtained using homogenized minced samples (Grunert et al., 2016; Huang et al., 2016). It should be noted that in that study, the best model developed with a high scientific and technical FT-NIR instrument is slightly superior to the one obtained with a portable LVF spectrophotometer.

As explained in section 3.1, the NIR spectra from fresh and frozen-thawed samples have similar pattern and show the same main peaks and valleys throughout the NIR range, and not noticeable absorbance differences at different wavelengths were observed (Fig. 1) depending on the conservation process (fresh and frozen-thawed). Therefore, it was undertaken the study of the PLS-DA loadings and VIP score values for the understanding of the basis of this classification (Fig. 6 and 7).

The most important wavelengths used by the PLS-DA models were observed in the vicinity of 900, 1100, 1200 and 1400 nm. Several authors stated that similar spectral regions are related to meat tenderness (Barlocco, Vadell, Ballesteros, Galietta, & Cozzolino, 2006; Prieto, Roehe, Lavín, Batten, & Andrés, 2009), water holding capacity (Bowker, Hawkins, & Zhuang, 2014) and discrimination of fresh and frozen-thawed poultry breast meat (Attanasova, et al., 2018).

However, the variable importance in projection (VIP) values provide a more quantitative information and are useful in understanding the contribution of each wavelength to the PLS discrimination model (Farrésa et al., 2015).

It was noted (Fig. 6 and 7) that the VIP values reveals the existence of relevant wavelengths in the same region than the observed in the PLS-loadings plots. An important remark is that those wavelengths were similar for the models developed in both evaluated instruments. As mentioned previously, VIP scores > 1.0 should be considered highly influential, 0.8 < VIP < 1.0 moderately influential and VIP < 0.8 less influential.

According to that, and for both instruments, wavelengths in the 930-960, 1130-1140, 1200-1230 and 1350-1450 nm, which showed VIP values much higher than 1, should be considered as informative of the discrimination between fresh and frozen-thawed samples.

Looking at Fig. 6 and Fig. 7, it can be observed that for any important wavelength regions, there are more relevant wavelengths with high VIP values when the models were developed using the Matrix-F than when the MicroNIR™ 1700 was used. This could be due to the major resolution of the Matrix-F instrument (1.1 nm vs 6.2 nm). The spectral ranges selected by the VIP (930-960, 1130-1140, 1200-1230 and 1350-1450 nm, and 1640-1670 only in the models developed using the MicroNIR™ 1700) are related to the well-known chemical changes occurring in frozen and thawed meats include water losses, protein insolubilisation and lipid hydrolysis (Giannakourou & Giannou, 2015). Workman & Weyer (2008) stated that region around 960 and 1400 nm could be associated with O-H bonds, mainly related to the moisture content in the muscle tissue. The water band of the fresh meat has a slightly higher absorption value, and this could be attributed to the moisture of the frozen-thawed meat. The region around 930 nm is related to the third overtones of C-H stretching modes while regions around 1100-1140, 1230 and 1640-1670 nm could be associated with the C-H second overtones. Lastly, band at 1430 nm represents the N-H stretch first overtone related to the $CONH_2$ group associated with the peptide bonds in

proteins. This band was lower for frozen-thawed meat samples, thus suggesting that protein denaturation occurred due to the freezing process.

Changes in the C-H bonds modes may be influenced by proteolysis and modification of the muscle lipids during thawing. Such changes may also affect the water interactions of the matrix with a different fraction of free or bound water species.

Freezing procedures, frozen storage and thawing procedures could affect meat quality attributes such as thaw loss, colour, and tenderness. Meat freezing process at different freezing rates forms ice crystals with different sizes and distributions. Slow freezing results in intercellular ice crystals, while quick freezing procedures intracellular ones. Fast freezing and low thawing seem to be the optimal combination, which can decrease thaw loss, cook loss and pressing loss, and can maintain the functional properties of frozen pork meat (Yu et al., 2010). Freezing and thawing processing has some bad effects on the structure of meat, which are more pronounced when the sample size is smaller. All that physical-chemical changes could affect to the log 1/R spectral signature. This fact should be studied in the future.

4. Conclusions

In this study, reliable classification PLS-DA models have been obtained for distinguishing fresh and frozen-thawed Iberian pig loin samples, using both a portable, low-cost spectrometer, based on Linear Variable Filter technologies and a FT-NIR spectrometer coupled to a fibre optic probe adapted to on-line analysis. The PLS-DA models obtained for both instruments show similar performance according to the different calculated scalars and graph metrics. The results illustrate that other scalars and graphical metrics, apart from just the estimation of the accuracy, are important for the evaluation of performance and interpretation of the classification models, as it is traditional in qualitative studies. It is strongly recommended to consider metrics such as those used in this work for food authentication, adulteration and integrity studies using non-targeted methods as NIRS.

That finding is important for potential users of the technology. The miniature and portable instrument can be more adapted for verification at retail level, and the FT-NIR instrument can be used at large meat processing plants, placing over a conveyor belt, to authenticate the meat cuts which were bought from others. In addition, the same instrument can be used for many other applications, by using different illumination and detection heads, located at different points in the plant, just with one single spectrometer. Although it has been demonstrated that even bandwidth seems to influence model performance, also other optical characteristics existing in the LVF instrument are of importance.

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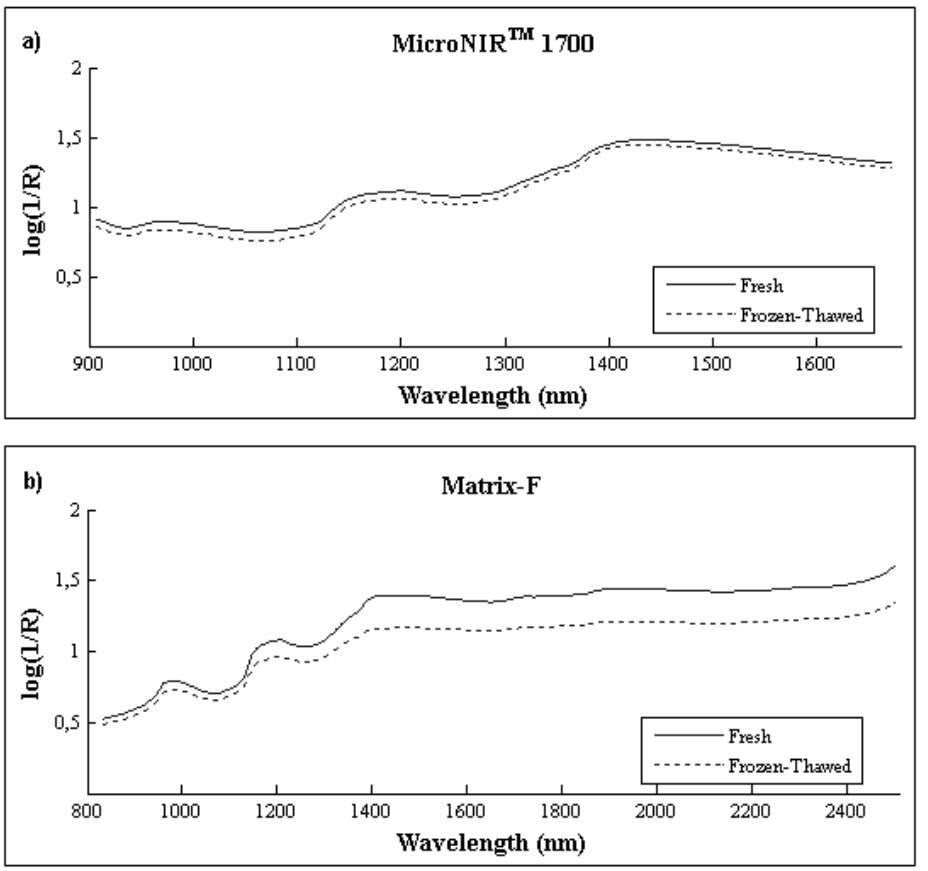

Figure 1. Average raw spectrum obtained from fresh and frozen-thawed Iberian pig meat samples. **a.** Average spectrum obtained with MicroNIRS. **b.** Average spectrum obtained with Matrix-F.

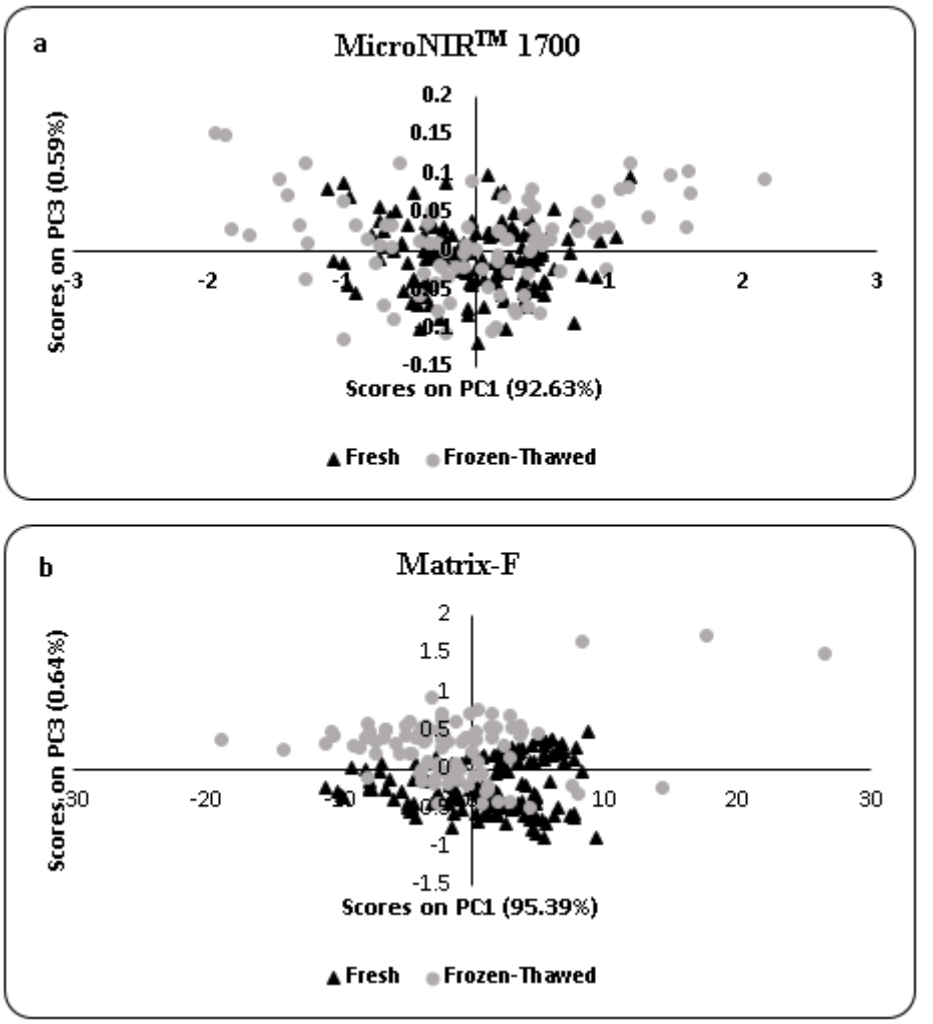

Figure 2. Scores plot for the first (PC1) and third (PC3) principal components using the MicroNIR™ 1700 in the spectral range 908.1-1676.2 nm (a), and Matrix-F in the spectral range 834.2-2502.6 nm (b).

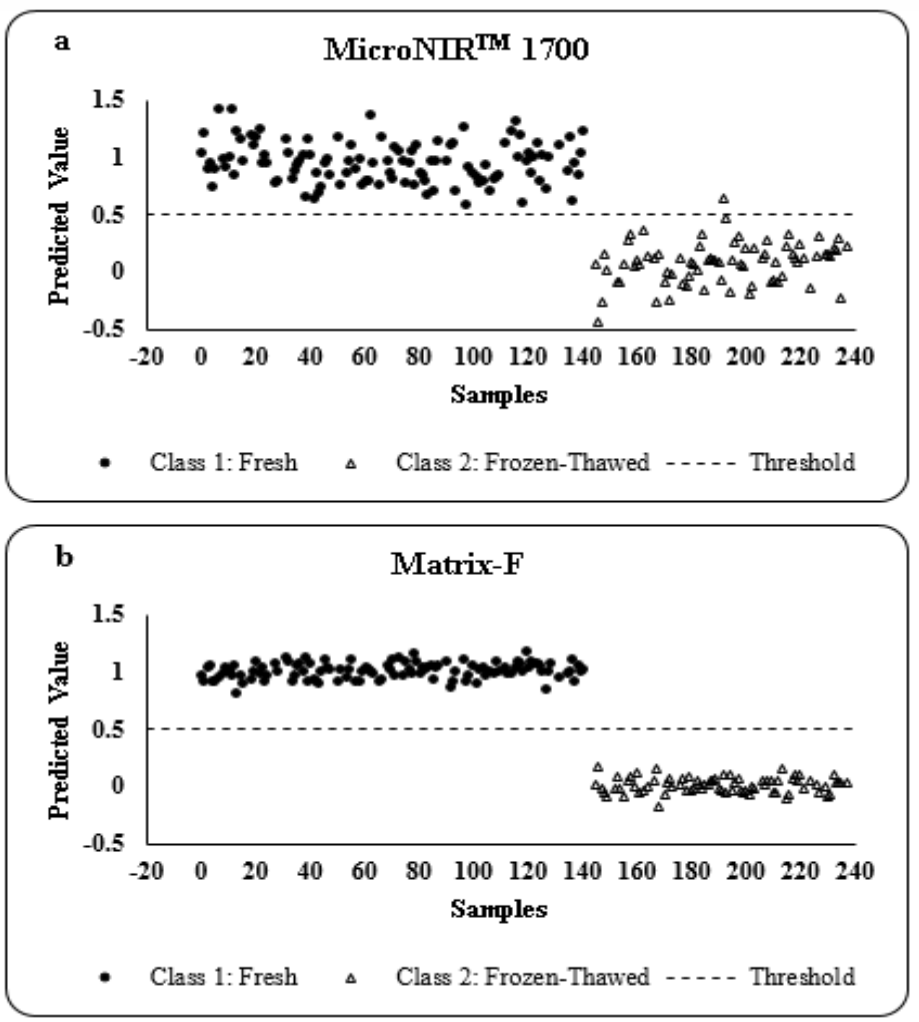

Figure 3. Distribution of predicted values for the training sets using MicroNIR™ 1700 (a) and Matrix-F (b). The dummy variable assigned for the fresh meat samples was 1 and for frozen-thawed meat samples was 0. The dashed line represents the classification threshold for fresh versus frozen-thawed samples.

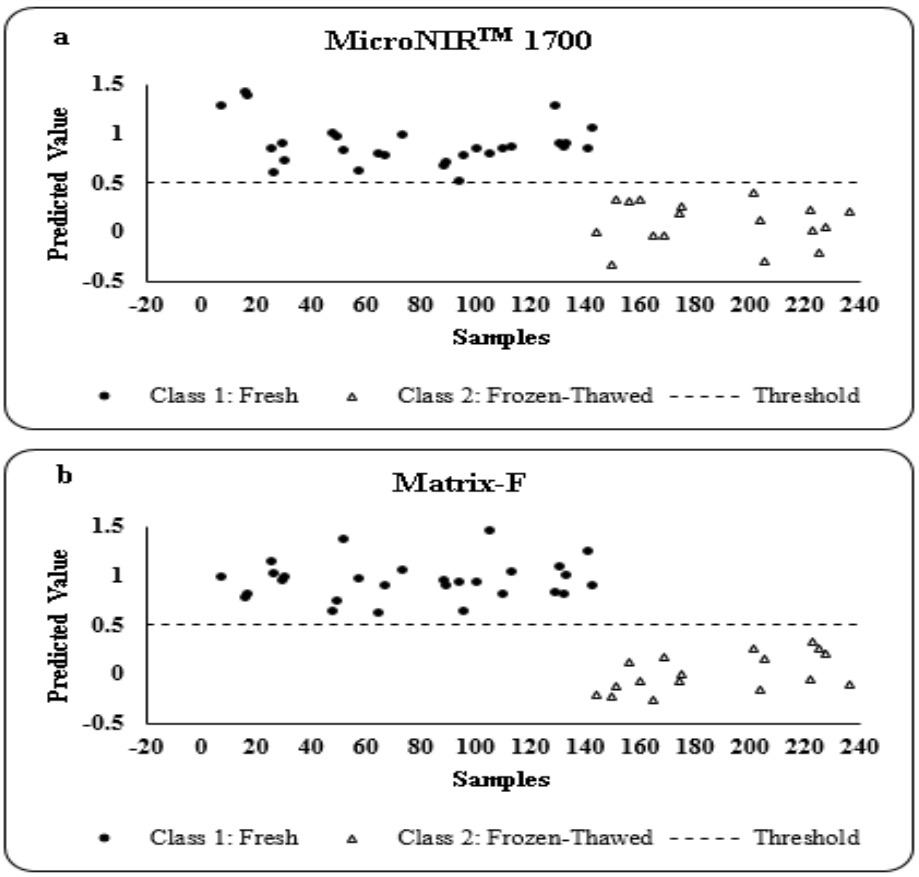

Figure 4. Distribution of predicted values for the test sets using MicroNIR™ 1700 (a) and Matrix-F (b). The dummy variable assigned for the fresh meat samples was 1 and for frozen-thawed meat samples was 0. The dashed line represents the classification threshold for fresh *versus* frozen-thawed samples.

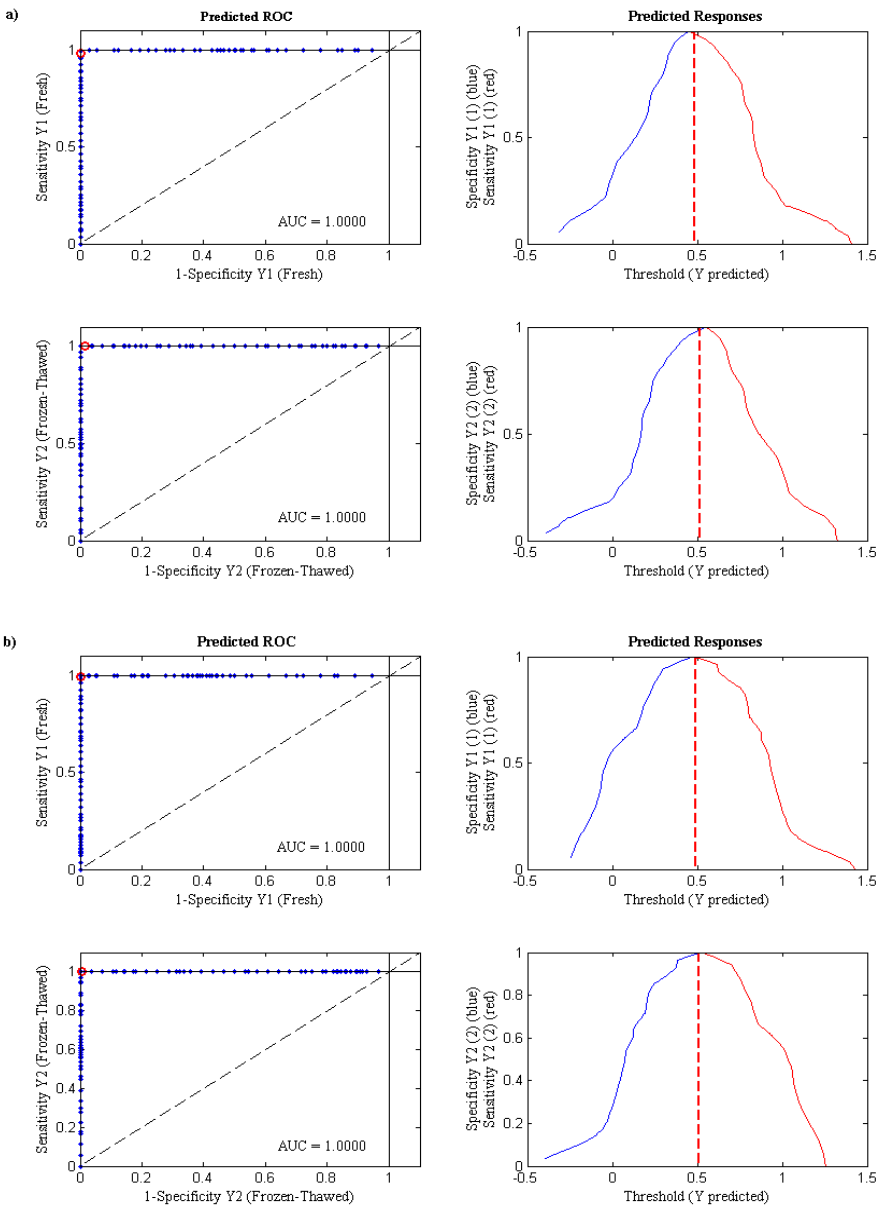

Figure 5. Left: ROC (Receiver Operating Characteristics) curves for the best PLS-DA model developed using the MicroNIR™ 1700 (a) and Matrix-F 1700 (b). Red circle indicates the umbral position of the threshold. Right: Diagrams of the threshold. The dashed line represents the value of the threshold selected by the model.

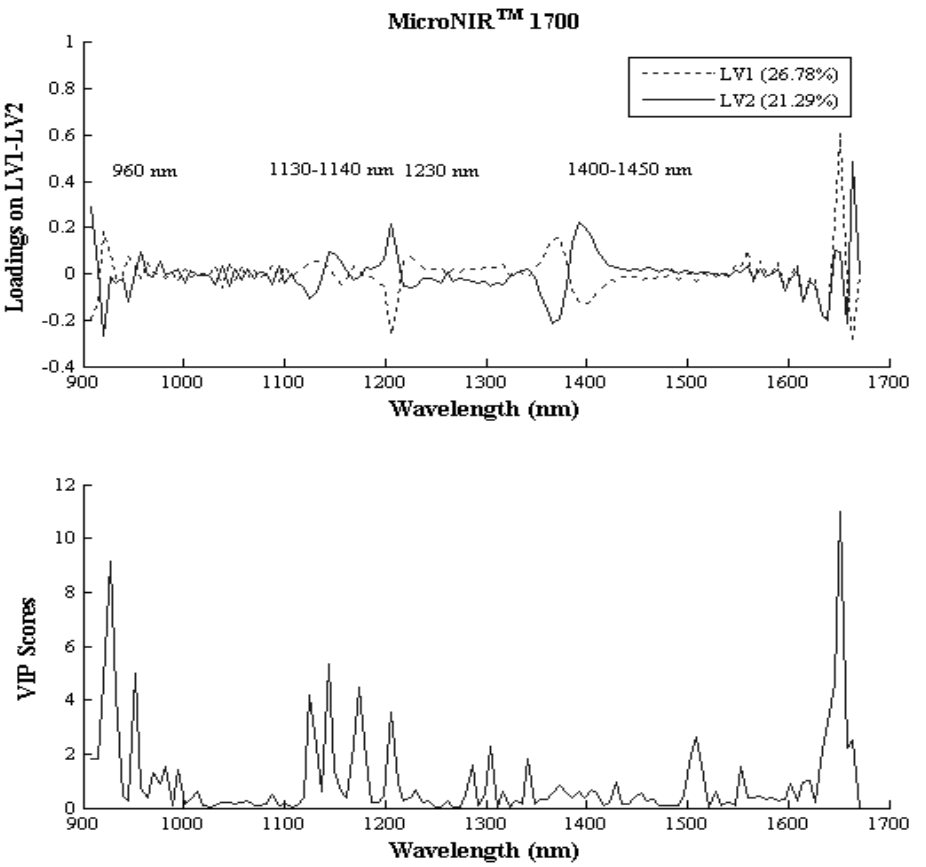

Figure 6. Loadings on LVs and Variable Importance in the Projection (VIP) of the best PLS-DA model developed using the MicroNIR™ 1700.

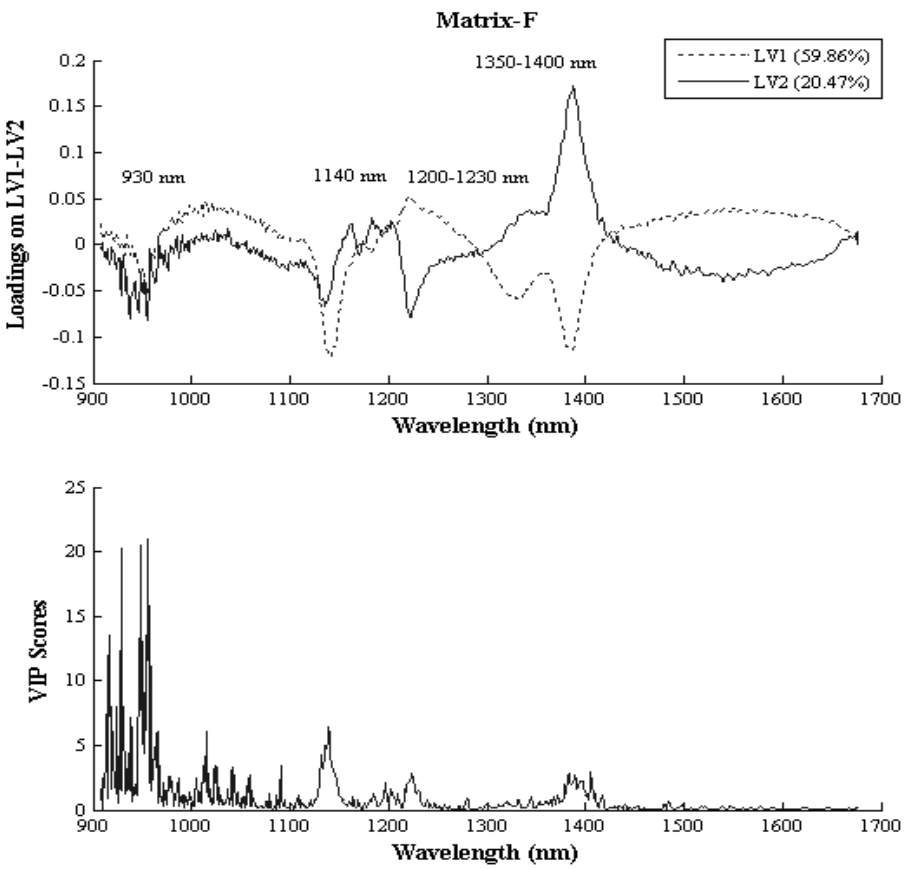

Figure 7. Loadings on LVs and Variable Importance in the Projection (VIP) of the best PLS-DA model developed using the Matrix-F.

Table 3. Statistics of the discrimination models developed using MicroNIR™ 1700 to differentiate fresh vs frozen-thawed Iberian loin steak samples.

Parameters	**Range (908.1-1676.2 nm) [c] DT, [d] SNV, [a] (1,5,5), [b] MC**		**Range (908.1-1403.6 nm) [c] DT, [a] (1,5,5), [b] MC**	
	Training Set	Test Set	Training Set	Test Set
Sensitivity (%)	100	100	100	96.43
Specificity (%)	98.70	100	97.4	94.44
Accuracy (%)	99.35	100	98.7	95.44
Matthew's correlation	0.99	1	0.98	0.91

[a] Savitzky-Golay derivative

[b] Mean Center

[c] Detrend

[d] Standard Normal Variate

Table 2. Statistics of the discrimination models developed using Matrix FT-NIR spectrophotometer to differentiate fresh vs frozen-thawed Iberian loin steak samples.

Parameters	**Range (834.4-2502.6 nm) [c] DT, [d] SNV, [b] MC**		**[e] Range (908.1-1676.26 nm) [c] DT, [a] (1,5,5), [b] MC**		**[f] Range (908.1-1676.26 nm) [c] DT, [a] (1,5,5), [b] MC**	
	Training Set	Test Set	Training Set	Test Set	Training Set	Test Set
Sensitivity (%)	99.13	96.43	100	100	96.15	53.57
Specificity (%)	100	100	100	100	92.98	100
Accuracy (%)	99.57	98.21	100	100	94.57	76.79
Matthew's correlation	0.99	0.96	1	1	0.88	0.56

[a] Savitzky-Golay derivative

[b] Mean Center

[c] Detrend

[d] Standard Normal Variate

[e] MicroNIR Spectral Range using the Matrix Resolution

[f] MicroNIR Spectral Range using the MicroNIR Resolution

CHAPTER V:

CONCLUSIONS

Chapter 5. CONCLUSIONS

Considering the planned objectives and the results obtained in this thesis, the following conclusions can be drawn:

1. The use of portable and handheld NIRS sensors allow the on-line and *in*-situ control of the quality of products and processes in the Iberian pig industry in a sustainable, efficient, fast, competitive, safe, low cost, objective, reliable and individual way. This allows the possibility of increasing the current quality controls that are performed routinely so that quality and homogeneity of Iberian pig products can be guaranteed. [*This conclusion was met in the research articles: 'Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of IBERIAN pork loins: Intact versus minced'. Meat Science 153, 86-93 (2019)*; *'NIR handheld miniature spectrometer to increase the efficiency of Iberian pig selection schemes based on chemical traits'. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 258, 119865 (2021)*].

2. The optimisation of the spectral range does not noticeably improve the precision and accuracy of the equations developed with loin samples using the Matrix-F spectrometer and OPUS Software. In addition, the time taken is higher than when the full spectral range is used. Such consideration makes advisable to calibrate with the full spectral range of Matrix-F. [*This conclusion was met in the research article: 'Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of IBERIAN pork loins: Intact versus minced'. Meat Science 153, 86-93 (2019)*].

3. The results showed that As regards with the Matrix-F spectrometer, which is ideally for on-line measurements, taking two spectra of the loin samples, locating the samples 10 cm away from the spectrometer head, selecting 32 scans per spectra at a speed of 10 kHz with a resolution of 16 cm^{-1}, would be enough to control the product's quality, which would facilitate the incorporation of the NIR instrument in the Iberian pig processing industry. [*This conclusion was met in the research article: 'Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of IBERIAN pork loins: Intact versus minced'. Meat Science 153, 86-93 (2019)*].

4. The results showed theta with the MicroNIR™ 1700 device, which is ideally suited for *in-situ* measurements, the models obtained using minced samples had a high accuracy, but the most important advance of this research is that the equations developed using intact samples enabled to predict with a good accuracy the intramuscular fat content in real time without destroying the samples. This is very important since it would allow to classify individual pieces of loins and, therefore, individual carcasses, enabling to take important decisions in selection schemes and breeding programmes. [*This conclusion was reached in the research article: 'NIR handheld miniature spectrometer to increase the efficiency of Iberian pig selection schemes based on chemical traits'. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 258, 119865 (2021)*].

5. Global MPLS algorithm was enough for predicting the composition of Iberian loin, both in intact and minced analysis modes, while .in these cases, the use of a non-linear algorithm did not give specific advantages for its application. It is important to stress that the maintenance of LOCAL algorithm is easier, since in this case only it needs to enlarge the spectral library. [*This conclusion was reached in the research article: 'NIR handheld miniature spectrometer to increase the efficiency of Iberian pig selection schemes based on chemical traits'. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 258, 119865 (2021)*].

6. Reliable classification using PLS-DA model has shown its potential to distinguish fresh and frozen-thawed Iberian pig loin samples, using both a portable, low-cost spectrometer, based on Linear Variable Filter technology, and a FT-NIR spectrometer coupled to a fibre optic probe adapted to on-line analysis. The handheld NIR sensor could be more adapted for verification at retail level, and the FT-NIR instrument could be used at large meat processing plants, placing over a conveyor belt, to authenticate the meat cuts which were bought from others. [*This conclusion was reached in the research article: 'Non-destructive Near Infrared Spectroscopy for the labelling of frozen Iberian pork loins'. Meat Science 175, 108440 (2021)*].

7. The two NIRS instruments tested (MicroNIR™ 1700 and Matrix-F) which are rapid, lightweight and user-friendly, provided a similar level of accuracy for the measurement of quality parameter in Iberian loins, intramuscular fat, protein and moisture. [*This conclusion was met in the research articles: 'Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of IBERIAN pork loins: Intact versus minced'. Meat Science 153, 86-93 (2019)*; *'NIR handheld miniature spectrometer to increase the efficiency of Iberian*

pig selection schemes based on chemical traits'. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 258, 119865 (2021)].

8. The results obtained suggest the importance of the optimization of the new generation NIRS instruments before their use for *in-situ* and online analysis of Iberian pig products. The results also showed the complementary use of the MicroNIR^{TM} 1700 and the Matrix-F instruments for global monitoring, allowing Iberian loins to be analysed throughout the whole food supply chain. [*This conclusion was met in the research articles: 'Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of IBERIAN pork loins: Intact versus minced'. Meat Science 153, 86-93 (2019)*; *'NIR handheld miniature spectrometer to increase the efficiency of Iberian pig selection schemes based on chemical traits'. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 258, 119865 (2021)*; *'Non-destructive Near Infrared Spectroscopy for the labelling of frozen Iberian pork loins'. Meat Science 175, 108440 (2021)*].

CHAPTER VI:

RECOMMENDATIONS FOR FURTHER RESEARCH

Chapter VI. RECOMMENDATIONS FOR FURTHER RESEARCH

There are a number of gaps in our knowledge around the consolidation of portable and transportable NIRS sensors in the Iberian pig sector that follow from our finding and would benefit from further research.

- ✓ Enlarging the existing data set of Iberian pig loins used in this PhD work, in order to increase the robustness of the models developed for their routine use at industrial level.

- ✓ Evaluation of other advanced chemometric strategies for structuring a largest population of samples and increasing the robustness of predictive models in cured Iberian products, mainly for chemical and quality traits (E.g. NaCl, breed, etc.).

- ✓ Follow the rapid evolution of NIRS sensors and face the study of cloning strategies to share the models between different instruments.

- ✓ Research aimed at considering NIRS sensors as IoT devices, which can be integrate to other important sectors in the production process of fresh and cured loins.

- ✓ Continue with the work initiated by the AGR-128 Group aimed to empower NIRS sensors as IoT devices for ensuring the integrity of Iberian pig products. That research should include the designs on interfaces, implementing multivariate algorithms for cloud computing and real time connectivity with process control software (e.g. SCADA), mobile phone, tablets and websites.

CHAPTER VII:

REFERENCES

Chapter VII. REFERENCES

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