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RAPID ON-SITE MONITORING OF FATTY ACID PROFILE IN RAW MILK USING A HANDHELD NEAR INFRARED SENSOR

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Highlights

- Monitoring in real time cow milk fatty acids in a farm or industry.
- Noninvasive FA analysis without sample pretreatment under outside ambient temperature.
- Multivariate regression of NIR data to quantify FA in raw cow milk.
- Proof of concept illustrating application of NIRS sensors to FA analysis

Abstract

This work is focused on development of a quick and simple to use analytical methodology for on-site monitoring the fatty acid (FA) profile in raw milk at farm level by using a near

infrared handheld spectrometer. This novel methodology was developed using a total of 108 liquid milk samples, scanned at room temperature by NIRS without pre-treatment, and analyzed by GC-MS for reference data. Calibration was carried out by multivariate regression combining math pre-treatments and Partial Least Square with internal and external validation. Calibration models displayed good predictive capacity for total saturated, monounsaturated (MUFA) and polyunsaturated FA (PUFA) with high coefficients of determination of cross validation (R^2_{cv} >0.8). Good results were also obtained for prediction of individual FA: caproic, capric, lauric, miristic, palmitic and arachidic as well as for unsaturated FA: oleic, conjugated linoleic acid and omega-6 acids with R^2_{cv} values ranged between 0.91- 0.73.. Validation statistics have confirmed that the highest R^2_v (coefficient of determination of external validation) values to quantify FA were for PUFA, linolenic acid (R^2_v = 0.92), caproic acid and MUFA (R^2_v = 0.87). These results establish that a profitable classification of milk can be carried out at farm level by including a fatty acid composition labeling.

Abbreviations

AI: Atherogenicity index

CLA: Conjugated linoleic acid

CV: Coefficient of Variation

CVD: Cardio vascular disease

FA: Fatty acid

FAMEs: Fatty acid methyl esters

FID: Flame ionization detector**GC:** Gas Chromatography

HPLC: High Performance Liquid Chromatography

LDL-cholesterol: Low density lipoprotein

MCFA: Medium chain saturated fatty acids

MEMS: Micro-electro-mechanical system

MIRS: Mid Infrared Spectroscopy

MS: Mass Spectrometry

MUFA: Monounsaturated Fatty acids

NIRS: Near Infrared Spectroscopy

PUFA: Polyunsaturated fatty acids

R^2_{CV}: Coefficient of determination for cross validation

R^2_v: Coefficient of determination for external validation

RPD: ratio of performance to deviation

SCFA: Short chain saturated fatty acids

SD: Standard deviation

SECV: Standard error of cross validation

SFA: Saturated Fatty Acids

SG: Savitzky Golay

SNV: Standard Normal Variate

TFA: Trans Fatty Acids

TI: Thrombogenicity index

Chemical Compounds List

Omega-3 Fatty Acids (PubChem CID: 56842239)

Capric acid (Decanoic acid, according to IUPAC guidelines from 1976) (PubChem CID: 2969)

Lauric acid (Dodecanoic acid, according to IUPAC guidelines from 1976) (PubChem CID: 3893)

Myristic acid (Tetradecanoic acid, according to IUPAC guidelines from 1976) (PubChem CID: 11005)

Palmitic acid (Hexadecanoic acid, according to IUPAC guidelines from 1976) (PubChem CID: 985)

Stearic acid (Octadecanoic acid, according to IUPAC guidelines from 1976) (PubChem CID: 5281)

Oleic acid (Cis-9-Octadecenoic acid, according to IUPAC guidelines from 1976) (PubChem CID: 445639)

Rumenic acid (9Z,11E-octadecadienoic acid, according to IUPAC guidelines from 1976) (PubChem CID: 5280644)

Linoleic acid, ((9Z,12Z)-octadeca- 9,12-dienoic acid, according to IUPAC guidelines from 1976) (PubChem CID: 5280450)

Linolenic acid ((9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid, according to IUPAC guidelines from 1976) (PubChem CID: 5280934)

Keywords: NIR sensor, multivariate calibration, on site analysis, fatty acid, milk, dairy cow, food composition, food analysis

1. Introduction

Fresh cow's milk is an important agricultural fluid product, both in its natural form and as a raw material for the dairy and food industries. Although there are several milk products, the general term milk should only refer to cow's milk, produced by healthy animals and excluding the lactic secretion between 15d before and 5d after or until it is almost completely free of colostrums (Pereira, 2014).

Cow milk composition can be influenced by several factors such as animal species and genetics, environmental conditions, lactation stage and animal nutrition status (González-Martín et al., 2017, Morales-Almaraz et al., 2017,). On average, bovine milk is composed of 87% water, 4% to 5% lactose, 3% protein, 3% to 4% fat, 0.8% minerals and 0.1% vitamins (Lindmark-Mänsson et al., 2003, Haugh et al., 2007,).

Cow milk fat composition consists primarily of triacylglycerols (97–98 percent of total lipids by weight), which are composed of fatty acids (FA) of various lengths (4–24 carbon atoms) and levels of saturation.

In milk fat more than 400 FA have been identified. Whole milk contains approximately 70% of saturated fatty acids (SFA), 25% monounsaturated fatty acid (MUFA), being oleic acid (C18:1 cis9) the most abundant unsaturated fatty acid in milk (about 8 g/l of whole milk), and approximately 5% polyunsaturated fatty acid (PUFA)/100 g (Haug, et al., 2007, Coppa et al., 2010,). Up to five percent of the fatty acids in cow milk may be ruminant-derived trans fatty acids (TFA), which are different from industrially-produced trans fats with respect to health outcomes.

Previous researchers have established the complex relationship between milk fat intake and health impact (German et al., 2009) and much has been written on the association between dairy and cardio vascular disease (CVD) risk factors. It is recommended that total intake of SFA should not exceed 10 percent of the daily energy intake, and these FA should be replaced in the diet with PUFA to reduce the risk of coronary heart disease. Individual SFA have different effects on blood lipids. For example, lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids are associated with elevated serum levels of low density lipoprotein (LDL)-cholesterol, whereas stearic acid (C18:0), which is poorly absorbed in the gut, has no effect on LDL-cholesterol (Shingfield et al., 2008; Gibson et al., 2011).

Other FA subject to intense research is conjugated linoleic acid (CLA). It consists of a collection of positional and geometrical isomers of octadecadienoic acid, with conjugated double bonds ranging from 6, 8 to 12, 14. The main isomer formed is C18:2, cis 9, trans 11 (Banni, 2002). There are two sources for C18:2, cis 9, trans 11

CLA synthesis in ruminant animals: 1) the rumen via incomplete biohydrogenation of C18:2, cis 9, 12 (linoleic acid), and 2) desaturation of C18:1, trans 11 by Δ9-desaturase enzyme. However, Mosley et al. have confirmed that the mammary gland was the major site for the conversion of C18:1, trans 11 to C18:2, cis9, trans11 in milk. The magnitude of the contribution of dietary C18:1, trans 11 is around 80%, to the synthesis of C18:2, cis9, trans11 CLA in the whole animal (Mosley et al., 2006). CLA isomers have been linked to health-promoting activities, including an ability to inhibit various types of cancer, hypertension, atherosclerosis and diabetes and improve immune function and body composition (Mills et al., 2011).

In this context, the demand by dairy farmers, dairy industry and consumers for healthy products and composition labelling could lead dairy companies to introduce the fatty acid composition in the payment of the bulk milk. Moreover, attending these considerations, some European countries (e.g. France and The Netherlands) have introduced FA composition among the parameters considered to determine milk price (Coppa et al., 2014). However, the reference method to quantify FAs involves multiple steps (extraction procedure, methylation, FA methyl ester extraction and Gas Chromatography (GC) determination) and it is tedious, time-consuming and requires skill staff (González-Arrojo et al., 2015).

A lot of efforts have been made to develop analytical methodologies that minimize this tedious and time consuming analytical procedure: extraction, derivatization and chromatographic analysis. Nowadays, GC with Flame ionization detector (FID) or Mass Spectrometry (MS) and Ag+-HPLC (High Performance Liquid Chromatography) with Ultraviolet Detector are the most popular (de la Fuente et al.,

2006) techniques for FA analysis. Although GC linked to Fourier Infrared or Nuclear Magnetic Resonance can also provide invaluable information (Mossoba 2001).

It would be of great interest for both, farmers and dairy industry to develop a fast and reliable method to monitor in real time FA and groups of FA in milk samples, which would allow to establish a payment system of milk according to its nutritional level, and to promote farmers to adapt their animals feeding systems accordingly, increasing milk quality and livestock farming profitability (Coppa et al., 2014).

Different methodologies based on Mid-Infrared Spectroscopy (MIRS) have been successfully used to predict C12:0, C14:0, C16:0, C16:1 cis9, C18:1, SFA and MUFA in cow's milk (Souyeurt et al., 2006, Coppa et al., 2014). Alternatively NIRS has also been successfully used to quantify FA in milk cows (Coppa et al., 2010, 2014) and milk goats (Andueza et al., 2013, Nuñez-Sánchez et al., 2016, Revilla et al., 2017) at laboratory level, with no possibility of moving to continuous on-site FAs monitoring. Samples were analyzed as liquid samples or after oven-dried (Stefanov et al., 2013, Coppa et al., 2014, Nieves-Nuñez et al., 2016;,). But in all these referred research works high performance NIRS laboratory instruments have been employed, with wide scanning window and wavelength range (400-2500 nm or 1000-2500 nm). These laboratory NIRS instruments are robust, but very expensive and make necessary to move the sample from the farm to the lab. Farms frequently are far away from the laboratory, making impossible the establishment of quality controls at farm level to take decisions focused to improve the milk quality, on the basis of modifying simple tasks such as animal nutrition or management (Morales-Almaraz et al., 2017).

In last decades, the evolution on NIRS instrumentation has made considerable progress in making, available low cost miniaturized, handheld near-infrared

instruments based on MEMS (micro-electro-mechanical system) (Pérez-Marín et al., 2010, Zamora Rojas et al., 2012, Modroño et al., 2017;,). These type of sensors offer significant advantages in terms of size, weight, robustness and low cost manufacturing process. They are highly resistant to mechanical stress (Cabassi, et al., 2015) and easy to use, which represents the evolution in the analysis of the samples from taking the sample to the Lab to taking the Lab to the sample (O'Brien et al., 2012). However these systems are limited by low sensitivity linked to small window and narrow wavelength range. These handheld devices allow the compilation of spectra data in the labor field with high contrast pixelate and the analyses of milk samples coming from individual cows. Several recent studies have evaluated this technology in the agro-food area with promising results for safety and quality controls in meat and feed (Soldado et al., 2013, Vega et al., 2013, de la Roza-Delgado et al., 2014, Modroño et al., 2017,).

Previous researchers have developed a methodology based on the use of hand-held portable NIRS for the analysis of major components (fat, protein and solids-non-fat) in raw milk, and the calibration models developed showed that the accuracy and precision by using the handheld instrument were similar, in terms of both calibration and validation statistics, to those of the equations obtained on high performance lab based instruments (de la Roza-Delgado et al., 2017).

Therefore, the aim of the present study was to develop a methodology based on the use of in situ NIRS sensors able to on-site monitoring the FA profile in raw milk samples from individual cows, without requiring sampling pre-treatment and at room temperature. This NIRS developed methodology will allow to establish a quality

control for milk labelling and real time decision making in the animal feed and dairy industries and at farm level.

2. Materials and methods

2.1 Milk Samples

A total of 108 individual milk samples were collected from two sources: Cows involved in feeding experiments at the Regional Institute for Research and Agrofood Development; and cows selected randomly from farms in Asturias (North West of Spain). Milk samples were collected from individual cow controls in order to obtain maximum variation. The same sample was used for NIRS and FA reference analysis. Firstly, the sample was scanned for NIRS analysis and after that the same sample was processed for reference data.

From the initial data set a sub-set of 12 samples (validation set) was separated randomly for external validation. It should be stressed that selection of calibration and validation sets was only performed on the basis of spectral information (Vega et al., 2013).

2.2 FA reference analysis

Gas Chromatography-Mass Spectrometry was used as reference method to quantify FAs to obtain reference data.

For each sample, 45 mL of milk were centrifuged (17,800×g, 30 min, 4 °C, Biofuge Stratos, Heraeus Instruments, Hanau, Germany) to obtain a top layer of fat. An aliquot of this layer was taken into an Eppendorf vial and centrifuged again

(19,300×g, 20 min, room temperature, Eppendorf Centrifuge 5415R, Hamburg, Germany), obtaining a lipid top layer (Feng et al., 2004).

The lipid layer was treated with a saturated oxalic acid solution (synthesis grade, Merck, Hohenbrunn, Germany), following the Chouinard et al. (1999) modification of a trans-esterification method based on ISO15884/IDF182. Forty mg of the lipid layer were weighted in a Pyrex tube with Teflon-lined screw cap, then 2 mL of hexane (95% HPLC grade, J.T. Baker, Mallinckrodt Baker, Inc., London, UK) were used to extract the FAs, and finally 40 μL of methyl acetate (synthesis grade, Merck, Hohenbrunn, Germany) and sodiummethylate (30% solution in methanol, synthesis grade, Merck, Hohenbrunn, Germany) were added to produce esterification. Allowing 10-min reaction time, 60 μL of saturated oxalic acid solution were then added, using a vortex for homogeneous mixing. After centrifuging the samples (1500 rpm, 5 min, room temperature), the top organic layer was filtered using 0.2 μm Teflon filters. The extract of fatty acid methyl esters (FAMEs) was diluted and subjected to GC-Mass Spectrometry (MS) to obtain the FA reference analysis, using a VARIAN 3800 GC equipped with a 4000 MS detector (Varian, Inc. Palo Alto, CA, USA) and a CP-Sil 88 column (100 m×0.25 mm, 0.20 μm i.d.; Varian, Inc.). The carrier gas flow was 1 mL/min Helium , and the temperature of injector and detector was fixed at 250 °C. The tempe-rature in the column was kept at 40 °C for 1.20 min; raised at a 30 °C min^{-1} rate to 140 °C, temperature at which was held for 25 min; raised at 1 °C min^{-1} to 190 °C and held for 15 min; raised at 1 °C min^{-1} to 215 °C, held for 8 min; and finally, raised at 30 °C min^{-1} until 240 °C, where it was held during 1 min. The MS detection system was operated at full scan from 50 to 500 m/z. FAME peaks were identified by comparing retention times and mass spectra from the samples with methyl ester standards (GLC-463 from Nu-Chek Prep Inc., Elysian, MN U.S.A., and

Methyl 9(Z), 11(E)-octadecadienoate from Metraya LLC, Pleasant Gap, PA, USA). The concentration of each FA in the sample (gFA/100g of total FA) was calculated comparing individual peak areas with the sum of all FAs peak areas as obtained from GC-MS. This reference methodology has been validated in our research group by González-Arrojo et al. (2015) using butter fat as Reference Material provided by Spanish Reference Laboratory for milk and milk products (EA Search Facility, 517/LE1040) (see Table 1). This reference Material has been developed by the Spanish Reference Laboratory for milk and milk products (Agro food Laboratory of Santander, Santander, Spain). It is a calibration Material employed in the interlaboratory proficiency tests organized by the cited official laboratory. It is traceable to CRM164 reference material (Community Bureau of Reference, Commission of the European Communities, Belgium)

Several groups of FA were calculated from the individual FA identified: SFA (total saturated FA), MUFA (total monounsaturated FA), PUFA (total polyunsaturated FA), SCFA (short-chain FA as sum of C6:0, C8:0 and C10:0 potentially beneficial fatty acids, C4 has not been included because the reference methodology does not quantify this FA, MCFA (medium-chain fatty acid as the sum of C12:0, C16:0 negative effects in human health) (Nuñez-Sanchez et al., 2016).). Two indexes proposed by Ulbricht & Southgate (1991) to measure the effect of FAs on coronary heart disease and to allow comparison of different foods and diets, the Atherogenicity Index (AI) and the Thrombogenicity Index (TI). Both indexes were calculated by equations 1 and 2, where n-6 are omega 6 FA and n-3 are omega 3 fatty acids (Nuñez Sánchez et al., 2016).

$$IA = \frac{C12{:}0 + 4 * C14{:}0 + C16{:}0}{MUFA + n{-}6PUFA + n{-}3PUFA} \quad \text{Eq [1]}$$

$$TI = \frac{C14{:}0 + C16{:}0 + C18{:}0}{0.5 * MUFA + 0.5 * n-6PUFA + 3 * n-3PUFA + {}^{n-3}/_{n-6}}$$ Eq [2]

2.3 NIRS analysis

The collection of NIRS spectra data from the 108 milk raw samples was carried with a handheld MEMS digital transform spectrometer (1.8 kg weight) from Polychromix PHAZIR™ (PhIR, Phazir 1624, Polychromix Inc., Wilmington, MA, USA). This instrument works in reflectance mode in the range between 1600 and 2400 nm with a non-constant interval of around 8 nm (pixel resolution 8 nm, optical resolution 12 nm), with a diameter window of 0.4 cm (sampling area of 0.13 cm^2).

Prior to spectra collection, samples were lightly shaken by hand and three subsamples were scanned immediately as fresh milk by NIRS, using an opaque liquid cup (Foss IH-0397), with dimensions of 4.5 cm height, 2.5 cm wide and 1.7 cm path length. This liquid cup has a quartz window in the front side and an aluminium in the back side to allow trans-reflectance measures (see Figure 1).. Each spectra is the average of 50 sub-spectra and one spectra was collected for each sub-sample. The final spectrum was the average of all of them. Spectra data were recorded as log (1/R).

2.4 Data processing and calibration development

The collected data were converted into a data matrix. The X and Y variables were defined as: X wavelength and Y log 1/R. Calibration development was performed in two parts; pre-treatments and mathematical treatments, and both were applied to the spectra using the Unscrambler v. 9.8 software (Camo Software Inc., Unscramble v.8.0, 2008)

As spectral pre-treatments were applied, the standard normal variate (SNV) together with first and second Savitzky and Golay (SG) derivatives as mathematical treatment, involving different segments 1.5.5.2; 1.10.10.2; 2.5.5.2 and 2.10.10.2, which indicate the polynomial order of the derivative (first number on the left), a smoothing factor with the number of data points in a running average on the second number on the left, and the secondary smoothing on the right, respectively (Nuñez et al., 2016, Stefanov et al., 2013). A total of four prediction models for each individual FA or FA groups were built.

After pre-treatment and before developing regression models, the calibration set was centered by principal component analysis to identify and remove spectral outliers. The regression model was performed using partial least squares (PLS). The optimal number of PLS factors used for the regression was determined from the minimum residual validation variance.

To select the best equations, the statistics evaluated were: the lowest standard error of cross validation (SECV), the highest coefficient of determination cross validation (R_{cv}^2). The external validation was evaluated in base of the coefficient of determination of prediction (R^2_v) value and the lowest standard error of prediction (SEP). These statistics have been calculated according the Unscrambles manual (The Unscrambler® Appendices: Method References, 2006).

3. Results and Discussion

The range, average values, standard deviation of the reproducibility of the reference methodology as estimated by Horwitz function (Thomson & Wood, 2006), and standard deviations for cow milk FA from the calibration and validation sets are shown in Table 2. As can be seen, the milk FA content showed a wide variability

going from 0.01 to 52 g FA/100g of total FA for minor fatty acids (C7:0, C20:3 cis 8, 11, 14; C20:4 cis 5, 8, 11, 14; C20:5 cis 5, 8, 11, 14, 17; C22:5 cis 7, 10, 13, 16, 19) and palmitic acids (C16:0) respectively. To remark the variability of the most abundant conjugated linoleic acid (CLA) found in dairy products, C18:2 cis9 trans11 (rumenic acid), ranged between 0.09-4.24 g FA/100g of total FA. This FA is an intermediate on the process of biohydrogenation of linoleic acid (C18:2 cis9 cis12) into stearic acid (C18:0) (stearic acid content range 5-90-16.62 g FA/100g) (Koba, Yanagita, 2014) and the final product of desaturation of C18:1 trans 11 in the mammary gland (Mosley et al., 2006).

This great variation in milk FA composition is a reflection of the wide variation in production conditions (feed and management) (Hernandez-Ortega et al., 2014).In this research work, we have observed that some minor fatty acids (content around 0.01-0.09 g/100g FA) were not found in all samples.

Additionally in Table 2, have been included the values of groups of FA: SFA, MUFA, PUFA, SCFA, MCFA. Our results are according with the averaged composition described by Pereira (2014): 70% *vs.* 77 % SFA, 30 % *vs.* 20 % as sum of MUFA plus PUFA, for previous research and our results respectively. Moreover, within SFA the most important from a quantitative viewpoint are palmitic (C16:0), miristic (C14:0) and stearic acid (C18:0). And the averaged composition described by previous researchers is agreeing with our reference results, 39% *vs.* 30% for palmitic, 14% *vs.* 11% for myristic and 11 *vs.* 12% for stearic acid (Pereira, 2014).

The averaged values of indexes established by Ulbricht & Southgate (1991), related to measure the effect of FAs on coronary heart diseases, were 5.28 and 4.62 for AI and TI respectively, not too much different to those obtained by Nantapo et al. (2014)

for cows' milk, with an AI index ranged between 4.08-5.13 depending on stage of lactation. Similar results were observed by Thanh & Suksombat (2015) for TI with an average value of 4.11.

In Figure 2 the regression coefficients obtained for SFA and PUFA are shown. As can be seen there is a dominant water band, around 1940 nm, related to O-H first overtone and O-H combination band, respectively (Osborne and Fearn, 1986). In addition small bands corresponding to fatty acids and fat contents appeared at 1726 and 1760 nm, associated with the first overtone from C-H stretching vibration of methyl ($-CH_3$), methylene ($-CH_2-$), and ethenyl (-CH=CH-).

After establishing the variability for all FA analyzed in the milk samples involved in this research work, the following step was to develop NIRS calibrations to predict the concentration of FAs related to the consumer demand of information and linked to consumption of functional foods that may exhibit health benefits beyond their nutritional value. The calibration statistics for individual FAs, groups of FA and indexes used to evaluate the fat nutritional properties of food (considering the potential effect, negative or positive, of some FA on the coronary heart disease) are detailed in Table 3The number of samples included in the calibration set was ranged between 96-80 because as detailed before, depending on feed and management some minor FA were not detected in all milk samples involved in the research work.

The differences in calibration performance among scatter correction and spectra mathematical treatment within each regression type were narrow, suggesting that the main source of spectra variability is due to milk composition. Thus, we have

presented in the table the statistical treatment that showed the best calibration performance for each individual, FA, groups of FA or AI, TI indexes. These treatments have been selected attending requirements detailed in section 2.4 (Data processing and calibration development).The calibration statistics for these several groups of FAs have had R^2_{cv} values ranged between 0.78and 0.89 obtained for MCFA and SCFA respectively and SECV ranged between 3.86-0.72, values achieved for SFA and PUFA total. Attending R^2_{cv} statistics, similar results to those obtained in this study for SFA, MUFA and PUFA were also reported by Coppa et al. (2014) using a laboratory instrument (Foss NIRSystem model XDS) with a wide wavelength (400-2498 nm) and narrow resolution (2nm). However, comparing results attending errors, we have observed that our SECV was higher, when calibrating with spectra coming from handheld instrument than using laboratory NIRS (for MUFA 3.56 *vs.* 1.06 respectively) (Coppa et al., 2014). The differences of performance could be due to overlap between water and fat adsorption bands, it could create interfering phenomena limiting the detection of FA when using liquid milk plus NIRS analysis (Coppa et al., 2014). These interferences are increased when the wavelength range is reduced, because is not possible to find other alternatives wavelength bands to minimize drawbacks. As established before, liquid milk is a very complex aqueous matrix for NIR analysis, consisting of proteins in colloidal dispersion, fat in emulsion and minerals in solution (Marinori et al., 2013), and in addition, it should be noted that these developed NIRS prediction models, have been developed with a portable NIR instrument with small scanning window and a limited sensitivity.

Some minor individual FAs, C22:5, C20:3,C18:1 trans9 and C16:1 trans9, were not included in the development of prediction models, because they were identified and quantified in a low number of milk samples analyzed (lower than 40 milk samples of

the total liquid milk samples).A small data set of an external validation could arise a concern.

For individual FA, attending to coefficient of determination for cross validation, the best R^2_{cv} values were observed for C6:0 (R^2_{cv} =0.91), followed closely by C18:1 cis9 with a R^2_{cv} value of 0.88 and C20:0 (R^2_{cv} =0.81). However, attending SECV some results show limitations for predicting the lowest values of the range (C18:1 trans11; C18:2 cis9 trans11; C18:3 cis9, 12, 15) due to SECV values are higher than the lower limit of the range. Nevertheless, R^2_{cv} values obtained to C18:2 cis9 trans11 and C18:3 cis9, 12, 15 were 0.85 and 0.89 respectively indicating the possibility of using the models above the low range of quantification. R^2_{cv} results obtained for other individual FA C10:0, C12:0, C12:1 cis11, C14:0, C16:0, C18:1 trans11, C18:2 cis9, 12, C20:4 cis5, 8, 11, 14 displayed very acceptable statistics with values from 0.8 to 0.7. R^2_{cv} values around 0.6 were observed for C8:0 and C18:0, these models allow to discriminate between high, medium and low values of FA concentrations. Attending to the ratio of performance to deviation (RPD), which is the ratio between the standard deviation of the sample population and SECV, the results are in accordance with those detailed above when comparing R^2_{cv} values. RPD values are ranged from 1.40 to 2.39. Those with RPD values lower than 1.5 are classified as poor calibrations (Cabassi et al. 2015).

Different authors have assayed the individual quantification of major FA by NIRS, scanning liquid milk (Coppa et al., 2014) or milk fat (Stefanov et al., 2013) and the obtained correlations (R^2_{cv}) are similar to those shown in Table 3, 0.85 for C18:2 cis9 trans11 and 0.78 for C18:1 trans11. SECV statistics obtained in this research work were higher than those presented by previous researchers, however, it is necessary to

emphasize that those researchers have used laboratory instruments (Stefanov et al., 2013, Coppa et al., 2014) with a wide wavelength range and higher resolution (2 nm) than the handheld device employed in this research work, easy to manage and able to give response on-site and in real time.

The repeatability of the NIR method was compared with that of the reference method (σ_R) through the S_r/σ_R ratio, being S_r the standard deviation of the FA NIR analysis and σ_R the standard deviation of reference values estimated by Horwitz function (Thomson & Wood, 2006). The repeatability of NIR methodology was considered to be comparable to that of the reference method if the value of the ratio is between a lower (A) and an upper (B) limit. A and B were obtained from Student's test, from n-1 degrees of freedom (n= number of spectra per sample) (Thomson & Wood, 2006). This condition is satisfied for methods with comparable repeatability. As can be seen in Table 3 the ratio is lower than 4.3 for all FAMES ($t_{\alpha=0.05,\ n=2}$= 4.3).For AI and TI Nuñez et al. (2016) have developed NIRS models to predict both indices on milk fat of goats using laboratory instruments and scanning milk samples in trans reflectance mode. The statistics comparison repeat previous findings with similar R^2_{cv} values, 0.85-0.89 vs. 0.75-0.68 for AI and TI with handheld and laboratory devices respectively); nevertheless the SECV values were lower for handheld than laboratory instruments (SECV 1.13-0.82 vs. 0.27-0.34 for AI and TI respectively).

In agro-food analysis different works have been carried out comparing the performance of a handheld micro-electro-mechanical system (MEMS) spectrophotometer with high performance laboratory instruments, analyzing different quality and nutritive parameters. In brief, these studies concluded that, although most comparison studies reported that the portable instruments had lower

performance scores than the laboratory instruments, the main conclusions were that their flexibility and possibility of field-use were major advantages that made the portable options the best solution (Teixeira et al., 2013).

After selecting the best math treatment to predict FAs, the following step was to validate the developed models with an external and independent group of 12 milk samples (set 2). In Table 3 are shown the external validation statistics in basis of standard errors, SEP and SEP(c) (Standard error of prediction corrected by bias). The SEP(c) was similar or lower than SECV. The ratio SEP/SEP(c) 0.9 to 1.6 for all the FA and sum of FA predicted, with the exception of palmitic acid (C16:0) with a ratio value of 3. An excessive difference between SEP and SEP(c) is related to high bias value and a systematic error in the developed model. For palmitic acid the ratio value could be linked with the lowest correlation observed for external validation statistics of palmitic acid ($R^2_v < 0.3$). For palmitic acid (C16:0), Coppa et al. (2010), scanning oven-dried milk, to remove the band water effect, obtained the highest bias of all individual FA predicted (-0.63). The highest R^2_v values to quantify individual FA were for PUFA, C18:3 cis 9, 12, 15 ($R^2_v = 0.92$), C6:0 and MUFA ($R^2_v = 0.87$), and C18:1 cis 9 ($R^2_v = 0.82$). Previous researchers established that the quality of NIRS prediction seems to be related to FA concentration, however the low correlation to accurately determine certain individual FA was probably due to similarities in their NIR absorption patterns, since different FA have the same absorbing molecular group (-CH_2-) (Windham and Morrison, 1998).

The low correlations measured versus predicted values (R^2_v) could show a tendency towards a non-linear relationship. The use of regression methods that take into account the risk of non-linearity (LOCAL regression or neural networks equations)

could eventually improve the NIRS calibration models for these determinations; however much more samples must be included in the calibration set.

The external validation statistics obtained for AI and TI indexes make possible that NIRs developed models can be used to establish the relationship between intake of milk SFA and the risk of heart disease, and could help to consider FA composition among the parameters to determine milk price.

4. Conclusions

We have focused this research work on the development of quick and easy methodologies to monitor the FA profile of milk coming from individual cows at farm level avoiding sampling manipulation and allowing the labeling of the cow milk. The results obtained showed that handheld NIRS can be used as a routine procedure to quantify FA sums and healthy indixes in individual cows milk at farm level. Good results were also obtained for prediction of individual FA, important saturated FA such as caproic (C6:0), capric (C10:0), lauric (C12:0), miristic (C14:0), palmitic (C16:0), stearic (C18:0) and arachidic (C20:0) as well as for unsaturated FA: oleic (C18:1 cis9), vaccenic (C18:1 trans11), rumenic (C18:2 cis9 trans11) and linolenic (C18:3 cis9, 12, 15)) and omega-6 (linoleic (C18:2 cis9, 12) and arachidonic (C20:4 cis 5, 8, 11, 14) acids. The speed of analysis and the proposed on-site methodology is an alternative in the dairy sector for providing indications of the FA profiles in cow milk. The estimation based on the use of cheap and portable NIRS sensors could become an useful prediction tool that will allow milk FA composition to be widely used as parameter for milk payment (application of supplementary

payments), for cow diet formulation and for breeding programs. In addition, Atherogenicity and Thrombogenicity index, could be help to establish the nutritional value of cow milk. Moreover it could be useful to test other calibration methods capable of combining linear and non-linear relations in an attempt to improve prediction statistics.

CONFLICT OF INTEREST: None

All authors were Ph D students or employees of the Regional Institute for Research and Agrofood Development.

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FIGURE CAPTIONS

Figure 1.- NIRS spectra collection using an opaque liquid cup

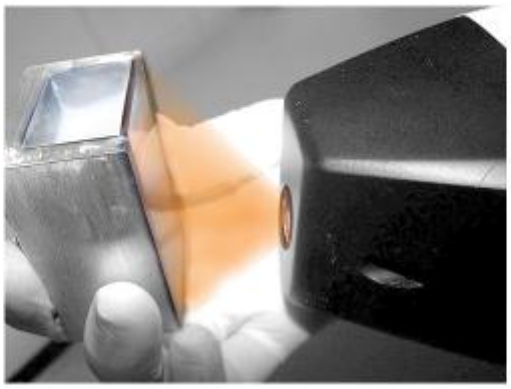

Figure 1

Figure 2.- Regression Coefficients obtained for Saturated (SFA) and Polyunsaturated (PUFA) Fatty Acids

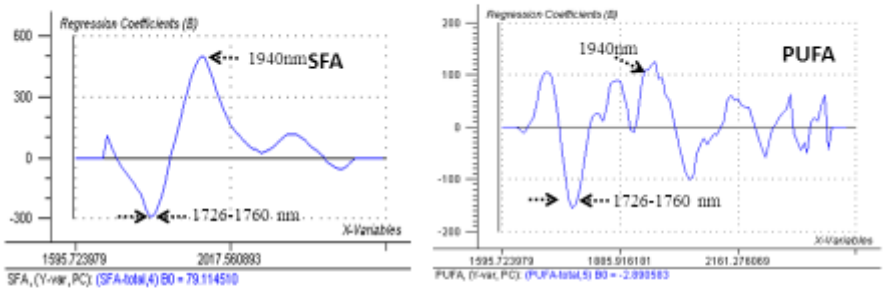

Figure 2

Table 1. Reference Material provided by Spanish Reference Laboratory for milk and milk products (EA Search Facility, 517/LE1040)

Fatty acid	g/100g of total FA
C8:0	1.40 ± 0.28
C14:0	10.9 ± 1.64
C18:2 cis 9, 12	3.02 ± 0.60
C18:3 cis 9, 12, 15	0.38 ± 0.08

Material employed in the interlaboratory proficiency tests organized by the cited official laboratory (Santander, Spain). It is traceable to CRM164 reference material (Community Bureau of Reference, Commission of the European Communities, Belgium)

Table 2.- Fatty acid (FA) composition (expressed in g/100g of total FA) in raw milk determined by Gas Chromatography analysis

	Calibration set (N=96)				Validation set (N=12)				
Fatty Acid	**Range**	**Mean**	**SD**	**CV**	**Range**	**Mean**	**SD**	**CV**	σ_R
C6:0	0.39-2.88	1.87	0.76	40.6	0.39-2.66	1.79	0.80	44.9	0.07
C7:0	0.01-0.07	0.03	0.01	48.4	0.02-0.04	0.29	0.08	28.5	0.01
C8:0	0.04-1.26	0.93	0.17	18.1	0.74-1.14	0.92	0.14	14.9	0.04
C10:0	1.56-5.77	3.50	1.00	28.5	2.00-4.78	3.46	1.01	29.2	0.12
C11:0	0.02-0.27	0.07	0.04	60.2	0.03-0.11	0.06	0.03	43.1	0.01
C12:0	2.10-6.47	4.26	1.03	24.3	2.65-5.71	4.20	1.07	25.4	0.14
C12:1 cis11	0.04-0.21	0.11	0.04	34.9	0.05-0.15	0.10	0.03	34.1	0.01
C13:0	0.03-0.25	0.10	0.04	38.5	0.05-0.15	0.10	0.02	24.9	0.01
C14:0	9.13-17.97	14.0	2.14	15.3	10.1-16.7	13.4	2.19	16.3	0.37
C14:1 cis9	0.42-2.25	1.07	0.30	27.8	0.67-1.17	0.92	0.17	18.6	0.04
C15:0	0.82-2.03	1.30	0.25	19.4	0.75-1.65	1.19	0.29	23.9	0.05
C16:0	27.6-50.8	39.4	4.84	12.3	32.9-52.3	41.4	5.40	13.1	0.93
C16:1 cis9	0.02-0.21	0.08	0.04	51.0	0.04-0.12	0.09	0.04	42.3	0.01
C16:1 trans9	0.24-3.04	1.11	0.53	48.1	0.60-1.86	1.00	0.43	42.7	0.04
C17:0	0.19-0.82	0.45	0.15	32.3	0.30-0.65	0.42	0.10	23.6	0.02
C18:0	5.90-16.62	10.9	2.17	19.9	8.45-13.6	10.6	1.92	18.1	0.3
C18:1 cis11	0.10-0.90	0.37	0.18	49.5	0.22-0.53	0.36	0.10	28.7	0.02
C18:1 cis9	7.55-33.9	15.7	6.97	44.4	6.41-27.8	15.1	8.10	53.8	0.41
C18:1 trans11	0.16-3.65	1.23	0.75	61.2	0.54-2.88	1.18	0.75	63.4	0.05
C18:1 trans9	0.02-0.24	0.08	0.05	63.1	0.02-0.14	0.08	0.05	56.2	0.01
C18:2 cis9 trans11	0.09-4.24	1.40	1.00	71.6	0.16-2.79	1.4	0.99	70.7	0.05
C18:2 cis9, 12	0.44-3.66	1.25	0.55	43.8	0.27-2.81	1.39	0.73	52.8	0.05
C18:3 cis6, 9, 12	0.01-0.04	0.02	0.01	40.0	0.01-0.02	0.02	0.01	39.2	0.01
C18:3 cis9, 12, 15	0.07-1.80	0.77	0.45	58.8	0.12-1.48	0.82	0.49	59.4	0.03
C20:0	0.02-0.17	0.07	0.04	54.0	0.02-0.11	0.06	0.03	48.0	0.01
C20:3 cis8, 11, 14	0.01-0.07	0.03	0.01	45.5	0.01-0.03	0.02	0.01	42.0	0.01
C20:4 cis5, 8, 11, 14	0.01-0.12	0.06	0.02	41.5	0.01-0.10	0.05	0.02	46.7	0.01
C20:5 cis5, 8, 11, 14, 17	0.01-0.05	0.02	0.01	53.2	0.01-0.04	0.02	0.01	57.6	0.01
C22:5 cis7, 10, 13, 16, 19	0.01-0.07	0.02	0.01	66.6	0.01-0.04	0.02	0.01	53.4	0.01
SFA total	59.7-86.6	76.8	7.17	9.33	63.9-87.5	77.6	7.74	9.97	---
MUFA total	9.86-38.2	19.6	7.53	38.4	9.30-32.6	18.7	8.36	44.8	---
PUFA total	1.21-7.20	3.51	1.43	40.6	1.58-3.55	3.70	1.11	30.0	---
SCFA	2.97-9.87	6.29	1.81	28.7	3.27-8.47	6.17	1.88	30.4	---
MCFA	40.61-71.77	57.63	6.83	11.9	47.9-70.3	59.0	7.07	12.0	---
AI	1.74-10.13	5.28	2.14	40.6	2.32-9.96	5.61	2.49	44.4	---
TI	1.54-8.31	4.62	1.76	38.1	1.98-8.30	4.73	2.05	43.3	---

SD: Standard deviation; CV: Coefficient of Variation; SFA: saturated Fatty Acids; MUFA: monounsaturated Fatty acids; PUFA: polyunsaturated fatty acids; SCFA: short chain saturated fatty acids (C6:0+C8:0+C10:0), MCFA: medium chain saturated fatty acids (C12:0+C14:0+C16:0); AI: Atherogenicity index, calculated as AI= (C12:0+4*C14:0+C16:0)/(MUFA+n-6PUFA+n-3PUFA); TI: Thrombogenicity index (Ulbricht & Southgate, 1991), calculated as TI= (C14:0+C16:0+C18:0)/(0.5*n-6PUFA+3*n-3PUFA+n-3/n-6) (Ulbricht & Southgate, 1991); σ_R=Horwitz standard deviation. Limit of Detection: 0.004g/100gFA.

Table 3.- Cross validation and external validation statistics for fatty acid composition and sums of FA of raw liquid milk samples scanned with a handheld NIR sensor in a range 1600-2400 nm.

		Calibration set							Validation set				
Fatty Acid	**Math pre-treatment**	**N samples**	**Slope$_{cv}$**	**N Terms**	**R^2_{cv}**	**Bias**	**SECV**	**RPD**	**R^2_v**	**SEP**	**SEP(c)**	**S_r**	**S_r/σ_R**
C6:0	SG 1.10.10.1	89	0.85	6	0.91	-8.0E-03	0.32	2.39	0.87	0.09	0.05	0.11	1.56
C8:0	SG 1.10.10.1	76	0.45	7	0.63	-2.0E-03	0.11	1.52	0.48	0.03	0.03	0.02	0.41
C10:0	SG 2.5.5.1 + SNV	89	0.65	5	0.79	8.0E-03	0.62	1.60	0.80	0.13	0.13	0.19	1.62
C12:0	SG 2.10.10.1 + SNV	84	0.58	3	0.75	3.0E-03	0.69	1.49	0.63	0.18	0.13	0.08	0.60
C12:1 cis 11	SG 2.10.10.1 + SNV	77	0.56	3	0.72	-2.0E-04	0.02	1.60	0.26	0.01	0.00	0.00	0.23
C14:0	SG 1.5.5.1 + SNV	88	0.61	9	0.77	2.0E-02	1.37	1.56	0.56	0.45	0.58	0.22	0.59
C16:0	SG 2.5.5.1 + SNV	80	0.54	2	0.73	3.6E-02	2.96	1.64	0.07	1.57	0.51	0.25	0.26
C18:0	SG 1.10.10.1 + SNV	80	0.35	6	0.54	2.1E-02	1.53	1.42	0.01	0.55	0.53	0.12	0.40
C18:1 cis 9	SG 1.10.10.1	87	0.82	6	0.88	-3.3E-02	2.95	2.36	0.82	1.04	0.96	0.63	1.53
C18:1 trans 11	SG 2.5.5.1 + SNV	72	0.60	4	0.78	-1.0E-02	0.49	1.52	0.37	0.19	0.25	0.09	1.82
C18:2 cis 9 trans 11	SG 1.5.5.1 + SNV	85	0.73	6	0.85	7.0E-04	0.55	1.81	0.57	0.22	0.26	0.21	4.24
C18:2 cis 9, 12	SG 1.10.10.1	94	0.63	11	0.78	-5.9E-03	0.35	1.57	0.17	0.19	0.19	0.11	2.12
C18:3 cis 9, 12, 15	SG 1.10.10.1 + SNV	77	0.82	6	0.89	-4.0E-04	0.21	2.11	0.92	0.04	0.05	0.07	2.39
C20:0	SG 1.10.10.1	80	0.68	5	0.81	1.0E-04	0.02	1.73	0.58	0.01	0.02	0.00	0.39
C20:4 cis 5, 8, 11, 14	SG 1.10.10.1 + SNV	74	0.51	3	0.70	3.0E-05	0.02	1.40	0.45	0.01	0.01	0.00	0.26
SFA total	SG 1.10.10.1	90	0.73	4	0.82	1.5E-02	3.86	1.86	0.72	1.15	1.11	----	----
MUFA total	SG 2.10.10.1 + SNV	85	0.80	7	0.86	-2.1E-02	3.56	2.12	0.83	0.97	0.87	----	----
PUFA total	SG 2.5.5.1 + SNV	78	0.70	5	0.82	-1.4E-02	0.72	1.97	0.55	0.22	0.14	----	----
SCFA	SG 2.10.10.1 + SNV	82	0.83	8	0.89	-7.0E-03	0.80	2.25	0.87	0.22	0.18	----	----
MCFA	SG 2.10.10.1 + SNV	84	0.62	2	0.78	1.1E-01	3.82	1.79	0.43	1.41	1.42	----	----
AI	SG 2.10.10.1 + SNV	81	0.73	5	0.85	1.1E-02	1.13	1.90	0.56	0.48	0.29	----	----
TI	SG 1.10.10.1	90	0.80	4	0.89	2.5E-02	0.82	2.16	0.55	0.39	0.38	----	----

SG N1 N2N3: Savitzky Golay, derivative order, smooth on the left, smooth on the right and polynomial order; SNV: Standard Normal Variate; R^2_{CV}: coefficient of determination for cross validation; SECV: standard error of cross validation; RPD: SD/SECV ; R^2_v: coefficient of determination for external validation; Sr: Standard deviation of NIR repeatability; σ_R: Standard deviation of laboratory reprodicibility; SFA: saturated Fatty Acids; MUFA: monounsaturated Fatty acids; PUFA: polyunsaturated fatty acids; SCFA: short

chain saturated fatty acids (C6:0+C8:0+C10:0), MCFA: medium chain saturated fatty acids (C12:0+C14:0+C16:0); AI: Atherogenicity index, calculated as AI= (C12:0+4*C14:0+C16:0)/(MUFA+n-6PUFA+n-3PUFA); TI: Thrombogenicity index (Ulbricht & Southgate, 1991), calculated as TI= (C14:0+C16:0+C18:0)/(0.5*n-6PUFA+3*n-3PUFA+n-3/n-6) (Ulbricht & Southgate, 1991)