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INSTRUMENTAL METHODS AS STRATEGIC SUPPORTING TOOLS FOR SYSTEMATIC BIOCHEMICAL ANALYSIS OF SERRA DA ESTRELA SHEEP MILK

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Abstract: Serra da Estrela sheep crude milk is mandatory for the PDO Serra da Estrela (SE) cheese production. The production system implies the need for systematic knowledge of the values of useful matter (fat and protein) to produce cheese. The knowledge of somatic cell count (SCC) allows to predict the mammary health status of ewes as well as make decisions on the use of crude milk for human consumption, through its transformation into cheese. The need for simple, fast and reliable methodologies for the determination of this parameters is fundamental for the sustainability of this productive sector through milk recording procedure for animal genetic evaluation, as well for the previous analysis of the bulk milk, before the process of elaboration of the PDO SE cheese. Two sets of milk samples were collected: 50 individual samples of Serra da Estrela ewes' milk from 2 farms for analysis of SCC and 53 samples of Serra da Estrela ewes' milk (16 individual samples and 37 samples of bulk milk) for analysis of fat and protein contents (Fat% and Prot%). Duplicates of samples were simultaneously analyzed by reference and instrumental methodologies (DCC De Laval optical reader and FT-NIR Master™ from Büchi) as reliable alternatives for parameters evaluation for the SE sheep milk. The results showed a significant agreement between the pairs of values (type of methodologies) for all parameters, with correlations between 0.925 (Prot%) and 0.960 (SCC) ($p<0.001$). The linear regressions for the pairs of data of the three parameters studied presented a strong adjustment (with the coefficients of determination between 0.856 and 0.921). The findings showed that both instrumental methodologies applied can be used as alternative to count somatic cells and evaluate fat and protein contents of SE sheep milk and will be useful as strategic measuring devices for milk farmers and cheesemakers.

Keywords: Instrumental analysis; somatic cell count; fat content; protein content; Serra da Estrela sheep milk; Portugal.

INTRODUCTION

The milk recording campaigns promoted by the Serra da Estrela Sheep Breeders Association (ANCOSE) since 1990 (Dinis, 2013) have allowed to know the milk production of the Serra da Estrela (SE) sheep breed, as well as increased and improved actions to dynamize this autochthonous Portuguese breed. However, this knowledge only refers to the amount of milk produced, and, for various reasons, there is no systematic information on other parameters such as the somatic cell count (SCC) and the fat (Fat%) and protein contents (Prot%).

Knowing the importance that the knowledge of these parameters has for the genetic evaluation of the animals, it is obvious the importance of including them, among other parameters, in the breeding scheme of this breed, leading to a new level of productive, technical and scientific development which is fundamental in the valorization of the Protected Designation of Origin (PDO) SE cheese.

Also, the evaluation of these parameters in the raw milk of bulk tanks at cheese factories of PDO SE cheese production is extremely important for the best strategy to increase efficiency and enhance the quality of this unique product.

Analyses of SCC, Fat% and Prot% can be carried out in laboratories using reference procedures, so that producers and associations can have this information to decide on the animal management and cheese making processes. However, these analyses are very time-consuming and expensive, which explains, at least in part, why there is limited information about these parameters.

Moreover, the standard methodologies used to determine the quality parameters for milk and cheese use chemical reagents which are highly polluting and represent not only an environmental risk but also a risk for the operator.

The optimization of methods that allow obtaining equally reliable results, more quickly and with lower costs will be very useful for producers, producers' associations and cheese factories. The near infrared spectroscopy (NIR) and cellular reader optical systems may contribute to achieve this goal, being an added value for the optimization of the response to a productive sector of great relevance at regional and national level.

With the development of this productive sector associated, in parallel, with the collection and generation of a large amount of data regarding these (and possibly others) parameters (De Marchi *et al.*, 2014), there is an urgent need to change the technical-scientific paradigm associated, directly and indirectly, with the PDO SE cheese production chain, allowing an increase in the associated economy.

Somatic cells, always present in milk, consist of epithelial cells (resulting from the desquamation of the alveolar epithelia and ducts of the mammary gland) and blood cells (macrophages; in ewes with healthy udder, they can reach between 45 and 88%) (Shah *et al.*, 2017). High SCC is a strong indication of udder infection, known as mastitis (Alhussien and Dang, 2018). One of the major consequences of increasing SCC is the milk quality drop, with implications on milk processing through productivity decline and the development of undesirable flavours in processed products. Jaeggi *et al.* (2003) reported that sheep milk with a SCC greater than 1 million reduces cheese yield and increases cheese rancification.

SCC analysis can be performed using the direct microscopy counting as the reference

method (IDF, 2008). However, their systematic implementation is impractical because of the time-consuming sample preparation and microscopic cell counting, which require both expertise and experience (Spanu, 2010). On the other hand, the secondary (instrumental) reference method, relying on the fluoro-optical-electronic principle, has played an important role in milk analyses massification (Berger and Luginbühl, 2016), despite the limitation of the time-delayed response.

The Fat% and Prot% of sheep milk are the ratios between the fat and protein quantities, respectively, to the milk yield. These parameters, with generically higher values in sheep compared to cow and goat milks (Gantner *et al.*, 2015; Vara Martínez *et al.*, 2018), are fundamental to guarantee profitability in the PDO SE cheese production. The protein matrix formed through the coagulation of the milk by the enzymatic action of the thistle (*Cynara cardunculus* L.) will aggregate the fat globules, providing a mass of unique textural and organoleptic characteristics, after ripening (Fogeiro *et al.*, 2020; Barracosa *et al.*, 2021).

The analysis of milk fat and protein yields (and consequently Fat% and Prot%) is carried out by reference laboratory methods (Kala *et al.*, 2019). However instrumental evaluation by near infrared spectroscopy (NIR) has allowed data incrementation of these parameters in a less costly way.

Fourier-transform near infrared spectroscopy (FT-NIR) is a technique widely used to obtain a near infrared spectrum of absorption or emission of a solid, liquid or gas (De Marchi *et al.*, 2018). The term FT-NIR originates from the fact that a Fourier transform (a mathematical process) is required to convert the raw data into the spectrum (Manuelian *et al.*, 2019).

This technic to assess values of the Fat% and Prot% parameters in PDO SE cheese sector is now starting to be used, like in cow's milk evaluation, allowing good perspectives to achieve better cheese productivity and quality and to implement novel models in the genetic program of the Serra da Estrela sheep breed (Dinis, 2013; Marina *et al.*, 2022).

The use of instrumental methodologies has demonstrated the sector's needs, due to the demand for faster and more expedite techniques to guarantee better decision-making support in the PDO SE cheese chain. In fact, the optimization of a methodology that allows equally reliable results, quickly obtained with controlled costs, will be very advantageous for producers, breeders associations and cheesemakers.

The aim of this work is to evaluate the results of crude milk SCC, Fat% and Prot% from Serra da Estrela sheep breed using instrumental methodologies in contrast with those obtained by reference laboratory methods and to show their importance in the value chain of PDO SE cheese.

MATERIAL AND METHODS

MILK SAMPLES

For this research, two sets of milk samples were collected from Serra da Estrela ewes, considering the different parameters analyzed.

For the SCC analysis, a set of 50 milk samples of 30 ml from 50 Serra da Estrela ewes in different lactation stages were collected in 2 farms.

For Fat% and Prot% analysis, were collected 53 milk samples from Serra da Estrela ewes, obtained in November and March, during a production season. Sixteen of these samples were collected in 4 producers from 4 animals (in different lactation stages), and 37 milk samples from bulk tanks of 10 cheesemakers where the QSE PDO is produced (1 to 8 samples for each cheesemaker).

The samples were conserved at 4ºC until being analyzed in a maximum period of 24

hours.

All samples were analyzed simultaneously with reference and instrumental methodologies.

ANALYTICAL PROCEDURES

The milk samples were submitted to somatic cell enumeration by microscopy direct counting (MDC) - gold standard method - and through a portable optical cell reading equipment (DCC, DeLaval, DeLaval International AB, Tumba, Sweden).

The reference method for milk somatic cell counting is the MDC method, recommended by ISO 13366-1/IDF 148-1 (IDF, 2008). In this work, this methodology was adapted from NP-460 (1985), based on the Breed-Brew technique. The dried and stained smears were observed under a photonic microscope with X100 objective, counting the somatic cells for each microscope field along 50 fields through a smear diagonal. The average number of cells, from counting the 50 microscope fields with the multiplicative factor 800000 was expressed as number of cells/ml milk.

The DeLaval cell counter (DCC; DeLaval International AB, Tumba, Sweden) is a portable optical system. The DCC counts somatic cell nuclei stained with a DNA specific fluorescent probe (propidium iodide). The milk is sampled by a piston and guided into a measuring window in a removable, non-reusable device (cartridge). Only a milk volume of 60 µl is used for counting. The stained nuclei are exposed to a LED light source and the fluorescent signals used to determine the SCC (Spanu, 2010). After 1 minute of reading, the SCC value is shown on the equipment's display.

The milk samples were also analyzed for Fat% and Prot% in an accredited laboratory with certified reference methodologies considered *gold standard* (determination of Prot% though Dumas technique [combustion] and Fat% using Pulsed Nuclear Magnetic Resonance) and simultaneously were assessed through FT-NIR technology, using a NIR Master™ FT-NIR spectrometer (Büchi, Flawil, Switzerland).

The analyses developed in FT-NIR were based on the chemometric references of the equipment database (Büchi, Flawil, Switzerland), allowing, among other parameters, the analysis of the milk Fat% and Prot%. The equipment is calibrated for each of the sample types to be analyzed, using internal and external standards for calibration. The milk samples are placed in the container (sample container and cover disc). The equipment makes a triplicate reading for each sample analyzed.

STATISTICAL ANALYZES

The statistical evaluation of differences between the means of SCC, Fat% and Prot% obtained by the two types of methodologies were performed using paired samples *t*-Student tests. Data were submitted to Pearson correlation and linear regression analyses. The exclusion of outliers was assessed using Cook's Distance method. SPSS v28 software was used, with a significance level of 0.05.

RESULTS AND DISCUSSION

For both sets of milk samples, considering the values obtained by both types of methodologies, the discrepancies between pairs of data were analyzed. We verified, for the SCC, that 4 observations do not belong to the prediction interval (95% confidence interval) and of these, 3 have extreme values higher than 3 million somatic cells by ml of milk, with great discrepancy between pairs of data for both methodologies. After outlier analysis of the residuals, it was confirmed that these observations have a great influence on the regression, with Cook's Distance values higher than 1 (observations with Cook's

Distance higher than 1 are over-influential). Following the same methodology, we found, for Fat% and Prot%, the existence of 3 and 5 pairs of data considered outliers, respectively.

Therefore, after the elimination of outliers, we observed a pattern of similarity between the values from the reference methods and those obtained with the instrumental methodologies for all parameters (Table 1; $p>0.05$). This fact is verified in other similar studies, for Fat% and Prot% (Aernouts *et al.*, 2015; Šustová *et al.*, 2007; Šustová *et al.*, 2014) and SCC (Hanuš *et al.*, 2009; Hanuš *et al.*, 2014b; Krupa Rose *et al.*, 2022).

Table 2 shows the very close relationship between the pairs of values, through the very high correlations: 0.960 (SCC), 0.931 (Fat%) and 0.925 (Prot%) ($p<0.001$).

Table 2 also shows the linear regression equations for the three parameters studied. We can state that there is a very strong adjustment between the pairs of data obtained from the two methods of analysis for any of the parameters studied. In fact, the regression lines almost match the main diagonal (Figures 1, 2 and 3), although the determination coefficients for Fat% and Prot% are slightly less than 0.900.

The graphical representation of the data pairs, through the regression lines (Figures 1, 2 and 3), as well as the high values of the determination and correlation coefficients show the close relationship between the two methodologies used and it can be stated that both instrumental methodologies are alternatives to the reference methods in the prediction of SCC, Fat% and Prot%.

The results of this study for SCC agree with those obtained by several authors (Gonzalo *et al.*, 2006; Kawai *et al.*, 2013) in milk sheep data, using the DCC equipment, considering some aspects inherent to sample handling and methodological particularities.

The limitation of the DCC to read values above 4 million somatic cells (observed in the outlier analysis) is a minor issue, as the problematic aspects, either in the predictability of udder health (intra-mammary infection) or in the decision to use the milk for cheese making, are limited to a lower value range. Both milk production and milk quality start to be compromised at 300,000 cells/ml milk. The increase of SCC from 300,000 cells/ml to 1,000,000 cells/ml milk, suggests that such secretion could be considered as a transition from normal to mastitic milk (Albenzio *et al.*, 2011). Kahinda (2021) reports that SCC in sheep milk can be used to define subclinical mastitis and a threshold of 200,000 to 400,000 cells/ml milk will accurately identify most sub-infected sheep. In sheep milk with SCC greater than 1,000,000 cells/ml milk there are differences in leukocyte production (Shah *et al.*, 2017) and provides lower cheese yield and enhances the development of rancified flavors and odors in cheeses (Jaeggi *et al.*, 2003). However, Leitner *et al.* (2016) note that SCC thresholds should be tested and determined for each breed, type of management and final dairy product.

In previous research of the same authors (Oliveira *et al.*, 2018) in the calibration of FT-NIR equipment for Serra da Estrela sheep milk samples, based on partial data used in this study, they verified that the analysis of Fat% and Prot% values through spectrometry are adequately represented in a linear regression with a 45º slope considering the same values analyzed by gold standard methodology. Šustová *et al.* (2014) had already verified this trend, presenting similar linear regression results for Fat% and Prot%, with determination coefficients of 0.984 and 0.994, respectively.

One of the great potentialities of these approaches is to allow the scale-up of the sample size in the procedures and methodologies validated in milk recording of Serra da Estrela sheep (ICAR, 2017), thus

Parameter		n	IM	GSM	Prob.	Sig.
SCC	47	274617.0±448546.7	311829.8±418479.2	>0.05		*ns*
Fat%	50	7.7±1.6	7.5±1.6	>0.05		*ns*
Prot%	48	5.7±0.8	6.2±0.9	>0.05		ns

GSM – Gold standard methodologies; IM – Instrumental methodologies; (ns – nonsignificant).

Table 1. Comparison of parameter values according to the methodologies adopted (GSM vs IM).

Parameter	n	Regression Eq.	R^2	R	Sig.
SCC	47	-46175.228+1.029*SCC(GSM)	0.921	0.960	***
Fat%	50	0.438+0.968*Fat%(GSM)	0.867	0.931	***
Prot%	48	0.498+0.848*Prot%(GSM)	0.856	0.925	***

GSM – Gold standard methodologies; IM – Instrumental methodologies; *** – prob <0.001.

Table 2. Relationship between pairs of values according to the methodologies adopted (GSM vs IM) through linear regression equations, and correlation and determination coefficients.

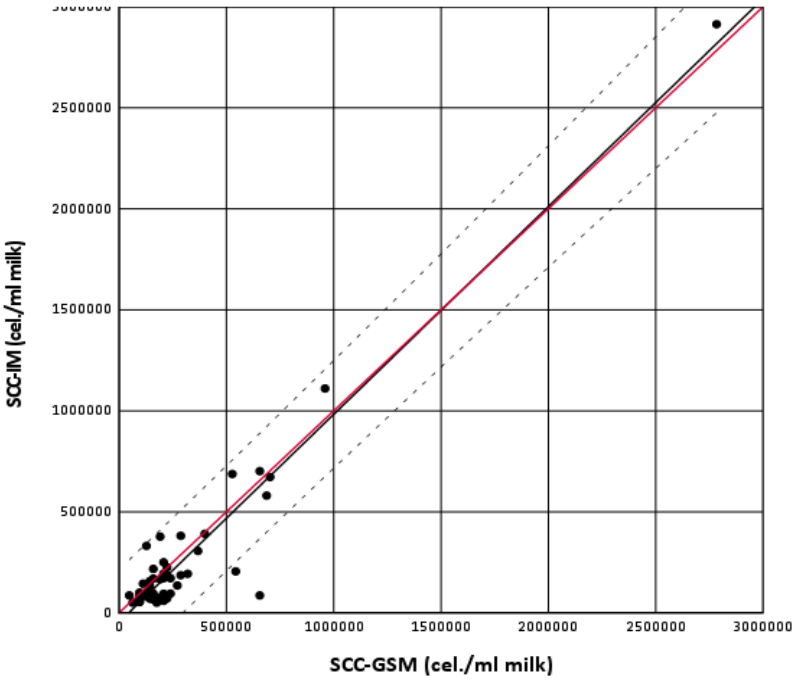

Figure 1. Scatter plot of the measured values (except outliers), the regression line (–) [SCC(IM)=-46175.228+1.029*SCC(GSM); R2=0.921], 95% confidence interval (--) for the values observed through IM (prediction interval) and optimal regression line (-), for SCC.

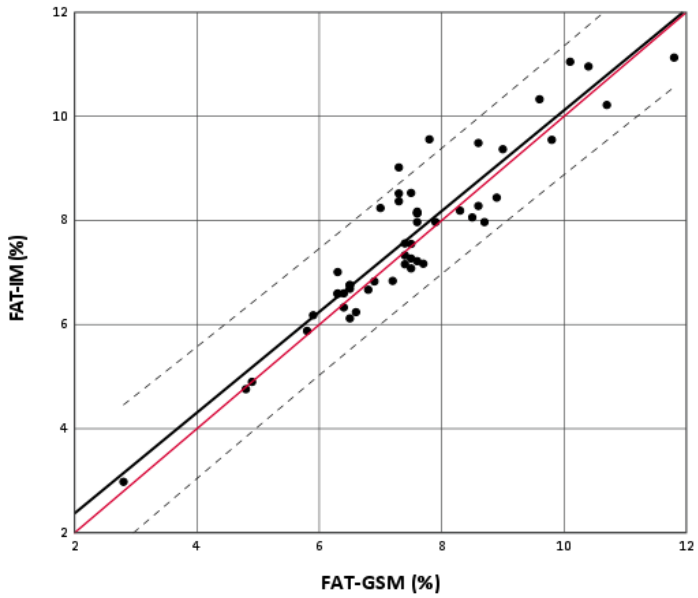

Figure 2. Scatter plot of the measured values (except outliers), the regression line (–) [Fat%(IM)=0.438+0.968*Fat%(GSM); R^2=0.867], 95% confidence interval (--) for the values observed through IM (prediction interval) and optimal regression line (-), for Fat%.

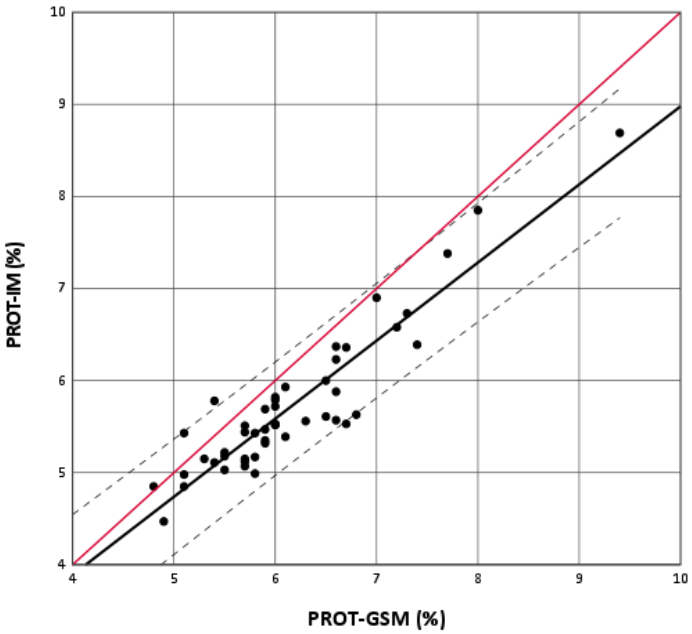

Figure 3. Scatter plot of the measured values (except outliers), the regression line (–) [Prot%(IM)=0.498+0.848*Prot%(GSM); R2=0.856], 95% confidence interval (--) for the values observed through IM (prediction interval) and optimal regression line (-), for Prot%.

being able to provide technical support to ANCOSE and directly to the milk producers of this breed. At the cheesemakers, due to their user-friendly operation, they can be fundamental equipment in supporting the production process and promoting the quality of the PDO SE cheese.

With the approach carried out and the results obtained, we are intending to develop future research for a more comprehensive evaluation of the multifactorial causes that contribute to the diversity of the organoleptic and yield features, as well as the chemical analysis of the PDO SE cheese. There are several studies that have confirmed the usefulness of these methodologies in the study of several parameters in various agri-food products, namely associated with milk and functional properties during cheese production (Čurda and Kukačková, 2004; Currò *et al.*, 2017; Hanuš et al., 2014a; Madalozzo *et al.*, 2013; Manuelian et al., 2019; Mlcek et al., 2016).

CONCLUSIONS

The use of this equipment as instrumental tools for biochemical analysis of milk samples has the following advantages: (1) minimum sample preparation; (2) evaluation of the sample in its natural state; (3) simultaneous analysis of different parameters (spectral information [FT-NIR]); (4) fast analysis (about 1 minute, excluding preparation); (5) reduced cost; (6) absence of reagents; (7) ease of learning and use.

The findings showed that the instrumental devices tested can be used to quantify Serra da Estrela ovine milk SCC, Fat% and Prot% on milk farmers and cheesemakers.

A great opportunity of this approach is the scalability of the validated procedures and methodologies to support the milk recording of Serra da Estrela sheep breed (ICAR, 2017). It is also undeniable the easiness of application in cheesemakers and can be fundamental equipment in supporting the production work, fostering to improve the quality of PDO SE cheese.

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