Energy-dispersive spectroscopy for the quantitative determination of the major chemical elements in milk

Espectroscopia por energia dispersiva para a determinação quantitativa dos principais elementos nas cinzas de leite

Espectroscopia por energia dispersiva para la determinación cuantitativa de los principales elementos en las cenizas de leche

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Abstract
The present preliminary study aims at the rapid quantitative determination of the major chemical elements in the ashes of raw milk samples, using energy dispersive X-ray fluorescence spectrometry. The precision of the method was satisfactory (variation coefficient ≤ 5%). The mean values (in mmol·kg-1) obtained from the analysis of 32 raw milk samples showed good agreement with data from the Brazilian and international literature: K (26.1 ± 4.6), Ca (33.8 ± 3.7), P (28.4 ± 2.7), Na (21 ± 3.3) and Mg (4.7 ± 0.5). Residual Cl concentration (19.0 ± 3.3 mmol·kg-1) was lower than expected due to incineration losses. The results suggest a good application potential of the method for other dairy products.
Keywords: Milk minerals; X-Ray spectrometry; Milk ashes; Milk analysis.

Resumo
O presente estudo preliminar visa a rápida determinação quantitativa dos principais elementos químicos nas cinzas de amostras de leite cru, utilizando espectrometria de fluorescência de raios-X por energia dispersiva. A precisão do método foi satisfatória (coeficiente de variação ≤ 5%). Os valores médios (em mmol·kg $^{-1}$) obtidos na análise de 32 amostras de leite cru mostraram boa concordância com os dados da literatura nacional e internacional: K (26,1 ± 4,6), Ca (33,8 ± 3,7), P (28,4 ± 2,7), Na (21 ± 3,3) e Mg (4,7 ± 0,5). A concentração residual de Cl (19,0 ± 3,3 mmol·kg^{-1}) foi menor do que o esperado devido às perdas por incineração. Os resultados sugerem um bom potencial de aplicação do método para outros produtos de laticínios.
Palavras-chave: Minerais do leite; Espectrometria de raios-X; Cinzas do leite, Análise de leite.

Resumen
El presente estudio preliminar objetiva la rápida determinación cuantitativa de los principales elementos químicos en las cenizas de muestras de leche cruda, utilizando la espectrometria de fluorescencia de rayos-X por energia dispersiva. La precisión del método fue satisfactoria (coeficiente de variación ≤ 5%). Los valores promedios (en

mmol·kg $^{-1}$) obtenidos de la análisis de 32 muestras de leite cruda mostraron buena concordancia con los datos reportados en la literatura nacional e internacional: K (26,1 ± 4,6), Ca (33,8 ± 3,7), P (28,4 ± 2,7), Na (21 ± 3,3) e Mg (4,7 ± 0,5). La concentración residual de Cl (19,0 ± 3,3 mmol·kg^{-1}) fue menor de la esperada debido a pérdidas durante la incineración. Los resultados sugieren un buen potencial de aplicación del método para otros productos lácteos

Palabras clave: Minerales de la leche; Espectrometria de rayos-X; Cenizas de la leche; Análisis de leche.

1. Introduction

Apart from the major milk constituents (water, lactose, fat, and protein), bovine milk contains a considerable amount of inorganic and organic substances in small quantities, the so-called milk ashes, making up less than 1% (m·m^{-1}) of the gross composition. The ash content varies typically between 0.68% and 0.78% (m·m^{-1}). It comprises the milk minerals, trace elements, and organic matter (e.g., citric acid), which were present in milk as ions or were in equilibrium with them, but excluding H$^+$ and OH$^-$ ions before the ash determination (Töpel, 1981). The ash content corresponds roughly to about 80% of the salt content of milk and is understood as the white residue of alkaline character left after milk's incineration at 500 – 550°C. In earlier times, it was recognized that the ash composition does not represent the state of the milk salts as they actually occur in milk because, e.g., oxidation and volatilization during the incineration alter their composition (Porcher & Chevallier, 1923; Rogers, 1935). For example, citrate is totally destroyed; from the minerals remains their oxides, carbonates, and sulfates; phosphor and sulfur from the proteins and lipid components are found in the form of phosphates and sulfates. Part of the carbonate results from the CO$_2$ from the combustion of organic materials (Töpel, 1981), and chlorine is partially lost during ashing (Porcher & Chevallier, 1923; Rogers, 1935; Sanders, 1939).

The milk salts and minerals (Ca, P, Mg, Na, citrates, etc.) and their distribution among the colloidal and soluble phases of milk are essential for all dairy technology processes. Some examples are milk acidification in the production of acid curds; rennet coagulation and natural cheese properties (Stocco et al., 2021); heat treatment of milk and stability; interactions with chelating agents in process cheese making; etc. (Broyard & Gaucheron, 2015). However, the determination of the major individual ash minerals (calcium, potassium, magnesium, sodium, phosphor) and other elements as chloride and oxygen is cumbersome and time-consuming. The energy dispersive X-ray fluorescence spectroscopy (EDS, used together with a scanning electron microscope, SEM) is a relatively quick method and has been recently used for the rapid determination of minerals in skim milk powder (McCarthy et al., 2020); infant milk powder formulae (Papachristodoulou et al., 2018); and dairy processing sludges after acid digestion (Daly et al., 2019).

According to literature research, it seems that the combination of the sample preparation and methodology used in the present study has not been previously used for raw milk analysis (Ali et al., 2014; De La Fuente et al., 1997; Habib-Ur-Rehman et al., 2012). Most sample preparation used freeze-drying, which is very expensive and time-consuming, or a digestion step to neutralize the interference of the organic fraction of the samples. Therefore, the present investigation presents our preliminary results on this spectroscopic technique's applicability for the rapid quantification of the major minerals and chloride in the ash fraction obtained from raw milk samples. The results are compared to the known composition of elements from bovine milk minerals.

2. Methodology

2.1 Sample preparation

A total of 32 (individual sand bulk) milk samples collected from a farm in Minas Gerais, located in the southeast of Brazil, were analyzed for their ash content and mineral distribution, including chlorine. Approximately 5 g of each sample was transferred into a crucible pre-weighted after thorough drying. The crucible with the sample was pre-dried for about 2 hours at

105°C in a gravity-flow convection oven before incineration in a muffle furnace at 550°C for 3.5 hours. The ash content was calculated by the difference between the sample weight and the residue (ash) remaining after incineration.

2.2 Analysis

Approximately 0.5 g of ashes was weighted for mineral analysis after mixing with a pestle in a mortar to ensure better homogeneity. The sample was taken to a pelletizer, transferred to a hydraulic press and compressed for 5 minutes at a pressure close to 7.4 x 10^8 Pa. This preparation was done to guarantee a flat surface, as it is known that the effect of absorption is strongly dependent on the tilt of the sample surface with respect to the X-ray detector (Garratt-Reed & Bell, 2005). The pellet was taken to a scanning electron microscope (SEM) (Hitachi, model TM3000 TableTop) coupled with an Energy Dispersive X-ray spectrophotometer (EDS) (Quantax 70 accessory - Bruker AXS Microanalysis set at Analysis mode). The EDS detector was a Silicon drift type (SDD); the energy resolution was 154 eV and the work distance 8.5 mm. The analysis was carried out in 5 fields of each sample. The chosen fields were in the center and in each corner of the pellet to guarantee a better representative sample.

In SEM/EDS equipment, the atoms (the electrons) of the elements in the pellet are excited by an electron beam and emit X-rays of a wavelength characteristic of the atomic structure of the elements in the sample. An Energy Dispersive X-ray Spectometer discriminates the X-ray energy, which is distinctive of each element, like a "fingerprint". It thus analyzes the emissions (the "fingerprints"), assigning them to the specific elements in the pellet's surface via comparison with reference spectra.

The fairly quantitative result generated by the measurement is then expressed as a percentual of normalized weight (%wt). The result was then transformed into mmol (Equation 1):

$$mmol \cdot kg^{-1} = \frac{(\%wt) \ \times RMF \ \times 100}{MM} \quad (Equation \ 1)$$

Where *%wt* is the normalized weight percentage provided by the SEM/EDS analysis; *RMF* is the fixed mineral residue; *MM* is the molecular mass of the element.

To check the precision of the method, i.e., the repeatability of the measurements under the same conditions, the ash content of three different raw milk samples was determined in duplicate. The corresponding pellets were prepared according to the above procedure and immediately analyzed each five times via SEM/EDS. The data average and its standard deviation were calculated for each sample and duplicate, and the variation coefficient was taken as an expression of the repeatability (precision). A t-test was applied to verify if the mean values of each pair have a statistical significance in the results for the selected elements.

2.3 Statistical analysis

The statistical analyses were conducted using software Excel® (Microsoft Office, 2016) and Statistica 8.0 for Windows.

3. Results and Discussion

The energy dispersive X-ray spectroscopy method (EDS) allowed the rapid identification and quantification of milk gross elements composition from the ashes. The results are obtained as illustrated in Figure 1. Despite the broad number of

elements possible to be detected with this technique (Sögut et al., 2013), the rapid analysis focused on the following elements: oxygen, calcium, potassium, phosphorus, sodium, magnesium, and chlorine, as showed in Figure 1. As stated before, there is a considerable loss of chlorine when the sample is incinerated (Sanders, 1939). Hence, the results presented hereunder for this element are considered as residual chloride. Therefore, the residual chloride determination from the milk ashes will not allow to establish correlations with a pathological change in the milk from animals with mastitic or with osmotic changes in the secretory cells, whereas Na and K concentrations as determined with the used method, along with lactose determinations, do.

Figure 1: Topography of the analyzed sample (A); general sample composition (B); X-ray dispersive energy spectrum (C); distribution of calcium (D), potassium (E), phosphorus (F), chlorine (G), sodium (H) and magnesium (I).
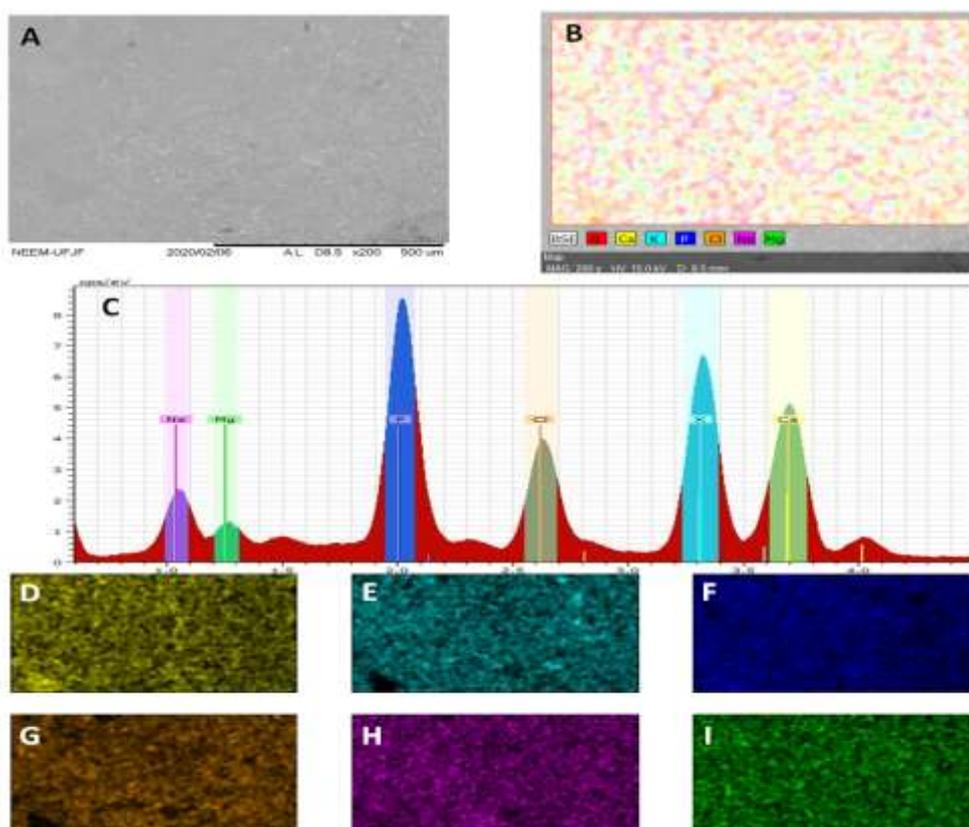
Source: Authors.

Figure 1 shows the graphic results obtained with the energy dispersive X-ray spectroscopy method (EDS), which are then converted to the numeric values statistically analyzed.

The results of three different raw milk samples determined in duplicate are presented in Table 1. The precision of the method expressed as the coefficient of variation (CV in %) shows both the samples' homogeneity and how repeatable the measurements are within the five analyzed fields of a sample and each element. Overall, most of the values are under 5% variation, which can be considered a good analytical result and a sign that the established protocol for the pellet preparation was adequate. Regarding the data analysis for the precision of the method, the statistical results of duplicates from three samples are presented in Table 1.

Table 1: Precision of the method: mean values (mmol·kg^{-1}) for K, Ca, Cl, P, Na, and Mg; standard deviation and variation coefficient CV (%) (n=3 milk samples, duplicate determinations, 5 fields analyzed).

Sample		1		2		3	
Duplicate		1	2	1	2	1	2
Element	Parameter						
K	Concentration (mmol·kg^{-1})	32.6±0.6[a]	33.3±0.8[a]	28.6±1.8[a]	27.8±0.5[a]	29.7±1.4[a]	32.0±0.8[b]
	CV (%)	1.84	2.40	*6.29*	1.80	*4.71*	2.50
Ca	Concentration (mmol·kg^{-1})	28.6±0.4[a]	26.6±0.5[b]	25.9±0.9[a]	28.3±0.8[b]	34.8±1.2[a]	33.4±0.2[a]
	CV (%)	1.40	1.88	3.47	2.83	3.45	0.60
Cl	Concentration (mmol·kg^{-1})	22.4±0.3[a]	23.0±0.5[a]	20.1±1.7[a]	20.5±0.6[a]	23.0±1.0[a]	26.3±0.9[b]
	CV (%)	1.34	2.17	*8.46*	2.93	4.35	3.42
P	Concentration (mmol·kg^{-1})	24.5±0.2[a]	23.6±0.3[b]	22.4±1.8[a]	23.4±0.4[a]	28.7±0.8[a]	29.2±0.2[a]
	CV (%)	0.82	1.27	*8.07*	2.52	2.79	0.68
Na	Concentration (mmol·kg^{-1})	16.3±0.2[a]	16.0±3.2[a]	16.5±0.7[a]	15.9±0.4[a]	20.7±0.8[a]	21.4±0.5[a]
	CV (%)	1.23	*20.00*	4.24	2.52	3.86	2.34
Mg	Concentration (mmol·kg^{-1})	4.4±0.1[a]	4.3±0.0[a]	4.0±0.1[a]	4.0±0.5[a]	4.9±0.1[a]	4.3±0.1[b]
	CV (%)	2.27	2.33	2.50	*12.50*	2.04	2.33

Within any row and sample (1, 2, or 3), duplicate values with the same superscript are not statistically different according to the T-test (p > 0.05). Source: Authors.

The repeatability of the determinations is given by the variation coefficient of the results within 1 sample (e.g., sample 1, duplicate 1, Potassium (K), CV = 1.84%). It can be initially observed that some samples presented a CV superior to 5% for some elements (e.g., for K, sample 2, duplicate 1 with 6.29%), which is not ideal and should be improved. A more detailed look at the results revealed an outlier value for few samples within the 5 repetitions. These outliers might be explained, considering the samples' final solid-state and the pellet preparation steps, which allow for tiny elements agglomeration only perceptible under the microscope. The expectation of this kind of error was one reason for adopting a security range protocol with five readings along the whole pellet. Therefore, considering that the results are the mean of five different fields, the outliers were excluded, and a new statistical analysis was carried out without these data (data not shown). In these few cases, the mean value represented four fields instead of the original five. The new calculated CV (in %) were within expectation ($\leq$ 5%): 3.24 (K); 3.64 (K); 4.64 (Cl); 2.78 (P); 2.84 (Na); and 2.63 (Mg), changing thus the CV (%) values (in bold, Tab. 1) from 6.29 (K); 4.71 (K); 8.46 (Cl); 8.07 (P); 20.00 (Na); and 12.50 (Mg), respectively. The mean values of those samples did not change meaningfully after deletion of the outliers; only the relative standard deviation improved. Papachristodoulou et al. (2018) reported relative standard deviations of 4.9% for Ca, and 5.5% for K, agreeing with our results (Tab.1).

Moreover, in a further examination of the differences between the mean values obtained for each element in duplicate determinations (Tab.1), which indicate how close to each other the mean values are, it was observed that in two cases (sample 1, Na and sample 2, Mg) a statistical significance appeared (table not shown) after deletion of the outlier. The biggest difference between mean values (~1.8 mmol·kg^{-1}) found for K in sample 3 makes less than 6% of the overall mean (30.1 mmol·kg^{-1}); for Ca with a maximal difference of 2.4 mmol·kg^{-1}, it makes 8.8% (sample 2); for Cl (3.3 mmol·kg^{-1}) it makes 13.4% (sample 3); for P (~1.8 mmol·kg^{-1}) it makes 8% (sample 2); for Na (1.3 mmol·kg^{-1}) it makes ~7.7% (sample 1), and for

Mg, the difference between mean values (0.6 mmol·kg^{-1}) in sample 3 makes 13% of the overall mean (4.6 mmol·kg^{-1}) of the duplicate.

Based on the statistical results, the spectrometric methodology was tentatively accepted as valid for the rapid milk elements analysis and applied to quantify and evaluate the 32 raw milk samples. The samples were treated as described in section 2.0 above. The results are presented in Table 2.

Table 2: Summary of the average concentration of the major elements of 32 raw milk samples.

Component	Ash[*]	K	Ca	Cl	P	Na	Mg
Mean	0.703	26.1	33.8	19.0	28.4	21.0	4.7
maximum	0.762	33.8±0.4	42.0±1.6	23.9±0.9	34.0±0.9	29.9±1.0	6.0±0.1
minimum	0.624	18.3±0.9	25.9±1.0	11.2±0.3	22.4±2.0	16.3±0.2	3.7±0.2
Standard deviation	0.04	4.6	3.7	3.3	2.7	3.3	0.5
Variation coefficient	5.7%	17.6%	10.9%	17.4%	9.3%	15.8%	11.3%

[*] Ash expressed as g·100g^{-1}; elements concentration in mmol·kg^{-1}. Source: Authors.

The mean values for the concentration of K, Ca, P, Na and Mg obtained from our results (Tab. 2) are in agreement with international literature data (Bijl et al., 2013; Gaucheron, 2005; Soyeurt et al., 2009). Only the phosphorus value might be seen to be slightly lower, compared to mean reported values elsewhere (31-32 mmol·kg^{-1}), albeit the value is within the broad milk composition range. There are also small differences between the minimum and maximum values found (Table 2) and those reported elsewhere. Still, the variations may be due to natural causes like the animal breed, time of lactation, season, fodder, and mastitis (Bijl et al., 2013; Gaucheron, 2005; Soyeurt et al., 2009).

Therefore, information about Brazilian's milk composition was searched for a better comparison with the present work results. Nascimento et al. (2010) used inductively coupled plasma optical emission spectrometry (ICP OES) to analyze for Ca, Mg, K, Na, P, Sr, and Zn in Brazilian milk in the north of the country. Our mean values of P, Na, and K are in the range of their results, while Ca and Mg presented higher values in our samples. Our Ca results are also higher than the values found in samples from the southeast of Brazil (Soares et al., 2010). On the other hand, the analyses using flame atomic absorption spectroscopy (FAAS) presented Mg values in agreement with the ones obtained in this paper (Soares et al., 2010). Overall, our results are also in the range of the ones obtained by Souza et al. (2018), employing inductively coupled plasma optical emission spectrometry (ICP OES) in samples treated with a microwave-assisted digester using HNO_3 and H_2O_2. The result obtained for (residual) chloride (19.0 ± 3.3 mmol·kg^{-1}) shown in Table 2 is about two-thirds of the normal chloride concentration in milk (~30 mmol·kg^{-1}) and can be explained by burning losses.

Some differences in the results published by different authors can also be attributed to sample preparation as, for example, substantial amounts of Ca, P, and Mg are strongly linked to the casein micelles. Ergo some elements may not be easily released, causing lower recoveries depending on the sample matrix and the process of eliminating organic matter interference (De La Fuente et al., 1997).

The present preliminary study results show that it is possible to determine the major minerals of raw milk utilizing the milk ashes, with the analytical energy dispersive X-ray fluorescence spectrometry. Comparable results to this methodology

were recently published in the analysis of milk powders (McCarthy et al., 2020) and infant food powder preparations (Papachristodoulou et al., 2018), where the reliability of the analytical methodology was proven. However, McCarthy et al. (2020) found a systemic bias in the Ca determination when compared to other analytical methods as inductively coupled plasma mass spectroscopy. The Ca content found in the present study agrees with the data from the literature; only the phosphorus content (28.4 ± 2.7 mmol·kg^{-1}) might be slightly low and might be related to the milk protein content. This aspect warrants further research.

4. Conclusion

The methodology and the sample preparation protocol presented in this study showed its potential application for the rapid determination of the major minerals (Ca, P, Na, K, and Mg) and residual chloride in the ashes of milk samples. The technique is relatively easy, not much time consuming, and did not utilize chemical reagents, which is aligned with the new tendency of more green chemistry analysis. Due to the CoronaVirus restriction and no access to labs, it was not possible to determine the method's accuracy, which is the next step in validating this method. However, the consonance of the results of the applied standardless approach with the literature data gives us the confidence to present this method as a valid option even to further the development of this analysis for other dairy products.

Further works should focus on comparing and validating this methodology against official methods and studying this sample preparation for other dairy matrices.

Acknowledgments

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