



Analytical potential of total reflection X-ray fluorescence (TXRF) instrumentation for simple determination of major and trace elements in milk powder samples

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ARTICLE INFO

Keywords:

Multielemental
Quantification
Foodstuff
Solid powder
Suspension
TXRF

ABSTRACT

In this contribution, the analytical potential of total reflection X-ray fluorescence (TXRF) instrumentation has been evaluated for the determination of major and trace elements in milk powder. TXRF allows the possibility of direct analysis of solid suspensions without the need for a digestion process and therefore it can be a potential analytical candidate for simple and cost-effective analysis.

A detailed study to select sample preparation and measurements conditions was carried out. Different quantification approaches (including internal standardization and empirical calibration) were also tested. Finally, the developed TXRF methods (W anode) were validated by a strict comparison with the data from the reference methods on a set of twenty-three samples using robust statistics. Results showed that acceptable results can be obtained for K, Ca, Fe and Zn determination if using adequate calibration approaches. Otherwise, only screening results can be obtained for light elements (P and Cl) in milk powder samples.

1. Introduction

Maintenance of optimal electrolyte balance by intake of major and trace elements via food products such as milk powder is of special interest because of the very important role that they play in the proper functioning of children body systems (Habib-Ur-Rehman, 2012). For this reason, the determination and control of specific elements in milk powder samples is important to check the right addition of mineral premix or mineral/vitamin premix during production as well as the compliance to specifications/claims of the final products (Habib-Ur-Rehman, 2012).

X-ray fluorescence spectrometry (XRF) is already a well implemented technique for rapid and simple measurement of major and/or trace elements, especially in matrices such as infant cereals and milk-based products (Pashkova, Smagunova, & Finkelshtein, 2018). One of the major benefits of XRF compared to wet chemical methods is that measurements can be carried out directly on solid samples without the need of preliminary sample mineralization (acidic or alkaline digestion). This fact is of special relevance considering that milk-based products are difficult matrices to mineralize because of their high content of organic

matter, especially fat, which is not completely decomposed by most conventional digestion procedures (Smagunova & Pashkova, 2013). Table S1 summarizes the main analytical parameters of the most cited XRF methods for milk powder analysis in the last fifteen years (2005–2020). As it is shown, for XRF analysis, milk powders can be directly poured into a sample holder with a support film (Chan & Palmer, 2013; Fernandes, Brito, & Gonçalves, 2015) or pressed into pellets (Ahmed et al., 2017; Hasan, 2020; Herreros-Chavez, Morales-Rubio, & Cervera, 2019; Habib-Ur-Rehman, 2012; Perring & Andrey, 2003, 2004; Perring and Blanc, 2008a; Perring and Blanc, 2008b; Smagunova & Pashkova, 2013) without any additional preparation. This fact avoids lengthy and laborious sample preparation steps, using corrosive and toxic reagents, and allows rapid analyses during production of such food products to follow and adapt process parameters on production lines. Moreover, less sample manipulation and elimination of time-consuming digestions also means savings in terms of chemicals, environmental impact, risks, costs and time. As reported in Table 1, XRF provides multielemental information with limits of detection of few mg/kg (for mid Z elements) and hundreds of mg/kg (for low Z elements). These limits of detection are not suitable to determine toxic elements

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<https://doi.org/10.1016/j.foodchem.2022.132590>

Received 18 November 2021; Received in revised form 28 January 2022; Accepted 25 February 2022

Available online 28 February 2022

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Table 1

Overview of publications on the use of XRF methods for the analysis of milk powder samples in the last fifteen years (2005–2020).

XRF system	XRF source	Sample preparation	Analytes	LOD (mg/kg)	Ref.
WDXRF	Rh X-ray tube (3.0 kW)	Pressed tablets (5 g)	Ca, Mg, K, P, Br, Cu, Fe, Rb, Zn	n.a.	Habib-Ur-Rehman (2012)
	Rh X-ray tube (4.0 kW)	Filling a sample holder with powder (5 g)	Al, Cu, Fe, Zn	0.2–7	Fernandes et al. (2015)
	Rh X-ray tube (2.7 kW)	Pressed tablets (3–6 g, Φ : 4.0 cm)	Na, P, Ca, Zn, Br, Sr	n.a.	Smagunova and Pashkova (2013)
	Rh X-ray tube (50 W)	Pressed tablets (4 g, Φ : 3.2 cm)	Na Mg P S Cl K Ca Fe Zn	2 (Cu)	Perring and Blanc (2008a) and Perring and Blanc (2008b)
EDXRF	Rh X-ray tube (3.6 kW)	Pressed tablets (10 g, Φ : 4.0 cm)	Na Mg P Cl K Ca Fe Cu Zn	n.a.	Perring and Andrey (2004)
	Pd (X-ray tube) (25 W)	Pressed tablets (4 g, Φ : 3.2 cm)	P, S, Cl, K, Ca, Fe and Zn	9–222*	Perring and Andrey (2003)
P-EDXRF	Rh X-ray tube (9 W)	Pressed tablets (4 g, Φ : 3.2 cm)	Na, Mg, P, Cl, K, Ca, Fe and Zn		Perring and Blanc (2008a) and Perring and Blanc (2008b)
	Pd/Co X-ray tube (50 W)	Pressed tablets (4 g, Φ : 3.2 cm)	Na, P, S, Cl, K, Ca, Mg, Al, Si, Fe, Mn, Co, Cr, Ni, Cu, Zn, Pb	n.a.	Hasan (2020)
Handheld EDXRF	¹⁰⁹ Cd	Pressed tablets (1 g, Φ : 2.5 cm)	Cl, K, Ca, Fe, Zn, Br, Rb, Sr	1.5–5848	Ahmed et al. (2017)
	Rh X-ray tube (4 W)	Filling a sample holder with powder	Ca	n.a.	Chan and Palmer (2013)
	Rh X-ray tube (0.75 W)	Pressed tablets (0.8 g)	Ca, K, Fe, Cu, Zn	1.7–178	Herreros-Chavez et al. (2017)

WDXRF: Wavelength dispersive XRF, EDXRF: Energy dispersive XRF, P-EDXRF: Polarized beam-EDXRF.

*Limit of quantification.

present at $\mu\text{g}/\text{kg}$ level but they are adequate to evaluate nutritional value and to ensure the quality control of milk powders (Pashkova, 2009).

A less explored technique for food analysis is Total Reflection X-ray Fluorescence spectrometry (TXRF). Over the past few years, TXRF is gaining acceptance over other spectroscopic techniques since it offers the possibility of simultaneous multi-element analysis of liquid and solid foodstuff samples in a wide dynamic range of concentrations with also a minimum sample treatment (Borgese, Bilo, Dalipi, Bontempi, & Depero, 2015). In this configuration, unlike XRF instrumentation, the primary beam strikes the sample at a very small angle and the detector is located very close to the sample, leading to an improvement of detection capability. To perform analysis under total reflection conditions,

samples must be prepared as (ultra)-thin films. This is achieved by depositing a small amount of sample (μL – μg range) on the surface of a reflective carrier with a subsequent drying step before TXRF analysis. Matrix effects are usually neglected and then element concentrations in the sample can be determined owing to the addition of a suitable internal standard (IS) and considering the instrumental sensitivities of respective analytes and the IS (internal standardization) (Marguá & Van Grieken, 2013). TXRF has been mostly used for the analysis of liquid samples (with or without dilution depending on the sample matrix complexity) or solid samples after a digestion step (De La Calle, Cabaleiro, Romero, Lavilla, & Bendicho, 2013; Marguá, Tapias, Casas, Hidalgo, & Queralt, 2010). In the field of liquid milk analysis, for instance, TXRF proved to be a suitable technique for mineral and trace elements quantification in milk samples as long as the samples were diluted with ultrapure water before the analysis to reduce the organic matrix and the fat contents (Smagunova & Pashkova, 2013).

TXRF also allows the use of small quantities (e.g., few milligrams) of solid samples to perform the analysis using a slurry sample preparation. This approach, which involves the suspension of a few milligrams of the sample in an adequate disperser, has been recently applied for multi-elemental analysis of different types of solid samples including placenta samples (Marguá, Ricketts, Fletcher, Karydas, Migliori, Leani, & Voutchkov, 2017), soils (Gallardo, Queralt, Tapias, Candela, & Marguá, 2016), cosmetics (Marguá, Dalipi, Borgese, Depero, & Queralt, 2019) and vegetables (Dalipi, Marguá, Borgese, & Depero, 2017). This sample preparation strategy is really suitable for the multielemental analysis of mass-limited samples (De Almeida, Sagbiero Montanha, Pereira de Carvalho, & Marguá, 2020) but one of the major drawbacks is the limited repeatability on the obtained results arising from the inherent heterogeneity of the sample at μg and mg level. Moreover, the use of internal standardization for the analysis of solid suspensions is not trivial. The main reason for that is the deposition of a thicker suspension residue on the reflector breaks the condition of total reflection (Dalipi et al., 2017).

In view of this premises in this study, the analytical capabilities of TXRF were explored using suspension preparation as a simple and cost-effective method for the determination of mineral and trace elements in milk powder products. To our knowledge, this is the first time that this analytical approach has been tested for milk powders analysis and the results derived from this study can be especially useful to evaluate its potential. A detailed study to select sample preparation and measurement conditions for milk powder TXRF analysis was carried out. Besides, different quantification approaches were evaluated and the validation of the developed TXRF methods was carried out by comparison of the results obtained by TXRF and the reference method in a set of 23 milk-powder samples using robust statistics.

2. Experimental

2.1. Reagents and materials

Stock solutions of 1000 mg/L (ROMIL PrimAg® Mono component reference solutions) were used to prepare V, Ga, Rh and Y internal standard solutions. Ultrapure de-ionized water for dilution of stock solutions and preparation of sample suspensions was obtained from a Milli-Q purifier system (Millipore Corp., Bedford, Massachusetts). Triton™ X-100 (laboratory grade, Sigma-Aldrich) was used for the preparation of sample suspensions. Silicone solution in isopropanol (Serva GmbH & Co, Germany) was used to coat all the quartz disc reflectors to obtain a hydrophobic film to facilitate sample deposition. In all cases, quartz reflectors with a diameter of 30 mm and a thickness of 3 mm \pm 0.1 mm were used as sample holders for introducing the sample into the TXRF equipment.

2.2. Milk powder samples

Samples were produced internally in one factory. They were

representing a wide range of milk-based products. Fortification was done during production according to commercial recipes.

All milk powder samples used in this study correspond to proficiency test samples, analysed by many laboratories with different analytical techniques. For each determination, the approach to determine the assigned value was to calculate a consensus value using the median of all participant results. The recommended method for the proficiency tests was to perform an acidic digestion of samples using high pressure microwave prior to analysis P, K, Ca, Fe and Zn by ICP-AES. Chloride was generally assessed by potentiometry. Both methods are official AOAC methods and additional details can be found elsewhere (Perring et al., 2017). All samples were systematically analysed in duplicate.

The aforementioned samples were splitted in two different sets, one was used as calibration standards ($n = 13$) and the other one as validation samples ($n = 23$), independent for the first one. Elemental compositions of samples are displayed in Tables S1 and S2.

2.3. Suspension preparation and TXRF analysis

A preliminary study was performed to select the most suitable experimental conditions to suspend the milk powder samples and analyse them by TXRF. According to the obtained results (see Section 3.1 for details), finally milk samples were prepared as follows: 40 mg of powdered sample was suspended in 1 mL of a solution of 1% (v/v) Triton X-100. Then, 50 μ L of a 200 mg/kg Y solution was added for quantification purposes (see Section 2.4). Homogenization of the resulting mixture was performed by vortex stirring for 1 min. Finally, 10 μ L of sample were deposited on a siliconized quartz reflector and dried under IR lamp for the later TXRF analysis.

In order to study the planar distribution of elements in the dried milk on the reflectors, mappings were performed by μ -EDXRF using a benchtop small-spot EDXRF spectrometer (XDV-SDD model, Helmut Fischer GmbH, Sindelfingen, Germany) equipped with a W X-ray tube. In previous studies, we showed the potential of such a system for organic-based sample matrices mappings (Bongiovanni et al., 2019; Marguí, Jablan, Queralt, Bilo, & Borgese, 2021). Mapping experimental conditions were: 50 kV, 1 mm Al foil as primary filter, 1 mm collimator, grid: 10 \times 10 points, time per point: 300 s. Spectral data from EDXRF analysis were evaluated using the WinFTM software, version 6.35 linked to the instrument.

TXRF measurements were performed using a benchtop TXRF spectrometer "S2 PICOFOX" (BrukerNano, GmbH, Germany) equipped with a low-power W X-ray tube (max power: 50 W). The characteristic radiation emitted by the elements present in the sample was detected by a silicon drift detector with a resolution of less of 150 eV at Mn-K α . One of the main advantages of this spectrometer is that is equipped with an air-cooled low-power X-ray tube and a Peltier cooled silicon drift detector and thus, no cooling media and gas consumption are required. These facts make this system very attractive for implementation in industrial quality control laboratories. For comparison purposes, limits of detection obtained using the same TXRF system but equipped with a Mo X-ray tube were also evaluated.

All samples were analyzed during 1500 s, since this time is necessary to obtain reliable results at trace levels when using low power TXRF systems equipped with W X-ray tubes. The evaluation of TXRF spectra and calculation of element concentrations based on mass ratio of sample to internal standard was performed using the software Spectra Plus 7.2.0 (Bruker Nano GmbH, Berlin, Germany) linked to the equipment. For the quantification in TXRF analysis, the software applies deconvolution routine which uses measured mono-element profiles for the evaluation of peak intensities.

2.4. Quantitative TXRF approaches

In TXRF, quantification is usually carried out by internal standardization. This method is based on the addition of an element named in-

ternal standard (IS) which is not present in the sample. Then, element concentrations are estimated using the following expression:

$$C_i = \frac{N_i \times C_{IS} \times xS_{IS}}{N_{IS} \times S_i}$$

where C_i : analyte concentration, N_i : analyte net peak area, C_{IS} : IS concentration, S_{IS} : instrumental sensitivity for the IS, N_{IS} : IS net peak area, S_i : instrumental sensitivity for the analyte.

This approach is only valid if the sample is presented as a thin layer on the carrier. However, in many cases, the resulting spot on the reflector is too thick to ensure the conditions of total reflection and matrix effects cannot be considered negligible, which is the premise for a proper use of internal standardization. In such cases, external calibration using a set of calibration standards can be used as a quantification approach alternative (Marguí & Van Grieken, 2013). In the present study both, internal standardization and external calibration were evaluated for the quantification of P, Cl, K, Ca, Fe and Zn in milk powder samples.

2.5. Validation and statistical details

Evaluation of TXRF performance was performed using robust statistics according to the formulas below. All the calculations were performed using for each sample the average duplicate concentrations of the reference method and the average concentrations of the triplicates analysed by TXRF.

Reference method value (ICP-AES or potentiometry)	Y_i
TXRF value	\hat{Y}_i
Number of samples	n
Difference	$d_i = \hat{Y}_i - Y_i$
Robust standard deviation of repeatability (from duplicates)	$SD_{rob}(r) = 1.4826 \times Med_{d_{i=1 \text{ to } n}}\{SD_i\}$
Bias	$\bar{d} = \frac{\sum_{i=1}^n (\hat{Y}_i - Y_i)}{n}$
Standard error of calibration = SEC	$SEC = \sqrt{\frac{\sum_{i=1}^n (\hat{Y}_i - Y_i)^2}{n - 1}}$
Difference standard deviation = SD(d)	$SD(d) = \sqrt{\frac{\sum_{i=1}^n (d_i - \bar{d})^2}{n - 1}}$
Standard error of prediction = SEP	$SEP = \sqrt{\frac{\sum_{i=1}^n (\hat{Y}_i - Y_i)^2}{n}}$

3. Results and discussion

3.1. Evaluation of experimental conditions for TXRF analysis of milk suspensions

First a set of experimental parameters that may affect the sample preparation process and subsequent analysis using TXRF were evaluated. In all experiments, a powdered milk sample (DDP-2-2004) containing [P]: 515 mg/100 g, [Cl]: 583 mg/100 g, [K]: 922 mg/100 g, [Ca]: 733 mg/100 g, [Fe]: 9.65 mg/100 g and [Zn]: 6.93 mg/100 g and a TXRF system equipped with a W X-ray tube were used.

The effect of the different parameters was evaluated calculating the recovery values ((TXRF concentration/Reference concentration) \times 100). TXRF concentration values were determined using internal standardization and K, Ca, Fe and Zn were used as element models to select the best analytical conditions.

3.1.1. Internal standard selection

As mentioned in Section 2.4, TXRF quantification can be performed by internal standardization. Element used as internal standard (IS) should not be present in the original sample, not interfere with the analytes and have an adequate analytical response (Marguí & Van Grieken, 2013). According to that, V, Ga, Y and Rh were tested as potential candidates. It is important to consider that the TXRF used in this

project is equipped with a W X-ray tube that allows the determination of Rh using its K-lines that it is not possible when using the most commonly used Mo TXRF systems. As it is shown in the TXRF spectrum for a milk powder spiked at 10 mg/kg of each IS (Fig. 1), the best candidates to be used as IS where Y and Rh since they present a better analytical response and they do not overlap with the target analytes in terms of fluorescence radiations. Finally, Y was selected as internal standard in further measurements.

3.1.2. Disperser agent

A suitable dispersing agent must be selected to obtain a homogeneous suspension of the solid sample for further analysis by TXRF. Besides, it is important to try to select a dispersing agent with low toxicity. Given that milk powder is soluble in aqueous solutions, three conditions were tested: Ultrapure water, an aqueous solution of 1% Triton X-100 and an aqueous solution of 5% Triton X-100. This non-anionic surfactant has been shown to be effective to increase the homogeneity of suspensions in previous similar studies (De La Calle et al., 2013). In all cases a milk concentration suspension of 40 mg in 1 mL of disperser agent was used.

To study the distribution of the milk powder sample on the quartz reflector using the different disperser agents, the spot residues obtained were analysed by μ -XRF (specific analytical details given in Section 2.3). In Fig. 2, images of the spot residues and the resulting two-dimensional element mappings are displayed. It was not possible to map Fe and Zn in the milk powder spots due to the low concentrations of these elements (<100 mg/kg) and the limited sensitivity of the benchtop μ -XRF system used.

It was found that with the addition of Triton X-100 to the aqueous solution, the diameter of the sample spot was larger resulting in a thinner layer on the carrier. However, when using a 5% of Triton X-100 solution, the background of the obtained TXRF spectrum increased considerably and thus, therefore it was considered appropriate to select a solution of 1% of Triton X-100 solution to suspend the milk powder samples. Moreover, using this surfactant concentration the drop on the reflector was better deposited on the carrier surface and a better homogeneity of the analytes within the residue was assessed (Fig. 2).

Finally, it is interesting to remark that since the distribution of the analytes of interest was not completely homogeneous in the deposited drop, it is especially relevant to irradiate and detect the entire area of the deposited sample when performing the analysis by TXRF to obtain reproducible results.

3.1.3. Sample amount

The amount of solid sample used for preparation of suspensions can significantly influence TXRF signals and therefore analysis. On the one hand, if the sample amount is too low, the detection of trace elements will not be possible. On the other hand, if the sample amount is too large, the sample deposited on the reflector will be too thick and matrix effects (mostly absorption) will take place. Considering previous studies regarding the analysis of suspensions by TXRF (Dalipi et al., 2017) the effect of the milk powder amount used to prepare the suspension was studied in the range of 5–40 mg (corresponding to 5–40 mg/mL in the resulting suspension). Obtained results are displayed in Fig. 3A. As it is shown, in all cases, K and Ca concentrations were underestimated. This fact can be explained probably due to absorption issues arising from the thickness of the milk sample. An improvement for K and Ca results could be surely assessed by using a lower sample amount to prepare the suspension but since the aim of the study was the development of an analytical TXRF method also for trace elements determination (Fe and Zn), it was not considered appropriate to decrease the sample amount used. Considering the results obtained in Fig. 3A, 40 mg of milk powder (corresponding to a 40 mg/mL suspension) were finally selected to prepare the suspensions. Using this amount of sample, a lower standard deviation between replicates and a better proximity of the TXRF results to references values was obtained, above all for elements present at trace concentrations.

3.1.4. Suspension homogenization

One critical issue when preparing solid suspensions in TXRF analysis is the sedimentation of the solid sample at the bottom of an Eppendorf tube, which leads to non-homogeneous sample suspension. For this reason, a mixing procedure is often required before depositing the sample on the reflector. Usually the sample is mixed by vortex agitation for several seconds before deposition. To increase the homogeneity of the suspension, in some cases, an additional mixing procedure using sonication is used. In the present study, several mixing strategies were explored including the use of a sonication process using a conventional ultrasonic bath (during 5 and 10 min) and using a sonication step of 2 min by a cup horn ultrasonicator device (suitable for small amounts of sample sonication) (DE UTR200, https://www.hielscher.com/utr2_p.htm) set at different amplitudes (20%, 50% and 75%). In all cases, samples were mixed by vortex agitation during 1 min before being deposited on the quartz reflector. As can be seen in Fig. 3B, there is no significant improvement in the results obtained with a previous sonication process before the vortex mixing. For this reason, it was decided

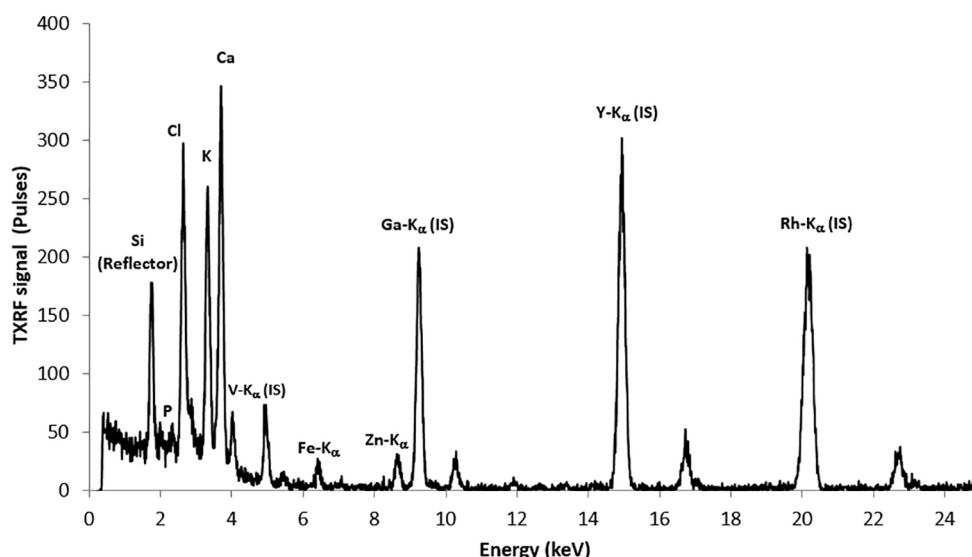


Fig. 1. TXRF spectrum for a milk powder sample, spiked with different internal standards (IS) at 10 mg/kg concentration level.

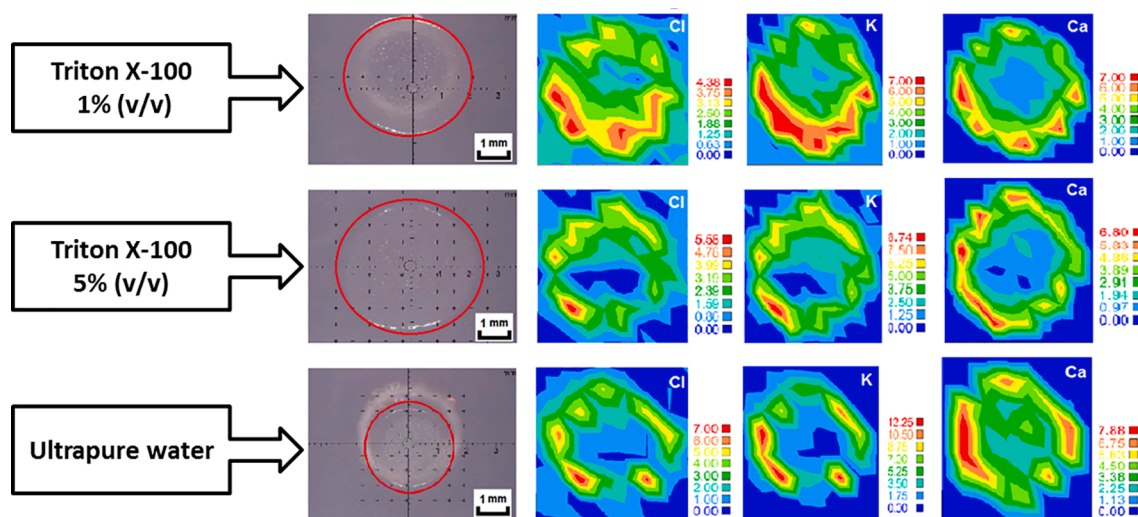


Fig. 2. 2D mappings for elemental distribution of Cl, K and Ca in milk powder sample (DDP-2-2004) deposited on a reflective carrier. Mapping conditions (μ -XRF): 50 kV, primary filter Al 1000 μ m, collimator: 1 mm, grid: 10 \times 10 points, time per point: 300 s. Red circles represent the sample area deposited on the reflector. Milk concentration suspension: 40 mg in 1 mL of disperser agent.

to prepare the suspensions without sonication process meaning more time, efforts, and resources.

3.1.5. Sample deposition volume

As aforementioned, in TXRF analysis is essential to deposit the sample as a thin layer on the reflector. Therefore, the sample deposition volume is a critical parameter to consider. In the case of suspensions, it is recommended that only a few microliters of solution are deposited on the carrier in order to avoid matrix effects and possible damage of the detector (De La Calle et al., 2013).

The influence of sample deposition was studied at different volumes: 2, 5, 10 and 20 μ L. Higher volumes were discarded given that the maximum sample diameter of the sample on the reflector is 10 mm to ensure a proper irradiation of the sample and detection of the characteristic elemental X-rays. Obtained results are displayed in Fig. 3C. As it is shown, the use of 2 and 5 μ L volumes lead to a higher deviation of the results in comparison with reference values, above all for trace element determination. For this reason, a volume of 10 μ L was finally selected since obtained Fe and Zn concentrations were close to the reference values and the dispersion of the results was lower.

3.1.6. Measurement time

Finally, the effect of measurement time was also evaluated since it can affect to the precision of the obtained results, above all for the determination of elements present in milk samples at trace concentrations. Thus, a suspension of the milk sample (DDP-2-2004) was analysed using the experimental conditions aforementioned and measured at different measurement times: 200, 500, 1000, 1500, 2000 s ($n = 6$). Higher measurement times were not considered for practical reasons. As it is shown in Fig. S1, for most elements, a clear improvement of precision (between 2 and 5 times) is assessed when increasing the measurement time from 200 to 1500 s. There was not a real difference between the results obtained by 1500 s compared to 2000 s. Thus, 1500 s time was fixed as suitable for TXRF measurements. It is also interesting to remark that the measurement time could potentially be significantly reduced by a factor of 3 if using a TXRF system equipped with a Mo X-ray tube, as we demonstrated in a previous paper dealing with the analysis of wine samples (Dalipi, Marguí, Borgese, Bilo, & Depero, 2016).

3.2. Validation of the TXRF method

To study the real analytical capability of the developed TXRF method

and the quality of the obtained results for the determination of P, Cl, K, Ca, Fe and Zn in milk powder samples, several figures of merit including limits of detection, accuracy and precision of the results were evaluated.

3.2.1. Limits of detection

Limits of detection (LDs) were evaluated directly from the TXRF spectrum obtained in the analysis of the milk powder sample (DDP-2-2004) containing [P]: 515 mg/100 g, [Cl]: 583 mg/100 g, [K]: 922 mg/100 g, [Ca]: 733 mg/100 g, [Fe]: 9.65 mg/100 g and [Zn]: 6.93 mg/100 g and using the following formula (Marguí & Van Grieken, 2013):

$$LD = \frac{3 \times \text{Analyte concentration} \sqrt{\text{Background}}}{\text{Net area analyte}}$$

In which the “net area analyte” corresponds to the area of the X-Ray fluorescence peak after background subtraction.

In Table 2, LDs estimated are summarized. For comparison purposes, LDs obtained using the same TXRF system but equipped with a Mo X-ray tube are also included. As expected, for both systems, LDs for low Z elements (P, Cl, K, Ca) were higher than those calculated for mid-Z elements (Fe, Zn). As it can be seen, with the Mo-TXRF system, LDs are around one order of magnitude lower compared to those obtained using the W-TXRF instrument. However, considering the analyte concentration ranges in milk powder samples, both systems seem to be adequate for the intended purpose.

3.2.2. Accuracy of the results

As already shown in Fig. 3, when using internal standardization (IS: Y) as quantification approach, K and Ca concentrations are systematically underestimated. This fact can be explained by the influence of self-absorption phenomena arising from the thickness of the milk sample. These findings are aligned with the results reported previously by Dalipi et al. (2017) and by Zarkadas and co-workers (Zarkadas, Karydas, & Paradellis, 2001) who demonstrated that for determination of elements with atomic numbers below $Z = 23$ in organic matrices such as biological fluids, TXRF quantification cannot be performed by using elemental sensitivities obtained from aqueous solutions (used in internal standardization approach, Section 2.4). For this reason, to improve results obtained for low Z elements, empirical calibrations were established using a set of 13 milk powder samples covering the whole range of element concentrations (see Table S1 for details). For all analytes, calibrations were based on a regression plot of corrected analyte intensity ((Net analyte peak area/Net IS area) \times [IS]) versus concentration

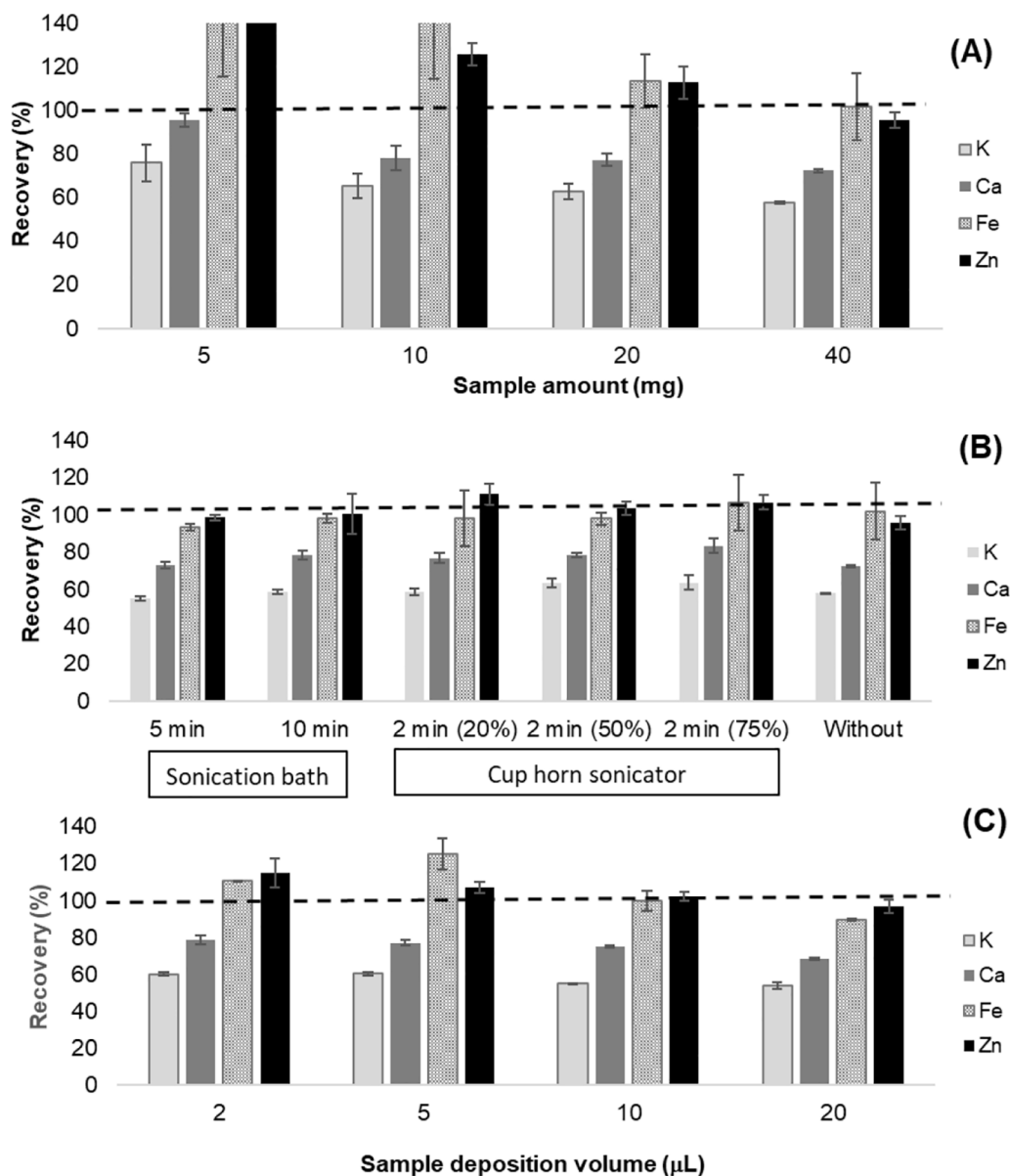


Fig. 3. Effect of: (A) sample amount, (B) sample deposition volume and (C) homogenization mode on analyte concentrations determined by TXRF method in milk powder samples. Error bars represent standard deviation of triplicate sample analysis.

Table 2

Limits of detection estimated for the determination of the analytes of interest in milk powder samples by suspension and TXRF analysis (using W and Mo X-ray tubes). Concentration values are expressed in mg/100 g.

	P	Cl	K	Ca	Fe	Zn
LD-W (TXRF)	97	45	16.5	4.9	0.42	0.18
LD-Mo (TXRF)	62	3	0.99	0.61	0.09	0.045
Concentration Ranges in milk	167–516	295–583	429–1125	255–757	5.12–9.68	3.39–9.46

$$LD = \frac{3 \times \text{Analyte concentration} \sqrt{\text{Background}}}{\text{Net area analyte}}$$

(reference value). Obtained results are summarized in Table 3. As it is shown, a good linearity was obtained for most of the studied elements, except for P (R^2 : 0.70). As observed in the TXRF spectrum displayed in Fig. 1, the analytical signal of P is really low, and the results obtained by TXRF are not accurate (see Fig. S2). Surely an improvement of the results for P determination could be assessed if using a TXRF system equipped with a Mo X-ray tube, considering that the analytical signal for light

elements is significantly higher in comparison with W-TXRF systems (Dalipi et al., 2016). In view of the obtained results, P was discarded of the list of potential elements to be determined by the developed TXRF method. As it is shown in Table 3, also for Fe the determination coefficient was slightly lower than 0.9. This fact can be explained considering Fe homogeneity in the milk samples (Fe is added in the form of a solid reagent in the milk powder) that is especially critical for microanalytical

Table 3

Concentration range of elements in calibration milk powder samples and values for linear regression (LR) analysis between TXRF data and reference values (External calibration).

Element	Min (mg/100 g)	Max (mg/100 g)	n	R ²	SEC ^a (mg/100 g)
P	167.0	516	13	0.70	0.77
Cl	295.0	583	10	0.95	1.98
K	429.0	1125	13	0.94	10.49
Ca	255.0	757	13	0.98	10.63
Fe	5.12	9.68	13	0.83	1.51
Zn	3.39	9.46	13	0.95	2.32

^a SEC: Standard error of calibration (see Section 2.5).

techniques such as TXRF, since only a very small amount of sample is finally analysed (10 µL of a 40 mg/mL suspension). Standard error of calibration values was also evaluated and in general they were acceptable.

A validation step was then performed using the obtained calibration models to determine analyte concentrations in a second set of samples totally independent of the calibration set (validation samples). Concentration values in the same samples were also estimated using internal standardization as quantification approach to compare the quality of the results obtained using both quantification models. Evaluation of the TXRF method performance was done by systematically comparing TXRF and reference method results applying the following linear regression model (LR): Concentration (TXRF method) = A + B × Concentration (Reference method) and using robust statistics (Section 2.5).

If the results from the reference and TXRF methods are strictly equal, these parameters should have the values R² = 1, A = 0 and B = 1. The numerical results of the analytical performance are given in Table 4. As it is shown, acceptable determination coefficients were obtained (R²: 0.80–0.96) for all analytes except for chlorine. For this element, R² ~ 0.6 for both quantification approaches and therefore, TXRF results obtained can be used only for screening purposes. This fact can be related to the poor analytical signal of light elements when working in atmosphere conditions (no vacuum) and TXRF systems equipped with W X-ray tubes.

It is interesting to mention that for a proper determination of K and Ca in powder milk samples it is necessary to use empirical calibration as quantification approach. When using internal standardization, the slope of the linear regression model is significantly lower than 1, and the results are systematically underestimated. However, when using a set of calibration samples with the same matrix of the milk powder samples for

Table 4

Comparison of validation performance characteristics obtained using internal standardization or external calibration as quantification approaches in comparison with reference values. Units in mg/100 g.

Internal standardization (TXRF method)										
Element	Min	Max	n	LR model			Bias	SEP		
				R ²	A (=0)	B (=1)				
Cl	305	544	23	0.64	2.67	No	0.246	No	-262	270.6
K	485	1079	23	0.85	25.71	Yes	0.473	No	-293	314.4
Ca	298	629	23	0.85	7.71	Yes	0.668	No	-160	165.9
Fe	5.14	8.78	23	0.78	0.13	Yes	0.903	Yes	-0.68	0.78
Zn	3.52	8.03	23	0.87	-0.68	Yes	1.130	Yes	-0.11	0.48
External calibration (TXRF method)										
Element	Min	Max	n	LR model			Bias	SEP		
				R ²	A (=0)	B (=1)				
Cl	305	544	23	0.57	-52.4	Yes	1.069	Yes	-24.0	57.1
K	485	1079	23	0.79	-101.3	Yes	1.060	Yes	-59.0	98.7
Ca	298	629	23	0.85	-29.4	Yes	1.043	Yes	-5.0	42.6
Fe	5.14	8.78	23	0.85	-3.12	No	1.358	No	-0.63	1.05
Zn	3.52	8.03	23	0.96	-1.06	No	1.137	No	-0.36	0.44

LR (linear) model: Concentration (TXRF method) = A + B × Concentration (Reference method).

Bias: median of differences, SEP: Standard error of prediction (see Section 2.5).

quantification purposes, self-absorption effects are compensated, and an acceptable linearity can be established between TXRF and reference values. On the contrary, it seems that for mid-Z elements such as Fe and Zn, slightly better results are obtained using internal standardization as quantification model (A = 0 and B = 1). The main reason for that is that quantification of these types of elements are not so influenced by matrix effects arising from the milk powder sample as in the case of low Z elements. In Fig. 4, as an example, graphical comparison between Ca and Zn concentrations determined by the reference and the TXRF method using internal standardization and external calibration is shown. This trend can be also deduced from the bias values displayed in Table 3.

To establish the prediction of performance the standard error of prediction (SEP) was also estimated (see Section 2.5 for details). SEP values obtained for all analytes (K and Ca determined by empirical calibration; Fe and Zn determined by internal standardization) were between 5 and 7 times lower than the lowest concentration value of the validation milk powder samples. Therefore, according to internal laboratory limits the TXRF method validation was found to be quite acceptable.

3.2.3. Precision of the results

Using the triplicate values of the validation samples, the relative repeatabilities CV_{rob(r)} (see Section 2.5 for details) were calculated for each analyte. Such a way of determination of repeatability data allows to obtain overall information and cover full range of concentrations met during validation. These values were calculated for the different quantification models evaluated and results are displayed in Table S3. As it is shown, in general, slightly lower CV_{rob(r)} values were obtained when using internal standardization as quantification approach, since in this case the uncertainty arising from the empirical calibration is not involved. Nevertheless, for both models CV_{rob(r)} values in the range of 2.5–11.3 % were obtained which are similar, for instance, to those obtained in other publications dealing with the determination of mineral elemental content in animal food samples (Perrig et al., 2017).

In order to study the different contributions of the analytical process to the global precision of the suspension preparation and TXRF analysis, six independent replicates of the sample DDP-2-2004 were prepared, analysed and the relative standard deviation (RSD) of the results was calculated. Besides, one of the replicates was measured six times and the RSD values were also estimated. This uncertainty is related to the instrument stability and counting statistics. Therefore, by means of error propagation ((RSD Total)² = (RSD Instrumental)² + (RSD Sample

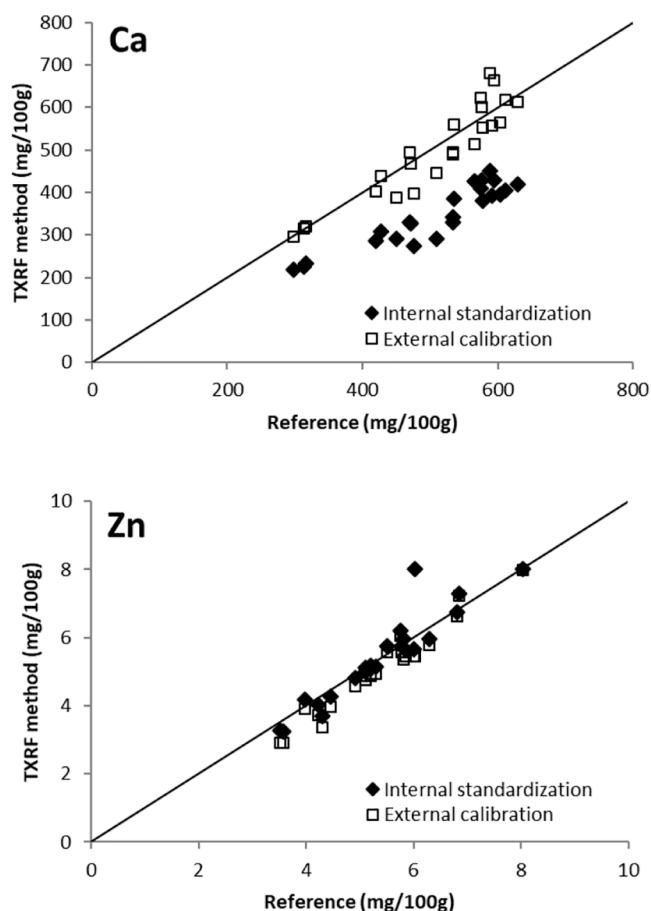


Fig. 4. Comparison of Ca and Zn concentrations determined by the reference and the TXRF method using internal standardization and external calibration as quantification approaches. TXRF results are expressed as mean of triplicate analysis.

preparation)²), the uncertainty arising from the sample preparation step can also be estimated. From the obtained results (Fig. S4) it was found that the contribution of the instrumental uncertainty was quite lower (18–40%) in comparison with the one related with the sample preparation step (60–82%).

4. Conclusions

In the present study, the potential of suspension preparation and analysis by TXRF instrumentation have been evaluated for the determination of mineral (P, Cl, K, Ca) and trace elements (Fe, Zn) in milk powder samples. The sample preparation was rather fast and consists of suspending 40 mg of the powder sample in 1 mL of a solution of 1% (v/v) Triton X-100. The real analytical time for each sample when using the proposed method was about 27 min (2 min for sample preparation + 25 min of measurement) which was shorter than those associated with acidic digestion of samples and ICP-AES (P, K, Ca, Fe and Zn) or potentiometry (Cl) analysis, corresponding to at least 4 h for ICP-OES and at least 2 h for potentiometry.

Limits of detection obtained were in the low mg/kg range and found to be adequate in view their concentration in this kind of samples.

Results obtained from the validation study demonstrated that trace elements such as Fe and Zn can be quantified by using internal standardization as quantification approach, without the need of any empirical calibration. This fact can be of special interest since the setting up of suitable calibration standards is a time-consuming step. However, for the determination of lower Z-elements such as K and Ca the use of a calibration procedure using suitable milk-powder standards is necessary

to compensate the influence of self-absorption phenomena arising from the thickness of the milk sample on the reflector. Finally, the reliable determination of very low Z-elements (i.e., P and Cl) was no longer possible using the developed method due to the lack due to unacceptable statistical performance characteristics ($R^2 \sim 0.70$ and $R^2 \sim 0.60$, respectively) and thus, only screening results can be obtained. This fact can be related with the poor sensitivity of the TXRF system used and working conditions in atmospheric air instead of vacuum.

Although the range of elements that can be quantified in powder milk samples with the TXRF method is lower than when using conventional EDXRF and WDXRF methods, it provides the possibility of easier quantification for the determination of trace elements (without the need of calibration standards) and also the use of a smaller sample amount (few mg instead of few g). Surely, better results by TXRF can be obtained using more sophisticated sample treatments of the milk-powder samples such as acid digestion. However, more complex sample preparation includes the risk of moving away from the simplicity and limited cost that made the solid-state techniques very attractive to be implemented in foodstuff production sites.

CRediT authorship contribution statement

E. Marguí: Conceptualization, Methodology, Data curation, Investigation, Writing – original draft, Writing – review & editing. **I. Queralt:** Methodology, Writing – review & editing. **D. Andrey:** Data curation, Writing – review & editing. **L. Perring:** Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Eduard Roura is acknowledged for his support in the laboratory work.

Ignasi Queralt also acknowledges the “Grant CEX2018-000794-S funded by MCIN/AEI/ 10.13039/501100011033”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2022.132590>.

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