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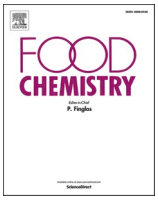

Assessment of energy dispersive X-ray fluorescence (ED-XRF) for quantification of iodine in non-lyophilized milk

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ABSTRACT

Iodine represents a fundamental element for human health, with particular regard to thyroid function. Dietary intake of milk naturally rich in iodine becomes of primary importance in the prevention of syndromes related to iodine deficiency. The concentration of iodine in milk is characterized by wide variability, mainly related to animal feed and level of mineral supplementation. Therefore, there is interest in the development of fast analytical techniques which are able to predict milk iodine concentration. The aim of the present study was to investigate the effectiveness of energy-dispersive X-ray fluorescence (ED-XRF) for the prediction of iodine in cow milk. Results showed moderate accuracy of the ED-XRF technique, with a coefficient of determination in cross validation of 0.60. This study represents a first contribution towards the possibility to discriminate milk with high or low iodine concentration, as an essential preliminary step for the introduction into the market of naturally fortified milk.

1. Introduction

Iodine is fundamental for human health, with particular regard to thyroid function and physiology. In humans, iodine can be obtained exclusively through the diet and cannot be replaced by other nutritional elements (Velasco, Bath, & Rayman, 2018). Recommended iodine intake ranges from 90 μg/d in children and 120 μg/d in schoolchildren, up to 150 μg/d in adults and 250 μg/d in pregnant and lactating women (World Health Organization, 2007).

Other than fortified salt and seafood, drinking milk represents the main fount of iodine in human nutrition (Censi et al., 2020; Herrick, Perrine, Aoki, & Caldwell, 2018). In this perspective, cow milk can be regarded as a functional nutraceutical food, which may attract the attention of consumers with particular high iodine requirements (Niero et al., 2023). The observed concentration of iodine in milk is highly variable and is regulated by several factors. It is well established that the main determinant for milk iodine concentration is the amount of iodine administered to lactating animals through the diet: the higher the level of iodine in the feed, the greater the level of iodine in the milk (Antaya, Ghelichkhan, Pereira, Soder, & Brito, 2019; Weiss, Wyatt, Kleinschmit, & Socha, 2015). On the other hand, goitrogen iodine antagonists which have been characterized on different plant species, are able to inhibit the sodium iodide symporter leading to lower milk iodine concentration (Flachowsky, Franke, Meyer, Leiterer, & Schöne, 2014). The adoption of iodized disinfectants during milking procedures has been reported to increase milk iodine concentration, which is likely due to iodine absorption at skin udder level and subsequent release into the milk (French et al., 2016). Scientific literature also demonstrated that milk iodine concentration is heritable. In other terms, a given dairy cattle population differs in respect to the breeding value of milk iodine concentration, including animals which are more prone to produce milk with greater iodine concentration thanks to their individual genetic predisposition (Costa et al., 2021; Denholm et al., 2019).

To date, milk iodine concentration is far from being standardized due to several hurdles. At upstream level, the exact quantification of iodine (and iodine antagonists) in feed ingredients and rations is not easily applicable neither on a large scale and nor on routine workflow due to high analytical costs and time demanding procedures. Similarly, at downstream level, the determination of milk iodine requires i) the application of specific and time demanding protocols for iodine extraction and ii) the use of costly analytical tools, such as inductively coupled plasma mass spectrometry or ion exchange chromatography (Niero et al., 2019). For these reasons, there is interest in the development of alternative methods aimed to quantify (or predict) milk iodine in reasonable time and at lower costs. Trying to meet this need, Niero et al. (2020) attempted to develop mid-infrared spectroscopy models to

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predict milk iodine concentration. The same authors concluded that such prediction models were not accurate enough to replace reference analysis; still, they proposed the same models as a tool to distinguish between high and low milk iodine concentration at relatively marginal costs (Niero et al., 2020).

Previous authors already reported the possibility to predict iodine content in milk powders through energy dispersive X-ray fluorescence (ED-XRF) technique (Crecelius, 1975; Hasan, Al-Saedi, & Jassim, 2020; Pashkova, Smagunova, & Finkelshtein, 2018). However, to authors knowledge, there is no information about the possibility to predict iodine concentration in liquid milk through ED-XRF technique, which indeed has been already proposed as a rapid and cost-effective alternative method for the quantification of other milk mineral elements (Perring & Andrey, 2003; Visentin, Niero, Cassandro, Penasa, & De Marchi, 2023). This research gap has been inspected through the present study, which aimed at investigating the effectiveness of ED-XRF for the quantification of iodine in non-lyophilized individual cow milk.

2. Materials and methods

2.1. Sample collection and gross chemical composition

The experiments were performed during routine milking procedures and were not invasive; therefore, animal welfare committee authorization was not required. Individual raw milk samples (n = 58) of Holstein Friesian (HF; n = 43), Brown Swiss (BS; n = 10) and Simmental (SI; n = 5) cows were collected in 15 commercial dairy farms located in Veneto region (Italy). After collection, each milk sample was divided into three aliquots. The first aliquot was transferred at 4 °C to the laboratory of the Breeders Association of Veneto Region (ARAV, Vicenza, Italy) and analyzed within 12 h for gross chemical composition (fat, protein, casein and lactose, g/100 g), pH and urea (mg/dL) using a MilkoScan FT6000 (Foss, Hillerød, Denmark). Somatic cell count (SCC, cells/mL) and differential somatic cell count (DSCC, %) were determined through Fossomatic (Foss, Hillerød, Denmark). To achieve normality of distribution, SCC was transformed to somatic cell score (SCS) using the formula proposed by Wiggans and Shook (1987): $SCS = \log_2 (SCC/100{,}000) + 3$. The second and third aliquots were used for inductively coupled plasma mass spectrometry (ICP-MS) and ED-XRF analysis, respectively.

2.2. ICP-MS and ED-XRF analysis

The second aliquot of milk was used for iodine extraction followed by ICP-MS analysis, which were performed in the laboratory of Eurolab S.r.l (Vicenza, Italy). Milk samples were diluted (1:24) in 0.6% ammonia in one-use 50 mL plastic tubes. Samples were heated in a water bath at 90 °C for 1 h to foster iodine extraction. After room temperature cooling, samples were filtered through 0.45 μm syringe filters. Afterwards, 5 mL of the filtered sample were diluted (1:1) in 0.6% ammonia, up to reaching a final volume of 10 mL. Finally, iodine was quantified through ICP-MS technique. Instrument settings together with method development, sensibility, repeatability and reproducibility have been extensively described by Niero et al. (2019).

The third aliquot was used for ED-XRF analysis, which was carried out in the laboratory of the Department of Agronomy, Food, Natural resources, Animals and Environment of the University of Padova (Legnaro, Italy) on untreated milk samples (i.e. samples without any preparatory steps), through Spectro Xepos 5P ED-XRF (Ametek, Kleve, Germany) characterized by an X-ray tube anode of Pd and Co (65:35), 50 keV voltage and 2 mA current. Iodine quantification was based on I L-α (3.94 keV), exploiting the potential of the X-ray tube anode of Pd and Co. Significant Pearson correlations coefficients (r) were observed between normalized impulses registered at I L-α 3.94 keV and Br L-α 1.48 keV ($r = 0.37$, $p < 0.01$), K K-β 3.59 keV ($r = 0.75$, $p < 0.01$), Ca K-α 3.69 keV ($r = 0.81$, $p < 0.001$) and Ca K-β 4.01 keV ($r = 0.81$, $p < 0.001$). Possible overlapping between these signals were accounted through the method of influence coefficients proposed by Rousseau (2006).

Samples were weighed (5 g) and placed in ED-XRF plastic cups (32 mm diameter, 24 mm height). Before the beginning of instrumental analysis, sample information was provided to the instrument (i.e. the type of matrix and the exact weight). This is a relevant step as the accuracy of the ED-XRF method depends on texture and weight of the sample that the X-rays have to pass through. The instrument took 10 min to analyze each milk sample. Fig. 1A depicts the ED-XRF spectrum of an individual milk sample (blue line) overlapped with ED-XRF spectrum of a water sample (black line), with energy (keV; x-axis) *versus* normalized impulses (counts per second; y-axis). The comparison between milk and water spectra in Fig. 1A and Fig. 1B allow to identify peaks associated to mineral elements and those related to the matrix effect.

2.3. Statistical analysis

Outliers for iodine concentration were defined as values deviating more than 3 standard deviations (SD) from the mean, and were removed from the dataset. Two outliers were detected, and the final dataset consisted of 56 records (41, 10 and 5 records from HF, BS and SI, respectively). Outliers for milk quality traits (fat, protein, casein, lactose, urea, SCS, DSCC and pH) were also defined as values exceeding more than 3 SD from the mean. No outliers for milk quality traits were detected.

Energy-dispersive X-ray fluorescence software reports raw data as energy (keV) *versus* normalized impulses (counts per second) and converts normalized impulses to iodine concentrations using the following formula, based on Extended Compton quantification, implemented in the XRF Analyzer Pro (Ametek, Kleve, Germany) software:

$$c_i = K_0 + \frac{K_1 * I_i}{I_{Comp}}$$

where c_i is the concentration of the element i, K_0 is the offset of the calibration, K_1 is the slope of the calibration, I_i is the fluorescence intensity of the element i and I_{Comp} is the intensity of the incoherent backscatter.

Energy dispersive X-ray fluorescence calibration phase was performed on a subset of 20 individual milk samples, as a regression equation between iodine concentration obtained through ICP-MS analysis and normalized impulses (counts per second) profiled by ED-XRF instrument. Samples included in the calibration set were selected to cover the entire range of measured milk iodine concentration. The accuracy of the method was expressed through the coefficient of determination in calibration (R^2_C). Energy dispersive X-ray fluorescence cross validation phase was carried out on the entire dataset (n = 56) as a regression equation between iodine measured through ICP-MS *versus* iodine predicted through ED-XRF using the calibration model obtained as previously described. The accuracy of the ED-XRF method in quantifying iodine was expressed through the coefficient of determination in cross validation (R^2_{CV}). Energy dispersive X-ray fluorescence external validation phase was performed excluding samples used in the calibration set (n = 20) and considering only the remaining samples (n = 36) as a regression equation between iodine measured through ICP-MS *versus* iodine predicted through ED-XRF using the calibration model obtained as previously described. The accuracy of the ED-XRF method in quantifying iodine was expressed through the coefficient of determination in external validation (R^2_{EV}).

3. Results and discussion

3.1. Descriptive statistics

Descriptive statistics for milk iodine concentration, production related traits and milk quality traits are presented in Table 1. In the present study, days in milk and parity varied from 16 to 587 d and from

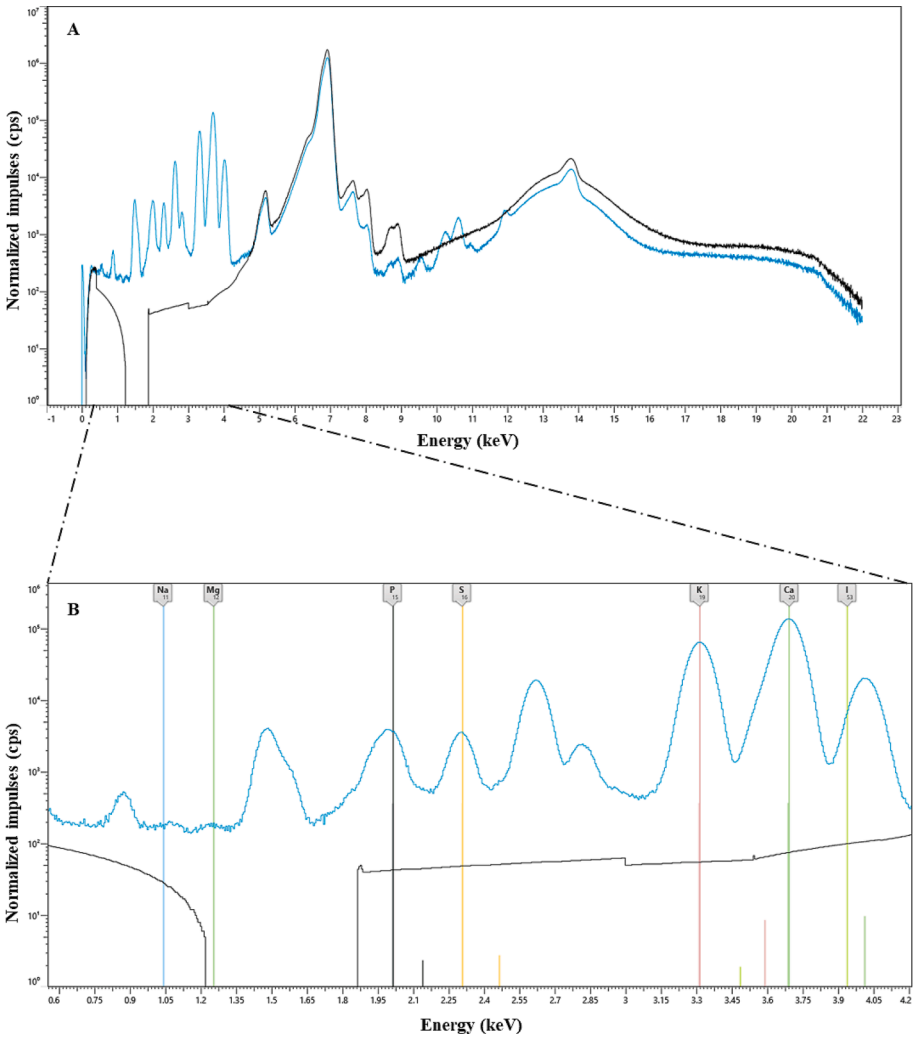

Fig. 1. A depicts energy dispersive X-ray fluorescence spectrum of an individual milk sample (blue line) overlapped with X-ray fluorescence spectrum of a water sample (black line); B depicts enlarged portion of the spectrum to show peaks related to milk minerals, including Na, Mg, P, S, K, Ca and I. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Descriptive statistics (n = 56) of milk iodine concentration measured through inductively coupled plasma mass spectrometer, production related traits and milk quality traits.

Trait[a]	Mean	SD[b]	CV[c] (%)	Minimum	Maximum
Iodine measured (μg/L)	645.09	489.55	75.89	100.00	2350.00
Production traits					
Milk yield (kg/d)	31.02	11.21	36.14	4.8	76.50
Days in milk (d)	234.95	137.58	58.56	16.00	587.00
Parity (n)	2.42	1.53	63.39	1.00	7.00
Milk quality traits					
Fat (g/100 g)	4.21	0.81	19.18	1.93	5.80
Protein (g/100 g)	3.61	0.49	13.55	2.64	4.91
Casein (g/100 g)	2.89	0.44	15.11	1.97	4.03
Lactose (g/100 g)	4.74	0.18	3.77	4.34	5.10
Urea (mg/dL)	24.69	5.12	20.74	11.30	37.20
SCS (units)	3.05	1.68	54.96	−0.64	7.21
DSCC (%)	63.63	15.51	24.37	24.10	87.80
pH	6.57	0.05	0.80	6.46	6.67

[a] SCS: somatic cell score, calculated as SCC = 3 + $\log_2$(SCC/100), where SCC is somatic cell count; DSCC: differential somatic cell count, calculated as the ratio of neutrophils plus lymphocytes on total milk SCC; [b]SD: standard deviation; [c]CV: coefficient of variation.

1 to 7, respectively. Milk yield averaged 31.02 kg/d, and means of fat, protein, casein and lactose were equal to 4.21, 3.61, 2.89 and 4.74 g/100 g, respectively. Observed average milk yield and quality traits are comparable with values reported by Penasa, Tiezzi, Sturaro, Cassandro, and De Marchi (2014), who studied milk coagulation traits of HF, BS and SI cows in multi-breed herds in the Veneto region. In the present trials, measured milk iodine concentration averaged 645.09 μg/L. Such value indicates that a glass of milk (125 mL) provides about 80 μg of iodine, which means nearly 90 and 30% of the recommended daily intake of preschool children and pregnant and lactating women, respectively. Average iodine concentration observed in the present study is considerably greater than values reported by Niero et al. (2020) and Walther, Wechsler, Schlegel, and Haldimann (2018). This is likely due to the samples considered in the present study, which indeed were selected to cover the greatest variability of milk iodine content (i.e. including also samples with extremely high iodine concentration), in order to enhance the robustness of ED-XRF prediction models. The relatively great amount of farms (with different feeding regimes and different levels of iodine supplementations) and the different breeds considered in the present study also contributed to enhance the variability of milk iodine concentration, which indeed was characterized by an elevated SD (489.55 μg/L) and coefficient of variation (75.89%).

3.2. ED-XRF calibration

Fig. 1A shows an ED-XRF spectrum obtained from the analysis of an individual cow milk sample. A portion of Fig. 1A was enhanced to show the region where Na (K-α, 1.04 keV), Mg (K-α, 1.25 keV), P (K-α, 2.01 keV), S (K-α, 2.31 keV), K (K-α, 3.31 keV), Ca (K-α, 3.69 keV) and I (L-α, 3.94 keV) were detected (Fig. 1B).

The calibration scatter plot of milk iodine measured through ICP-MS *versus* ED-XRF normalized impulses is depicted in Fig. 2A. The calibration model was implemented on 20 individual milk samples selected to cover the variability of the entire dataset, as a regression between iodine concentration measured through ICP-MS and normalized impulses of

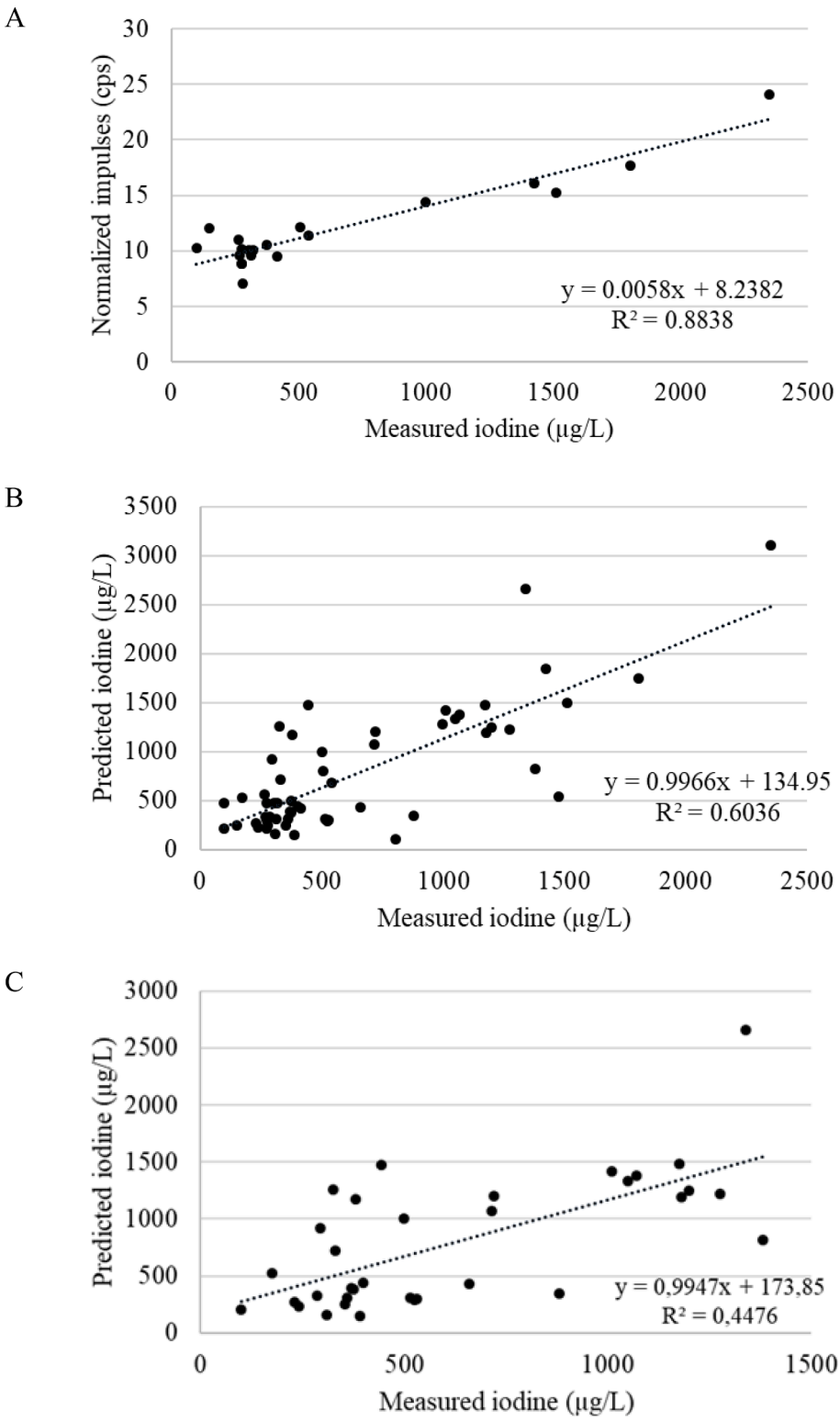

Fig. 2. A depicts the calibration scatter plot of measured iodine concentration (µg/L; x-axis) *versus* energy dispersive X-ray fluorescence normalized impulses (counts per second, cps; y-axis); B depicts the cross validation scatter plot of measured iodine concentration (µg/L; x-axis) *versus* predicted iodine concentration (µg/L; y-axis); C depicts the external validation scatter plot of measured iodine concentration (µg/L; x-axis) *versus* predicted iodine concentration (µg/L; y-axis).

ED-XRF tool. The R^2_C obtained for iodine is equal to 0.88. Such value is considerably greater than R^2_C obtained by Visentin et al. (2023) who studied the effectiveness of ED-XRF technique to quantify major mineral elements in non-lyophilized milk samples. Also, R^2_C obtained in the present study is greater than those reported by Niero et al. (2020) in a work aimed to predict milk iodine through mid-infrared spectroscopy (R^2_C = 0.65 and 0.69 using partial least square and backward interval partial least square, respectively).

3.3. ED-XRF validation

Cross validation and external validation scatter plot of milk iodine measured through ICP-MS *versus* milk iodine predicted through ED-XRF are depicted in Fig. 2B and Fig. 2C, respectively. The cross validation was carried out on the entire dataset (n = 56), whereas the external validation was carried out excluding samples used in the calibration and considering only the remaining samples (n = 36). The R^2_{CV} and R^2_{EV} were equal to 0.60 and 0.45 (respectively), which translates into a correlation coefficient of 0.78 and 0.67 (respectively). Results highlight the ability of ED-XRF to predict the concentration of iodine in individual milk samples with moderate accuracy, despite the relatively low concentration in the milk matrix. Similar results were obtained by Niero et al. (2020) who reported R^2_{CV} equal to 0.51 (using partial least square algorithm) and 0.60 (using backward interval partial least square algorithm) and R^2_{EV} equal to 0.47 (using partial least square algorithm) and 0.57 (using backward interval partial least square algorithm). Results of the present study are in line with R^2_{EV} obtained by Visentin et al. (2018) in a work aimed to predict major milk minerals through mid-infrared spectroscopy in cow milk (R^2_{EV} from 0.40 to 0.69 for Na and K, respectively) and greater than those reported by Visentin et al. (2023) who predicted the content of major milk minerals through ED-XRF (R^2_{CV} from 0.02 to 0.39 for Na and K, respectively). Greater R^2_{EV} (from 0.92 to 0.98 for S and P, respectively) were obtained by Perring et al. (2003) who predicted major milk mineral contents after a lyophilization step which conversely was not performed in the present trials.

4. Conclusions

In the present study, individual milk samples of HF, BS and SI cows were analyzed for iodine concentration through ED-XRF technique, avoiding any preliminary preparatory step. Results indicate that ED-XRF technique has moderate accuracy in the prediction of milk iodine concentration (R^2_{CV} = 0.60; R^2_{EV} = 0.45). In conclusion, even if the ED-XRF analysis cannot replace gold standard method, it can be assumed that this technique may be effectively adopted by dairy companies as a supporting decision tool for the discrimination of milk batches with high or low iodine concentration. In perspective, further research should be addressed to improve the accuracy of ED-XRF prediction models by increasing the number of samples in calibration, or by considering alternative chemometric approaches. Findings of the present study also represent a preparatory step for the monitoring of iodine concentration in functional fortified milk products.

CRediT authorship contribution statement

E. Visentin: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. **G. Niero:** Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. **M. De Marchi:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, 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.

Data availability

Data will be made available on request.

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