Manuscript Details

Manuscript number	TIFS_2020_77
Title	ANALYTICAL ADVANCES IN THE DETERMINATION OF CALCIUM IN BOVINE MILK, DAIRY PRODUCTS AND MILK-BASED INFANT FORMULAS
Article type	Review Article

Abstract

ABSTRACT Background: Milk, dairy products and milk-based infant formulas (MIF) are major sources of calcium in humans' diet, expecially in certain groups of population. The knowledge of the amount of this essential mineral in such complex matrices is a major concern to several areas such as health, quality control and research. Scope and approach: Through years, laboratory methods for calcium measurement have evolved into instrumental techniques, which brought to significant enhancements in analytical performances and sample throughput. Standardization bodies encourage keeping updated methods of analysis for nutrients. In this review, we gather knowledge on both the conventional and the promising analytical approaches of calcium measurement, highlighting their advantages and limitations. Key findings and conclusions: A wide array of techniques is available for calcium measurement and official international bodies have standardized several procedures. Classic sample pre-treatments, consisting in ashing or wet digestion, were joined by other effective and green-friendly solutions such as slurry sampling or direct solid sampling, when made feasible by the subsequent detection technique. Atomic spectrometry methods, complying with several standardized acceptability criteria, are nowadays largely used. In particular, flame atomic absorption spectrometry is well established together with inductively coupled plasma techniques, the latter being advantaged in terms of sensitiveness and multi-analyte capability. More recently, the interest in other approaches, such as instrumental neutron activation analysis and infrared techniques, has increased. X-ray fluorescence spectroscopy and laser-induced breakdown spectroscopy are suitable alternatives for routine at-line applications.

Keywords	calcium; bovine milk; dairy products; infant formula; analytical technique.
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DeFENS

January 24th, 2020

Dear Editor Prof. Fidel Toldrá,

On behalf of all Authors, I have the pleasure to submit the manuscript "Analytical advances in the determination of calcium in bovine milk, dairy products and milk-based infant formulas" to be considered for publication in *Trends in Food Science & Technology*.

The knowledge of calcium levels in bovine milk, dairy products and infant formulas is a pressing issue in health, research and routine control areas. In view of the ongoing progress in analytical approaches to measure minerals, standardization official bodies promote a steady updating activity to renew techniques.

This review aims at giving an overview on advances in both traditional and emerging approaches to measure calcium also in view to satisfy the request of dairy industry for high throughputs and at-line measures.

Even though the text alone is 7900 words, the manuscript exceeded the limit of 10,000 words.

We hope that the manuscript could be considered for the publication in *Trends in Food Science & Technology*.

Thank you for your consideration.

Sincerely,

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Highlights

- Calcium measurement in bovine milk, dairy products and infant formulas is reviewed
- Several official techniques are available for calcium measure in dairy matrices
- The demand for accurate calcium measures in milk and dairy products is growing
- Dairy industry is pressing for at-line measures of calcium content

ABSTRACT

Background: Milk, dairy products and milk-based infant formulas (MIF) are major sources of calcium in humans' diet, expecially in certain groups of population. The knowledge of the amount of this essential mineral in such complex matrices is a major concern to several areas such as health, quality control and research.

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Key findings and conclusions: A wide array of techniques is available for calcium measurement and official international bodies have standardized several procedures. Classic sample pre-treatments, consisting in ashing or wet digestion, were joined by other effective and green-friendly solutions such as slurry sampling or direct solid sampling, when made feasible by the subsequent detection technique. Atomic spectrometry methods, complying with several standardized acceptability criteria, are nowadays largely used. In particular, flame atomic absorption spectrometry is well established together with inductively coupled plasma techniques, the latter being advantaged in terms of sensitiveness and multi-analyte capability. More recently, the interest in other approaches, such as instrumental neutron activation analysis and infrared techniques, has increased. X-ray fluorescence spectroscopy and laser-induced breakdown spectroscopy are suitable alternatives for routine at-line applications.

ANALYTICAL ADVANCES IN THE DETERMINATION OF CALCIUM IN BOVINE MILK, DAIRY PRODUCTS AND MILK-BASED INFANT FORMULAS

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Background: Milk, dairy products and milk-based infant formulas (MIF) are major sources of calcium in humans' diet, expecially in certain groups of population. The knowledge of the amount of this essential mineral in such complex matrices is a major concern to several areas such as health, quality control and research.

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Keywords: calcium; bovine milk; dairy products; infant formula; analytical technique.

1 Introduction

Calcium, an essential mineral for humans supplied through the diet, is mainly present in bone and teeth being involved in several biological processes (Burrow, Young, McConnell, Carne, & Bekhit, 2018). Body requirements for bone development and maintenance vary throughout life, resulting higher during childhood/adolescence, pregnancy/lactation, and in the elderly (Kruger, Awan, Poulsen, & Kuhn-Sherlock, 2017). In western countries, bovine milk and dairy products are the main food group contributing to calcium intake in humans' diet. Cheese represents the main source of calcium in the elderly, whereas milk-based infant formulas (MIF) are the prevailing alternative to breast-feeding during infancy. Concerns about the nutritional adequacy of commercial MIF have triggered the research addressing the calcium content and the compliance with declared values (Papachristodoulou, Tsiamou, Sakkas, & Papadopoulou, 2018). To pursue this goal, among the others, a continuous development of more robust, sensitive and cost-efficient techniques is required. In addition, the number and variety of dairy products and beverages are on the rise in the marketplace and represent a serious challenge to current analytical methods, requiring versatile and time-saving approaches for high-throughput screening of the mineral content (International Dairy Federation, 2019). The accurate and precise measurement of calcium levels in milk, dairy products and MIF is a pressing demand for quality control and compliance to labeling legislation. Nowadays, several criteria are involved in the selection of a method to determine mineral concentration in milk and dairy products (Figure 1). Among these, applicability under usual laboratory conditions, accuracy, sensitivity, specificity, speed of analysis, high throughput, labour and infrastructure costs, as well as recommendations from standardization bodies are crucial. The multi-analyte capability is an additional criterion of choice of paramount importance for analytical laboratories. The availability of reliable data allows public health authorities and pediatric professionals to establish dietary recommendations for infancy. This knowledge is relevant also for food operators developing adequate fortified formulas to satisfy body requirements (Dubascoux, Andrey, Vigo, Kastenmayer, & Poitevin, 2018). In research laboratories, besides nutritional and quality control aspects, the availability of accurate data on calcium concentration and partitioning during dairy products' processing is useful to understand the changes in the equilibrium between soluble and micellar fractions and the subsequent relationship with the properties of the final product. Furthermore, the continuous revision of methods is a new ambition oriented both to solve real analytical problems and to integrate principles, priorities and strategies of green analytical chemistry. This review presents an overview on advances and trends of analytical techniques to measure calcium in milk, dairy products and MIF. Strengths and weaknesses, as well as main developments, of analytical strategies in use are discussed.

2 Calcium content in milk, dairy products and MIF

Minerals represent a small fraction of bovine milk (approximately 7–9 g L^{-1}), and calcium is one of its main cations. Calcium ions and salts in milk are distributed between aqueous and micellar phases in dynamic equilibria at 9 mM and 21 mM concentration, respectively (Gaucheron, 2011). Approximately 33% of total calcium is found in serum in the ionic/free form (~ 2 mM, i.e., 7% of total calcium) or associated with citrates (~ 6 mM, i.e., 23% of total calcium), phosphates (~ 0.6 mM, i.e., 2% of total calcium) or chloride (~ 0.2 mM, i.e., < 1% of total calcium) (Oh & Deeth, 2017). Only a negligible concentration of calcium (0.1 mM) is associated to α-lactalbumin (Gaucheron, 2011). Most of total calcium is in the micellar/colloidal phase, where it is bound to casein phosphoseryl residues (~ 16 mM, i.e., 50% of total calcium) or associated to inorganic phosphates (~ 5 mM, i.e., 16% of total calcium). Calcium in micellar phase, also named micellar calcium phosphate, is an integral part of the casein micelle affecting its structure and properties (e.g., hydration and heat stability). Calcium plays a role in several phenomena including rennet and acid gelation, firmness of coagulum and sediment formation (Nian, Chen, Aikman, Grandison, & Lewis, 2012). Also the processing history of dairy products and variations in processing conditions (i.e., milk pH, thermal treatments, microfiltration and mineral addition) modify calcium partitioning, and, as a consequence, they can promote changes in properties of the final products (Renhe, Zhao, & Corredig, 2019; Zulewska, Kovalik, Lobacz, & Dec, 2018).

Overall, calcium content in milk and dairy products (Table 1) exhibits a variation of approximately two orders of magnitude, ranging on average from 180 mg kg^{-1} in butter to 12800 mg kg^{-1} in skim milk powder (SMP). The wide variability observed in milk (Table 1) is explained by several factors, including breed, feeding system, stage of lactation, cows' nutritional and health status and, to a less extent, by season (Akkerman, Larsen, Sørensen, & Poulsen 2019; Gaignon, Gelé, Hurtaud, & Boudon, 2018; Gulati et al., 2018). Furthermore, analytical procedures, characterized by different calcium recoveries, may represent an additional source of variability (Table 1). Cheese is recognized as a rich source of bioavailable calcium, with most hard varieties containing approximately 8000 mg calcium kg^{-1} and up to 12000 mg kg^{-1} in Parmesan type cheeses. In comparison to rennet coagulated cheeses, sour varieties contain about tenfold lower amounts of calcium due to the solubilization of colloidal calcium phosphate. Also commercial MIF are characterized by variable calcium contents (Table 1) as a function of the recipe used by manufacturers who have to comply with both requirements laid down at national level and with minimum and maximum levels reported by EU Commission, i.e., 50 mg and 140 mg of calcium per 100 kcal, respectively (EU Regulation, 2019). These threshold levels were set up to meet nutritional requirements of infants and on the basis of an established history of apparent safe use.

3 Calcium determination

Several collaboratively studied and validated methods to measure calcium in bovine milk, in dairy products as well as in MIF are available (Table 2). Standardized methods enable consistency of data among different laboratories adopting the same procedures and act as a benchmark for comparing results with respect to new techniques. International Dairy Federation (IDF) collaborates with other international bodies, including Codex Alimentarius, the International Organization for Standardization (ISO) and AOAC International (AOAC), to the development of standard methods (Cruijsen, Poitevin, & Brunelle, 2019). Each procedure offers specific merits and shortcomings depending on the analyzed matrix and required attributes (Table 3). Most of the AOAC, ISO, European Norm (EN) and IDF official methods ratified for the determination of minerals in dairy products and infant products are multi-analyte and feasible in case of regulatory or trade disputes (Table 2) (Pacquette, & Thompson, 2018). Poitevin (2016) provided a thorough review of Official methods currently available for the determination of minerals and trace elements in MIF and milk products. In 2018, to reflect analytical advances, the Codex Committee on Methods of Analysis and Sampling (CCMAS) was asked to re-type or revoke the existing methods for minerals in MIF (FAO/WHO, 2018). Furthermore, some techniques are not fit-for-purpose for new commercialized milk-based formulas (Poitevin, 2016). It is worth to note the case of AOAC method 984.27 for mineral measurement in MIF following acid digestion by $HNO_3/HClO_4$. This method was modernized into the AOAC 2011.14 version characterized mostly by the use of time-saving microwave digestions using safer HNO_3/H_2O_2 solutions, new instrumental configurations, improved recoveries and improved stability. A further collaborative study established the precision of this method also for some dairy samples, and thereafter it became equivalent to ISO 15151| IDF 229 (2018).

3.1 Sample preparation

Milk, dairy products and MIF are complex matrices, and their mineral determination is a challenging task due to difficulties to achieve representative mineral dispersions matching the selected detection technique. Several sample preparation procedures for mineral determination have been developed and improved over the past decades, with the objectives to reduce overall analysis time, cost and to improve analytical performances (Figure 2). In this regard, the understanding of calcium partitioning between aqueous and colloidal phases is of utmost importance. Typically, to monitor the calcium content in the aqueous phase, three sample treatment procedures are mainly described in the literature, namely ultrafiltration, ultracentrifugation and rennet coagulation of milk (Gaucheron, 2011). Calcium measurement may be carried out in the permeate obtained by ultrafiltration typically with membranes at molecular weight cutoff of 10,000 Da. In this aqueous fraction, soluble calcium concentration is underestimated because it precipitates during the process (Franzoi, Niero, Penasa, Cassandro & De Marchi, 2018). Ultracentrifugation (usually carried out at 100,000 g for 1 h) may be applied as an alternative technique to obtain a good estimation of the amount of soluble minerals. An easy sample preparation characterizes this technique, but the equipment is rather costly. Furthermore, the high pressure can promote solubilization of minerals from casein micelles and the supernatant in addition to soluble calcium ions and salts also contains the calcium bound to α-lactalbumin. The third strategy to recover soluble calcium consists in milk coagulation by rennet, whey recovery and its subsequent filtration. This method is simple, rapid and less expensive than the first two approaches. Also in this case, the analyst should consider that the calcium content includes also the fraction linked to α-lactalbumin (Gaucheron, 2011).

Traditional analytical techniques for total calcium determination imply a preliminar decomposition of the organic matrix and the dissolution of the minerals (Figure 2). This is usually carried out by dry or wet decomposition of the samples in open or closed systems, using thermal, ultrasonic or microwave energy. In milk and dairy products, dry ashing is common, and it is carried out in a muffle furnace by calcination, generally at 525 °C under atmospheric pressure. In the case of milk powder, after digestion, ashes are dissolved in a small volume of acids resulting in high sample concentration and, consequently, low limits of calcium detection. Anyway, following these conditions, volatile elements are likely lost, and contamination may take place. Another common pretreatment is based on the sample acid digestion carried out by heating to obtain a homogeneous mineral dissolution. Wet acid digestion methods employ one or more concentrated acids. Nitric acid is typically preferred because of its oxidant activity and the availability at high purity. The complete oxidation of a sample through HNO_3 is advantageously achieved by the combined use of temperature and pressure (Muller, Souza, Muller, Muller, Mello et al., 2016). In recent decades, sample preparation procedures based on closed pressurized devices, either microwave-assisted or conventionally heated, have been reported in the literature. Undoubtly, microwave-assisted wet digestion (MAWD) proved to be a simple, clean and rapid digestion procedure. Anyway, the presence of a security system to avoid explosions due to the pressure exceeding the safety level and an exhaust system are necessary. These requirements make the system rather expensive. In a study undertaken to compare different sampling procedures (i.e., dry ashing, wet-digestion by high-pressure ashing system and MAWD system) MAWD allowed the best agreement between measured and certified calcium levels in reference samples of whole milk powder (WMP) (NIST 84359) and SMP (BCR-063) with recovery rates of 99.6% and 97.8%, respectively (Herwig, Stephan, Panne, Pritzkow & Vogl, 2011). A relatively new instument innovation introduced on the market is a reflux system device, consisting of a cold finger inserted in the digestion vessel heated with conventional heating. This solution is preferred over open systems offering reduced risk of analyte loss. Sample preparation through such device was adopted to determine calcium and other minerals in milk powders and MIF (n = 4) by inductively coupled plasma optical emission spectrometry (ICP-OES) (Oreste, de Souza, Pereira, Lisboa, Cidade et al., 2016). Results fitted well (at 99% confidence level) with those obtained by conventional open-vessel heating system. The sample preparation by reflux system was advantaged for ease of use and low operating cost. Moreover, it proved to be an alternative for laboratories that do not have microwave oven devices.

Despite less used than MAWD, digestion is efficiently carried out also by focused microwave-induced combustion (FMIC), a green digestion method using oxygen as an oxidant with diluted acid and ammonium nitrate as igniter. Through FMIC, samples are combusted in vessels under pressurized oxygen, and ignition step is performed under microwave radiation. The advantages consist in high efficiency of sample oxidation, low blank values due to oxygen purity, low reagent consumption and use of diluted acids for analyte absorption. In addition, this sytem allows minimization of spectral interferences on mineral measure by ICP techniques (Rocha, Batista, Rocha, Donati, & Nóbrega, 2013).

Currently, sample preparation strategies are moving towards greener approaches. One trend is based on the reduction of acid volumes for sample digestion. In addition, the use of diluted acid solutions, leading to safer digestion conditions, lower residual acidities and, consequently, smaller residue amounts and reduction in costs, has been suggested. A green approach in sample digestion is based on the simultaneous use of diluted HNO_3 and pressurized O_2 as an auxiliary reagent. In this case, nitrogen oxides (deriving from oxidation of organic matter) react with O_2 and regenerate HNO_3 in the reaction vessel, thus avoiding incomplete digestion of organic matter. This sample pre-treatment coupled with ICP-OES was applied for calcium determination in milk powder (Bizzi, Barin, Garcia, Nóbrega, Dressler et al., 2011). Under these conditions, good accuracy was observed in certified reference material SMP (NIST-1549) (recovery equal to 102%), and the calcium levels in commercial WMP fitted well (at 95% confidence level) with those obtained by digestion in concentrated HNO_3. An effective strategy to overcome the partial mineralization following the use of diluted acid solutions consists in the combined use of diluted HNO_3 and H_2O_2 to boost the oxidant power (by regeneration of HNO_3), thus achieving a low residual carbon content (Bizzi et al., 2014).

The direct analysis of solid samples or suspensions is gaining importance, as it offers reduced times, high sensitivity, reduced sample amount, overcoming the use of acids and other aggressive reagents. Advantages and challenges of solid sampling for mineral determination have been recently highlighted in the literature (Machado et al., 2020). Direct analysis of powder samples (compact material or pellets) or suspensions is well established for different atomic spectrometry techniques including electrothermal atomic absorption spectrometry (ET-AAS), ICP techniques, laser-induced breakdown spectroscopy (LIBS) and X-ray fluorescence spectroscopy (XRF). A successful alternative to circumvent time-consuming mineralization protocols, offering the advantage of direct solid or liquid sampling, is slurry sampling. Minerals in organic matrices can be dissolved by simply using reagents at room temperature, thus providing solutions or slurries of samples. A variety of solvents and stabilizers such as HNO_3, H_2O_2, tetramethylammonium hydroxide, ethanol and Triton X-100 are successfully used in the preparation of slurries (Alegría, Barberá, Lagarda, & Farré, 2010; Diniz, Carrasco, Medina, Ribeiro, & Nunes, 2017; Sola-Larrañaga & Navarro-Blasco, 2009). A further option coupled to slurry preparation is the ultrasound-assisted extraction. The mechanism of this technique has been overviewed and described as a green alternative to conventional approaches adopted for mineral extraction (Rocha et al., 2013). Action of the ultrasound waves promotes the breakage of bonds between minerals and the organic matrix. The application of ultrasound is normally combined with the use of acids or H_2O_2 to intensify the sonication power. The ultrasound-assisted extraction was successfully employed to determine the presence of micro- and macro-minerals in MIF, and it resulted in a reduction of acid consumption and sample preparation time (Gamela et al., 2019).

3.2 Analytical techniques for calcium determination

A variety of techniques are available for calcium determination in milk, dairy products and MIF. A list of those currently adopted is detailed in Figure 2.

3.2.1 Titrimetry

Complexometric titration is one of the oldest techniques adopted for calcium determination in the analysis of milk (Table 2). It has been mostly used for routine analysis of milk due to its simplicity and lower cost than ICP-based techniques (de la Fuente & Juárez, 2015). The titrimetric approach is described in the ISO12081 | IDF36 Standard (2010). It gained popularity in research laboratories requiring basic equipment (i.e., balances and burets). This titration method consists in a preliminar step of protein precipitation by trichloroacetic acid and the subsequent precipitation of total calcium as oxalate followed by the separation of the calcium oxalate. Skilled titrations with potassium permanganate of the dissolved precipitate should provide results with repeatability (absolute difference between two single tests) lower than 0.002%. An alternative approach is based on precipitation of proteins by salicylic acid followed by calcium titration directly with ethylenediamine tetraacetic acid, in the presence of indicator palladiazo. These complexometric methods are single-analyte and no longer in use for routine application, being laborious and time-consuming (Alegría et al., 2010).

3.2.2 Atomic absorption spectrometry (AAS)

Basic principles of atomic absorption spectrometry (AAS), instrumentation components as well as applications in the food analysis have been overviewed in the literature (López-García & Hernández-Córdoba, 2015). This technique has been used since the end of the 20th century for mineral and trace mineral analysis (Fernandez, Lobo, & Pereiro, 2019) being advantaged for (i) simple instrumental arrangement, (ii) high selectivity and specificity, (iii) reduced spectral interference and (iv) robustness of atomizers (Machado et al., 2020). Anyway, by AAS the measurement of a single analyte at a time is possible. Main variants of AAS, classified as a function of the type of atomizer, include flame AAS (FAAS) and ET-AAS, the latter having an electro-thermal graphite furnace replacing the flame/burner arrangement. ET-AAS has been mainly adopted for trace mineral determination, due to its sensitiveness at ppb levels (i.e., up to two orders of magnitude lower than FAAS).

3.2.2.1 Flame atomic absorption spectrometry

FAAS is a popular technique applied extensively for measuring minerals in different food matrices due to its relative simplicity (López-García & Hernández-Córdoba, 2015). It is the least expensive and most suitable for the determination of calcium and other macro-minerals among atomic spectrometric techniques. To overcome chemical interferences (mainly phosphates) in the determination of calcium, the sample is usually supplemented with lanthanum as a releasing agent (matrix modifier). Through FAAS, liquid samples are easily introduced as aerosols into the analyzer after a preliminar treatment by conventional ashing or wet digestion. To increase the sample throughput rate, digestion with pressure control and MAWD is often recommended (Sola-Larrañaga & Navarro-Blasco, 2009). This procedure coupled to micro-sampling FAAS detection was validated for the determination of calcium and the other macro-minerals in foodstuffs within the French Total Diet Study to estimate dietary exposure to these nutrients (Chekri et al., 2010). Such approach was judged the most suitable and provided accurate results (recovery rate for calcium of 96%) with repeatability and reproducibility coefficient of variations of 5.6% and of 7.5%, respectively.

High-resolution continuum source FAAS (HR-CS FAAS) is a variant of FAAS with multi-analyte capabilities, characterized by specific instrumental solutions, such as high intensity xenon short-arc lamp and bidimensional detector (Brandao, Matos, & Ferreira, 2011). Generally, following the use of secondary line of the analyte instead of the resonance line, through HR-CS FAAS it is feasible to avoid high dilution of the sample (typical of FAAS), thus increasing sensitivity, which results useful to quantify micro-minerals (Lopez-García & Hernandez-Córdoba, 2015). Brandao, Matos and Ferreira (2011) by HR-CS FAAS applied slurry sampling technique (in presence of HNO_3 and subsequent sonication) to measure calcium levels in commercial samples of milk, yoghurt and milk powder. The LOD value was 12 mg kg^{-1}, and precison parameters expressed as repeatability standard deviation (RSD_r) and reproducibility standard deviation (RSD_R) accounted for 2.8% and 2.9%, respectively. Accuracy, verified in certified reference material SMP (NIST 1549), resulted in a calcium recovery of 94.3% in comparison to the declared level. Figures of commercial samples fitted well (at 95% confidence level) with those recorded by conventional wet digestion sampling and were judged in agreement with literature data. Recently, Gamela et al. (2019) by HR-CS FAAS determined calcium and other minerals in commercial MIF by using ultrasound-assisted extraction. Slurry of samples by mixing with HNO_3 was agitated by ultrasonic device operating with a frequency of 37 kHz at a temperature of 80 °C for 10 min. Calcium contents in two commercial MIF almost overlapped the results obtained with traditional MAWD, i.e., 4246 ± 86 mg kg$^{–1}$ *vs* 4258 ± 86 mg kg$^{–1}$ and 2827 ± 50 mg kg$^{–1}$ *vs* 2806 ± 96 mg kg$^{–1}$, respectively. Following the optimization of analytical conditions for each element, this approach proved to be promising for multi-mineral analysis.

Among instrumental techniques used for measuring calcium in milk, dairy products and MIF, FAAS has been adopted and internationally validated by regulatory bodies (AOAC, CEN, ISO/IDF) (EN 15505, 2008; ISO 8070 | IDF 119, 2007) (Table 2). The Technical Committee of Joint Action Team "Minor Compounds" evaluated precision parameters of FAAS method for calcium determination in milk and milk products (ISO 8070 | IDF 119, 2007). The study involved 18 laboratories, and calcium determination was performed in whey protein concentrate, WMP, processed cheese, whey powder, freeze-dried cheese and SMP. Two mineralization techniques, i.e., MAWD and dry ashing, were used for sample digestion. Calcium results demonstrated the equivalence to the two procedures (at 95 % confidence level), and precision parameters were acceptable, being overall RSD_r and RSD_R in the ranges 0.9–5.5% and 2.1–7.4%, respectively.

3.2.3 Atomic emission spectrometry

In atomic emission spectrometry (AES), analyte atoms in solution are aspirated into an excitation area, where they are desolvated, vaporized and free atoms are created under high temperature by different atomization sources (mostly flame or plasma). The excited atoms from high-energy levels decay back to lower levels by emitting light. The analyte concentration is determined by the spectrometric measurement of the emitted photons. Although, flame AES is consolidated for the determination of alkali metals in solutions, this technique is affected by chemical, matrix or spectral interferences (Lyra, Almeida, Cunha, Diniz, Martins et al., 2014). These limitations may be overcome by correcting the background in combination with the use of high-resolution spectrometers and/or mathematical and multivariate procedures (Moldovan, 2019).

Digital image-based flame AES (DIB-FES), a variant of traditional FES, is based on the use of a video camera along with digital image analysis to obtain an analytical signal. A procedure based on DIB-FES in combination with chemometry was developed for the determination of calcium in milk powder (Lyra et al., 2014). In this case, calcium in the flame emits radiation, which is revealed in the digital images captured by the detector (i.e., the video camera). Through multiple linear regression models calcium levels were predicted in commercial milk powder samples. Results fitted well (at 95% confidence level) with those recorded by FAAS as a reference, with recoveries in the range 99–103%. The authors suggested this procedure as a valid alternative to FAAS for calcium determination in milk powder.

3.2.3.1 Inductively-coupled plasma optical emission spectrometry

ICP-OES is a powerful and versatile analytical approach well established since 1980s, currently widespread for routine analysis of minerals in milk, dairy products and MIF (López-García, & Hernández-Córdoba, 2015; Manuelian, Currò, Penasa, Cassandro, & De Marchi, 2017b). Considering the presence at high levels of calcium in milk and dairy products, ICP-OES offers an excellent compromise between speed, cost, and simplicity. The popularity of ICP techniques has to be ascribed to overall analytical performances, including high precision, selectivity and sensitivity as well as low matrix effects, particularly when ICP is compared with alternative approaches such as AAS (Table 3). Samples (liquid or solid) may be analysed by nebulization, slurry or laser ablation, the latter being a powerful tool for direct solid analysis (Fernández-Sánchéz, 2019).

Several studies demonstrated the feasibility of ICP-OES-based procedures to measure calcium and micro-minerals in complex matrices such as milk, dairy products and MIF (Bilandžić, Sedak, Đokić, & Božić, 2015a;

Bilandžić, Sedak, Đokić, Božić, & Vrbić, 2015c; Luis, Rubio, Revert, Espinosa, González-Weller et al., 2015) (Table 1 and 4). ICP-OES was used to compare the efficiency of MAWD and FMIC to digest milk powder and MIF samples (Pereira, Pereira, Schmidt, Moreira, Barin et al., 2013). These two sampling procedures agreed (at 95% confidence level) in terms of accuracy for calcium and other minerals with respect to certified reference materials i.e., SMP (NIST 1549) and WMP (NIST 8435), showing a mean recovery rate of 98%. Calcium concentrations in MIF samples (n = 2) with the two pre-treatments fitted well (at 95% confidence level) too. Overall, the digestion procedure based on FMIC was advantaged for use of diluted acid solutions, shorter digestion time, higher sample amount digested and lower LOQ values (0.1 mg instead of 0.6 mg kg^{-1}). In OES an alternative to ICP technology is microwave-induced plasma (MIP). This excitation source is in line with the principles of green analytical chemistry. By MIP-OES, an electrical field coupled with microwave energy produces stable plasma using nitrogen gas (Poitevin, 2016). The use of nitrogen to sustain the plasma is preferred, because this gas is cheaper than argon used in ICP procedures, and simpler spectra are generated. Furthermore, MIP-OES, differently from FAAS, does not use toxic gases, and it is claimed to be of wider linear ranges, greater detection limits, lower operational costs and improved analysis speed (Diniz et al., 2017). Williams et al. (2017) suggested the coupling of dry ashing (with a prototype apparatus) and MIP-OES for measuring minerals in cheese, butter and MIF samples. The authors highlighted the pros of this technique, including the short warm-up times (approximately 20 min) and the higher sensitivity (LOD value for calcium of 2 µg L^{-1}) over FAAS. Calcium concentrations, obtained following dry ashing, were in agreement ($p<0.05$) with those by conventional MAWD. Despite this, the proposed procedure coupling dry ashing and MIP-OES was judged simple, fast and accurate for mineral determination in complex dairy matrices.

3.2.4 Inductively-coupled plasma mass spectrometry

A description of ICP mass spectrometry (ICP-MS) principles (including its applications for mineral analysis in foods and beverages) has been reported in the literature (García Alonso, Marchante-Gayón, & Moldovan, 2015). Procedures based on ICP as an excitation source associated with MS are capable of ultra-trace detection, and for this reason they are mainly exploited to measure trace minerals and to carry out isotopic analysis in milk and dairy products. Through years, ICP-MS instruments evolved, and to date the modern configuration adopted for mineral analysis in routine testing laboratories incorporates collision/reaction cell technology capable to remove interferences, but with reduced multi-element capabilities (García Alonso et al., 2015). Calcium-dedicated applications are scarcely reported in the literature (Reykdal, Rabieh, Steingrimsdottir, & Gunnlaugsdottir, 2011; Zwierzchowski & Ametaj, 2019). A fast and accurate screening method to determine simulteneously macro- and micro-minerals in milk was developed through sector field (SF) ICP-MS (Herwig et al., 2011). This variant offered the capability to separate interfering molecules from the analyte ion maintaining the multi-analyte capability. Method validation revealed good agreement between measured and declared calcium values in two certified reference materials SMP (BCR 063) and WMP (NIST 8435) with recoveries of 97.8% and 99.6%, respectively. This multi-element screening method was applied to monitor the mineral variability in milk samples taken from cows reared at two different feeding regimes. The authors revealed that the diet did not affect significantly calcium contents of milk samples.

In the context of the first French Total Diet Study for infants and toddlers, undertaken by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), an ICP-MS method enabling the simultaneous determination of macro- and micro-minerals in foodstuffs was developed and validated (Chevallier, Chekri, Zinck, Guérin, & Noël, 2011). The procedure, based on ICP-MS after MAWD in closed vessels, fulfilled several criteria of analytical performances (such as linearity, specificity, precision and limits of quantification) and resulted feasible to be used in the routine analysis for dietary assessment in several food matrices mainly consumed by infants and toddlers.

Recently, ICP-MS has been collaboratively applied to develop Standard ISO 21424 | IDF 243 (2018) /AOAC Official method 2015.06. Twelve macro- and micro-minerals were measured in a variety of dairy products, MIF and adult nutritionals. Samples underwent MAWD in presence of a HNO_3/H_2O_2 solution. Measured calcium levels fulfilled Standard Method Performance Requirements (SMPR), thus the method resulted fit-for-purpose. In particular, LOQ was on average equal to 5.3 mg kg^{-1}, and precision data expressed in terms of RSD_r and RSD_R were 2.0% and 5.6%, respectively. The same samples, concurrently analyzed by ICP-AES with the AOAC Official method 2011.14, showed RSD_r and RSD_R values of 3.7% and 8.5%, slightly higher than those above mentioned by ICP-MS. Anyway, both techniques were judged as interchangeable to determine calcium concentrations.

3.2.5 Instrumental neutron activation analysis

Instrumental neutron activation analysis (INAA) is a non-desctructive spectrometry technique applied to multi-analyte determination in milk, dairy products and MIF. This approach is based on the conversion of stable nuclei to radioactive nuclei via nuclear reactions and the subsequent measurement of the reaction products through an equipment at high energy resolution (Greenberg, Bode, & de Nadai Fernades, 2011). In general, a procedure by INAA consists in (i) freeze-drying and homogenization of the sample, (ii) activation by irradiation with reactive neutrons, (iii) measurement of the induced radioactivity emitted at different decay intervals via a gamma-ray spectrometer and (iv) interpretation of gamma-ray spectra. The main advantage of this approach is that the preliminar sample dissolution is not required (Table 3). The sample portion is simply weight, encapsuled (in foils, vials or ampoules) and directly analyzed without any mineralization step. The use of INAA proved to be effective for calcium determination in milk (Potočnik, Nečemer, Mazej, Jaćimović, & Ogrinc, 2016; Santos, de Nadai Fernandes, Tagliaferro, & Bacchi, 2008) and dairy products (Wasim, Rehman, Arif, Fatima, & Zaidi, 2012). Different drawbacks, including the necessity to have access to a nuclear reactor, the expensive equipment and the required expertise, limit the routine application of this technique (Table 3) (Chajduk & Polkowska-Motrenko, 2017). Anyway, this sophisticated approach remains an important asset in specialized laboratories and research institutes for the analysis of standard reference materials.

3.2.6 X-ray fluorescence

X-ray fluorescence is a comparative technique requiring a calibration approach to perform quantitative measurements. The principle of XRF consists in submitting the sample to a beam of X-rays, which results in the emission of secondary (fluorescent) X-rays specific for each mineral from the sample surface (Klockenkämper & von Bohlen, 2015). The intensities of emitted X-rays are proportional to the concentration of the element. As a function of the mode of detection of emitted X-rays, two XRF configurations are available, namely wavelength-dispersive (WD) XRF and energy-dispersive (ED) XRF. In the WD system, the emitted polychromatic beam emerging from the sample surface is dispersed into its monochromatic components (wavelengths) with an analyzing crystal, whereas the ED approach measures the intensity of the photon energy of the individual X-rays generated. It is well accepted that WD-XRF has superior sensitivity (LOD of 8.0 mg kg^{-1} *vs* 1 mg kg^{-1} of ED-XRF) and resolution, fewer spectral overlaps and lower background intensities (Pashkova et al., 2018). Anyway, the WD-XRF configuration is reported to be less employed in milk and dairy product analysis due to the higher price compared with ED-XRF (Habib-Ur-Rehman, Rehana & Yawar, 2012). Although not so widespread as ICP-OES, this approach has become popular for the mineral analysis in milk and dairy products (Pashkova, Smagunova, & Finkelshtein, 2018) and MIF (Papachristodoulou et al., 2018). The reasons rely on the fact that solid samples are directly analyzed without any chemical treatment. Other factors promoting the use of XRF consist in the current easier accessibility to the equipment, the simplicity of use, the short analytical times (about 1 min) and, therefore, subsequent higher output. XRF has been implemented in milk-based powder processing plants close to production lines to ensure their quality control (Perring & Monard, 2010). The advantages of XRF include, among the others, the implementation of the principles of green chemistry (i.e., no use of solvents), miniaturization, automation as well as reduced energy consumption (Rossmann, Zaichick, & Zaichick, 2016) (Table 3). In comparison to other spectroscopic techniques (i.e., AAS and ICP), measurements of calcium by XRF are carried out directly on the sample portion (liquid, loose powder or compressed into pellets) without additional chemical treatments. Pashkova, Smagunova and Finkelshtein (2018) highlighted advances in XRF instrumentations, sample preparation, calibration and quantification procedures for the analysis of macro and micro minerals in milk and dairy products.

Nowadays, user-friendly benchtop and portable ED-XRF analyzers are successfully applied for at-line analysis of mineral content in milk powders (Panalytical, 2020). In a recent paper, focusing on multi-mineral composition of milk measured by INAA, ICP-MS and ED-XRF, the latter approach resulted the cheapest, simplest and most environmental-friendly (Potočnik et al., 2016). McCarthy et al. (2018) used ED-XRF spectrometry as a tool for the rapid determination of calcium in SMP. The authors highlighted the strength of ED-XRF for rapid mineral analysis during dairy processing but observed a systematic bias with an average calcium recovery of 104% in comparison to data obtained with ICP-MS (n = 27). To minimize the discrepancy of data ascribed to matrix effects, the authors suggested increasing the number of samples in the calibration set to improve the library matching.

A variant of ED-XRF system is the total reflection X-ray (T-XRF) spectrometry. In this case, the primary beam strikes the sample at a very low incident angle leading to improvement of detection limits. T-XRF can be successfully applied as a screening tool for chemical composition and quality control in the analysis of milk. Smagunova and Pashkova (2013) provided recommendations in sample pre-treatment of liquid milk with different fat contents (1.5–4.0%) for the analysis of minerals by T-XRF. The authors recorded an increase in the repeatability of calcium outcomes, when milk was diluted with water (to 5% total solid content) prior to the lyophilization step. This outcome was explained as the result of an inhomogeneous distribution of the dried undiluted sample, which led to pellet areas of different thickness and, as a consequence, of variable absorption effects. Benchtop T-XRF instrumentation equipped with low-power X-ray tubes is available on the market, and it allows to reach detection limits below 10 ppb in milk for most minerals (Pashkova et al., 2018).

3.2.7 Laser-induced breakdown spectroscopy

Laser-induced breakdown spectroscopy is a form of emission spectroscopy technique triggering growing attention for the assessment of multi-mineral composition in biological samples. Generally, in LIBS a small amount of sample (few μg) is vaporized and excited by a high-energy pulsed laser beam. The excited atomic and ion species turn to their ground state emitting light signals with specific wavelenghts, which are measured by a spectrophotometer (Markiewicz-Keszycka, Cama-Moncunill, & Casado-Gavalda, 2017). The emission wavelenght determines the type of element, whereas the emission intensity is correlated to the amount of the element. This technique gained attention in the dairy industry for *in situ* analysis of nutritionally important elements and heavy metals. In particular, Cama-Moncunill et al. (2017) recently underlined the potential of LIBS as at-line validation method for the determination of calcium in the MIF manufacturing industry. The benfits of LIBS over other techniques (such as AAS, ICP-OES and ICP-MS) rely on the real-time monitoring capabilities, its simplicity and lack of time-consuming sample preparation (Table 3). LIBS is applied for either qualitative or quantitative analysis of minerals in milk and dairy products. Quantitative multi-analyte analysis can be carried out either with or without calibration methods. Nervertheless, several limitations may take place, specifically the signal fluctuation of laser source, which reduces sensitivity and accuracy, and the occurrence of matrix effects. A further drawback of LIBS technique applied to liquid samples is the splashing of liquid due to the high laser energy, and the subsequent formation of shock waves (Sezer et al., 2018). To overcome this drawback, Abdel-Salam, Al Sharnoubi and Harith (2013) converted liquid milk to solid phase by absorption on a filter paper, and carried out a qualitative study concerning the mineral composition of commercial MIF. Differences in calcium content of commercial samples were recorded by means of normalization of spectra intensities to reduce matrix effects and to compensate for experimental fluctuations.

Sezer et al. (2018) turned liquid milk into a gel form ready for LIBS analysis by applying gelatin over the milk sample. In this way, they avoided difficulties related to liquid sampling and the time-consuming step of lyophilization. Overall, LIBS demonstrated to be a powerful, fast (few minutes), safe, simple and reliable approach for *in situ* use. Cama-Moncunill, Markiewicz-Keszycka, Cullen, Sullivan et al. (2020) illustrated the potential of LIBS to predict calcium content in liquid ready-to-nourish IF. In this case, a thin film of liquid sample, formed by a rotating wheel partially submerged, was presented to the laser beam. The film was stabilized with the use of a gas purge jet directed to the wheel surface. The authors used multivariate analysis by partial least square regression (PLSR) to correlate FAAS data with LIBS spectra. The model exhibited a good predictive accuracy of calcium contents of the validation set (n = 45), as supported by the value of coefficient of determination (R^2_p) of 0.91 and the root-mean square error of prediction (RMSEP) value of 64.5 mg L^{-1}. The authors assessed the at-line application with real-time monitoring capabilities of this LIBS approach in the MIF manufacturing industry to determine calcium content.

3.2.8 Infrared technologies

Infrared (IR) technologies are widely used in dairy industry for milk standardization, payment and phenotyping as well as to determine quality features in dairy products. The basis of infrared spectroscopy as well as applications to dairy foods have been reviewed (De Marchi, Penasa, Zidi, & Manuelian, 2014). In the last years, traditional applications of IR technologies, focused on the determination of major components in milk, paved the way also for the evaluation of other traits, such as the mineral content. Infrared technologies are a predictive approach based on the coupling with statistical programs for spectra interpretation (spectral chemometric analysis). The spectra regions of interest at dairy industry level are both the near infrared (NIR) and mid-infrared (MIR). One criterion widely used to determine the proficiency of predictive models is the ratio performance deviation (RPD). It is a nondimensional statistic parameter useful for quick assessment of infrared calibrations. In the analysis of difficult matrices such as cheese, a threshold range of RPD between 2.4 and 3.0 is considered as adequate for a rough screening (De Marchi et al., 2018), whereas prediction models with RPD smaller than 1.5 are considered unsatisfactory (Karoui et al., 2006). Several reports pointed out the potential of NIR/MIR spectrometry for prediction of calcium content in milk and dairy matrices using ICP-OES as the reference method (González-Martín, Hernández-Hierro, Revilla, Vivar-Quintana, & Lobos Ortega, 2011; Manuelian, Currò, Penasa, Cassandro, & De Marchi, 2017a; Manuelian, Currò, Visentin, Penasa, Cassandro et al., 2017c; Toffanin, De Marchi, Lopez-Villalobos, & Cassandro, 2015). In particular, Manuelian et al. (2017c) by NIR spectroscopy reported RPD values for calcium in Mozzarella and Stracchino cheeses of 1.51 and 1.50, respectively. The authors judged these outcomes as sufficient for screening criteria. The same research group in another study involving 19 different varieties of soft, semi-hard and hard cheeses recorded an optimal RPD value for calcium (i.e., 3.73) (Manuelian et al., 2017a). This improved result was explained by the fact that, when a large variability of reference data is considered, more accurate calibrations are obtained. Overall, this IR procedure resulted fast (no sample treatment required) and feasible for the at-line implementation of calcium (and mineral) determination in cheeses.

Malacarne et al. (2018) investigated the ability of MIR spectroscopy to predict micellar and diffusible (by ultrafiltration) calcium (and other minerals) in bulk milk samples (n = 147). The RPD values for total, soluble and colloidal calcium were 1.12, 1.24 and 1.25, respectively. This poor performance of MIR spectroscopy prediction models was likely due to the low variability of reference datasets. The authors emphasized the necessity of further improvements in the use of MIR spectroscopy to predict calcium (and mineral) content in raw bulk milk. More recently, an investigation on the suitability of MIR spectroscopy to evaluate the partitioning of calcium fractions in individual raw milk samples was carried out by Franzoi, Niero, Penasa and De Marchi (2019) on 93 samples of Holstein cows. The adopted predictive statistical approach, i.e., backward interval partial least squares' algorithm, allowed to build an improved prediction model for total, diffusible and micellar calcium contents in comparison to the partial least square model. Despite the optimized fitting ability, the accuracy of the MIR prediction was moderate, with RPD values for total, diffusible and micellar calcium of 2.16, 2.09 and 2.04, respectively.

3.2.9 Potentiometry by free calcium ion selective electrode (ISE)

Potentiometry is a simple electrochemical technique applied in research laboratories for the determination of free calcium ion activity in milk and dairy products (Chan & Palmer, 2013). The widespread use of this technique is attributed to the fulfilment of several criteria of choice, namely, the simplicity of the involved procedures, relatively fast responses, reasonable selectivity, wide linear dynamic range, acceptable mechanical stability, as well as the simple instrumentation, low cost and suitability for continuous monitoring

(Yáñez-Sedeño, & Pingarrón, 2015) (Table 3). This method is timesaving (response time in the order of seconds), easy to use and sensitive (limit of detection, i.e., LOD, in the range 10^{-5}–10^{-9} M) (Chan & Palmer, 2013; Kormali Ertürün, 2018). A great variety of ISE devices is commercially available, and their development is addressed to the achievement of lower detection limits than previously thought possible. A potential problem affecting the accuracy of the analysis is the lack of day-to-day reproducibility and the existence of matrix effects. Solutions to these problems are the adoption of proper configuration of ISE device and the selection of suitable standards for calibration to equilibrate the ionic compositional differences between milk sample and calcium solutions (Gao, van Leeuwen, van Valenberg, & van Boekel, 2011). This technique is currently adopted in research laboratories to investigate physicochemical (Akkerman, Larsen, Sørensen, & Poulsen, 2019; Nian et al., 2012), nutritional (Lorieau, Le Roux, Gaucheron, Ligneul, Hazart et al., 2018) and technological properties (Ho, Murphy, Drapala, O'Callaghan et al., 2018; Koutina, Knudsen, Andersen, & Skibsted, 2015) of milk and rennet gels. In the preparation of conventional calcium sensors with internal solution, the main component responsible for its selectivity is the ionophore incorporated in the ion-selective membrane. An alternative to electrodes with internal solution is represented by solid-state sensors, the transducer of which is a coating layer mainly made of nanostructured materials, such as multi-walled carbon nanotube (MWCNT), acting as ion-to-electron transfer promoters. Kormali Ertürün (2018) developed a solid-state vitreous carbon electrode coated with MWCNT and titanium oxide nanoparticles, as a transducing layer for the direct determination of total ion calcium in a commercial UHT milk. The ion calcium ISE characterized for a Nernstian behaviour over a wide working range (1×10^{-7}–1×10^{-1} M), short response time (4–5 s), sensitivity (LOD of 6.9×10^{-8} M) and stability. The milk samples, after acid digestion (by HNO_3 in presence of H_2O_2 for 30 min under boiling conditions) to ionize colloidal calcium, were left to stand for 12 h in refrigerator. The calcium determination was carried on the supernatants, and the results were in good agreement with those obtained by FAAS (1097.6±72.8 *vs* 1070.4±81.2 mg L^{-1}, respectively). This sensor proved to be selective for calcium and useful for routine quality control analyses.

3.2.10 Other techniques

Other approaches for calcium determination based on gravimetry, capillary electrophoresis, coulometry, voltammetry, ultraviolet-visible spectrophotometry and spectrofluorimetry are described in the literature (Lyra et al., 2014). Anyway, their application to milk, dairy products and MIF is lacking or dating back in the past (de la Fuente & Juárez, 2015). Ion chromatography (IC) for calcium determination in milk received little interest in the literature, likely not fulfilling all criteria required to be considered analytically attractive in comparison to conventional approaches. Actually, in the last two decades, less than 10% of scientific reports on the application of IC in food matrices were dedicated to the analysis of cations. An IC column-switching method with non-suppressed conductivity detection to measure, among the others, free calcium ions in milk and MIF appeared in the literature (Wei, Wang, Wang, & Zhu, 2017). The sample treatment prior to column-switching system was required, and it consisted in milk protein precipitation, centrifugation and filtration of the supernatant. The column-switching technique allowed to carry out the on-line clean-up through an ion excluding column and the simoultaneous chromatographic separation and determination of calcium by two ion exchange columns. The authors judged the method as simple and reliable in terms of analytical performance. However, results for calcium content were not compared with those of other spectrometric methods.

4 Conclusions

Routine analysis, product screening, fundamental research, suitability of processing conditions as well as compliance with declared nutritional labels are purposes making the determination of calcium in milk, dairy products and MIF a topic of primary interest. Most of research efforts are addressed to improve sample preparation, which, in case of complex matrices such as milk and derivatives, represents the bottleneck of these methods. In particular, the target is the minimization of pre-treatment steps and the development of automation and mechanization of techniques in view of further improve analytical performances. Several analytical techniques are available, including potentiometry by ISE being cost-efficient and time-saving. FAAS is a well-established tool also due to the relatively low costs of instruments and their maintenance. Anyway, the sequential mode of operation has led laboratories to substitute FAAS apparatus with ICP-based techniques. Currently, the appeal of other approaches is triggering more and more attention due to a variety of factors including, among the others, instrumental innovations, cost reduction and the improvement of performances following combination with chemometric methods. In this scenery, XRF and LIBS are demonstrating their effectiveness to fulfill requirements of dairy industry and MIF manufacturers. These techniques might become more prevalent in use in the coming years for *in situ* determination of calcium and other minerals.

Funding sources

The preparation of this article was not subjected to any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

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Figure Captions

Fig. 1

Selection criteria for the choice of the analytical technique for calcium determination in bovine milk, dairy products and milk-based infant formulas.

Fig. 2

Main steps of analytical methods for the determination of calcium in bovine milk, daiy products and milk-based infant formulas.

Table 1 – Calcium levels in bovine milk, dairy products and milk-based infant formulas (mg kg^{-1}) reported in the recent literature.

Sample matrix	Calcium[a]	Samples (n)	Recovery (%)	Reference
Butter	130–190	NS[b]	NS	Food composition table (2020)[c]
Cheese, hard	10700±1900	4	98.6	Bilandžić et al. (2015c)
Cheddar	5710 (540)	4	NS	Manuelian et al. (2017b)
Montasio PDO[d]	6960 (320)	5	NS	Manuelian et al. (2017b)
Grana Padano PDO	8450 (610)	10	NS	Manuelian et al. (2017b)
Parmigiano Reggiano PDO	7950 (500)	4	NS	Manuelian et al. (2017b)
Cheese, semi-hard	6970±910	13	98.6	Bilandžić et al. (2015c)
Maasdam	6600 (620)	7	NS	Manuelian et al. (2017b)
Emmentaler PDO	7710 (850)	10	NS	Manuelian et al. (2017b)
Fontina PDO	6330 (460)	5	NS	Manuelian et al. (2017b)
Provolone PDO	6600 (620)	7	NS	Manuelian et al. (2017b)
Cheese, soft	1350±245	9	98.6	Bilandžić et al. (2015c)
Pasta filata	5450 (580)	3	NS	Manuelian et al. (2017b)
Gorgonzola PDO	3020 (670)	9	NS	Manuelian et al. (2017b)
Mozzarella TSG[e]	3260 (390)	10	NS	Manuelian et al. (2017b)
Cheese whey	349±12	3	101.9	Reykdal et al. (2011)
Cream	676±85	3	101.9	Reykdal et al. (2011)
MIF[f] (0–6 months)	4740–4780	2	99.5	Habib-Ur-Rehman et al. (2012)
	3170–5470	5	>93	da Silva et al. (2013)
MIF (6–12 months)	6390–6430	2	99.5	Habib-Ur-Rehman et al. (2012)
	5520–7840	5	>93	da Silva et al. (2013)
MIF (>12 months)	7650–7880	2	99.5	Habib-Ur-Rehman et al. (2012)
Milk, raw	800–2100	45	98.3	Bilandžić et al. (2015a)
	1262–1275	NS	NS	de la Fuente and Juárez (2015)
	1223–1504	60	NS	Gulati et al. (2018)
	1094–1237	147	NS	Malacarne et al. (2019)
	1078–1922	156	98.3	Zwierzchowski and Ametaj (2019)
	924–1467	93	NS	Franzoi et al. (2019)
Milk, pasteurized	1390±370	16	98.6	Bilandžić et al. (2015c)
	1025–1609	9	98.3	Bilandžić et al. (2015b)
	1066–1314	8	NS	Sezer et al. (2018)
Milk powder, skim	10500–12100	27	104.5	McCarthy et al. (2019)
UHT milk, fortified	1741–1772	NS	NS	de la Fuente and Juárez (2015)
Yoghurt	1027–1351	9	98.3	Bilandžić et al. (2015b)
	796–1270	72	100.8	Luis et al. (2015)
	1317–1570	NS	NS	de la Fuente and Juárez (2015)
	1080±110	36	100.8	Luis et al. (2015)
	957±1259	63	97.0	Khan et al. (2014)
	1290–1550	4	116.3	Souza et al. (2018)
Yoghurt, flavoured	952±70	36	100.8	Luis et al. (2015)
	971±1304	71	97.0	Khan et al. (2014)
Yoghurt, fortified	1793–2310	NS	NS	de la Fuente and Juárez (2015)

[a]: calcium levels expressed as min – max, mean ± standard deviation or mean (standard error of the mean)
[b]: not specified
[c]: available at https://frida.fooddata.dk/food/lists/grouped?lang=en; accessed January, 2020
[d]: protected designation of origin
[e]: traditional specialties guaranteed
[f]: milk-based infant formula

Table 2 – Official methods for calcium analysis in bovine milk, dairy products and milk-based infant formulas.

Official method	Sample preparation	Technique	Validated matrix	Codex type or status type[a]	Reference
ISO 12081 | IDF 119	Extraction	Titrimetry	Milk	Second edition	ISO 12081 | IDF 36 (2010)
AOAC 985.35	Ashing	FAAS[b]	Whey-based formula, casein-based enteral formula, whey powder	Status III[c]	Poitevin (2016)
GB 5413.21 part 1 (China)	Ashing	FAAS	Milk, dairy products, infant formula	Adopted 2010	China Standard (2010)
EN 15505	MAWD[d]	FAAS	Food (Cheese)	Approved 2008	EN 15505 (2008)
ISO 8070 | IDF 119	Ashing, MAWD	FAAS	Butter, buttermilk, casein, caseinate, cheese, cream, infant formula, milk, whey, yogurt	Status III Second edition	ISO 8070 | IDF 119 (2007)
AOAC 984.27	Acid digestion	ICP-AES[e]	Whey-based formula	Status III	Poitevin (2016)
GB 5413.21 part 2 (China)	Ashing	ICP-AES	Milk, dairy products, infant formula	Adopted in 2010	China Standard (2010)
AOAC 2011.14	MAWD	ICP-AES	Fortified food products	Final action 2013	Poitevin (2016)
ISO 15151 | IDF 229	MAWD	ICP-AES	Butter, cheese, dietetic milk powder, infant formula, milk, milk powder, whey powder, whey protein concentrate	First edition	ISO 15151 | IDF 229 (2018)
EN 16943	MAWD	ICP-OES[e]	Milk, infant formula	Approved 2017	EN 16943 (2017)
AOAC 2015.06 | ISO21424 | IDF243	MAWD	ICP-MS[f]	Butter, cheese, infant formula, milk, milk powder, whey powder, whey protein concentrate	Status II[g]. First edition	ISO 21424 | IDF 243 (2018)

[a]: status type according to FAO/WHO

[b]: flame atomic absorption spectrometry

[c]: method used for control, inspection or regulatory purposes

[d]: microwave assisted wet digestion

[e]: inductively-coupled plasma (optical)atomic emission spectrometry

[f]: inductively-coupled plasma mass spectrometry

[g]: reference method (selected from Type III methods) and recommended for use in cases of dispute and for calibration purposes

Table 3 – Pros and cons of major techniques adopted for calcium determination in bovine milk, dairy products and milk-based infant formulas.

Technique	Pros	Cons	Reference
Calcium ion selective electrode	- Direct analysis - Ease of use and low cost - Short response time (tens of s) - No sample preparation - Wide linear dynamic range - Routine application - Continuous monitoring - High sensitivity (limit of detection 10^{-8}–10^{-5} M)	- Single-element technique - Matrix effects, sensor-dependent day-to-day reproducibility and stability	Yáñez-Sedeño and Pingarrón (2015)
Flame atomic absorption spectroscopy	- Fast and less expensive than ICP techniques - Simple to operate - Good analytical performances - Cost-saving in comparison to ICP techniques	- One analyte at a time - Poor sensitivity for trace minerals - Cost of lanthanum as releasing agent - Time required for sample mineralization - Destructive technique	García Alonso et al. (2015)
Inductively-coupled plasma optical emission spectroscopy	- Multi-mineral analysis - Simpler to operate and less expensive than ICP-MS - Low detection limits and high accuracy - Rapid analyses over wide concentration ranges	- Complex matrices can cause blockage of cones, deposition of organic matter in the injector tube of the torch and interferences - Time required for traditional mineralization of sample - Destructive technique	de la Fuente and Juárez (2015)
Microwave-induced plasma optical emission spectroscopy	- Wider linear dynamic range, lower detection limits and faster analyses in comparison to FAAS - Multi-mineral technique - Simpler spectra than ICP-OES - Nitrogen-based plasma (green technology) - Reduced running costs in comparison to ICP-OES - No flammable or toxic gases - Lower consumable costs in comparison to FAAS and ICP-OES	- Time required for sample mineralization - Destructive technique	Poitevin (2016)
Inductively-coupled plasma mass spectrometry	- Fast multi-mineral analysis - Ultra-trace detection capability - Steady improvements in cost reduction and simpler use - Higher sensitivity in comparison to ICP-OES	- High cost of analysis - Blockage of cones, deposition of organic matter in the injector tube of the torch and interferences - Time required for sample mineralization - Destructive technique	Poitevin (2016)

Technique	Pros	Cons	Reference
Instrumental neutron activation analysis	- Direct analysis - Multi-mineral technique - No sample pre-treatment - No contamination from reagents - Sensitive technique - Analysis of reference standard materials - Non-destructive technique	- Necessity of a nuclear reactor and a radiochemical laboratory - Long turn-around time - Labour intensive - High cost of analysis	Potočnik et al. (2016)
X-ray fluorescence spectroscopy	- Direct analysis - Multi-mineral technique - Rapid screening measure (1 min) - Minimal sample preparation - High sample throughputs - Accurate quantitation - At-line measures - Ease of use - Non-destructive technique	- Cost of instrument - Poor sensitivity for determination of trace elements (ED-XRF) - Dependence to reference methods for equipment and method calibration, validation and monitoring	Pashkova, et al. (2018); Rossmann, et al. (2016)
Laser-induced breakdown spectroscopy	- Real-time technique - Multi-mineral technique - Rapid measure (1–3 min) - Environmental-friendly technique - Minimal sample preparation - Ease of use and safe for the operator - Lower cost in comparison to other OES methods - At-line measures	- Difficulty of plasma formation and decrease in atomic emission intensity in liquid samples - Low sensitivity and repeatability with liquid samples - Matrix-dependent interaction between laser and sample (matrix-effect) - Reduced sensitivity and accuracy by laser source signal fluctuation - Destructive technique	Cama-Moncunill et al. (2017); Cama-Moncunill et al (2020); Sezer et al. (2018)

Table 4 – Analytical performances of major techniques for the determination of calcium in bovine milk, dairy products and milk-based infant formulas reported in the literature (2010–2019).

Sample matrix	Technique	Reagents	Target analyte	Reference material for calibration	Precision (%)	Recovery (%) or performance parameter	LOD[a] (mg kg^{-1})	Reference
Milk powder and infant formulas	WD-XRF	None	Multi-analyte	Milk powder (IAEA 153)	NS[b]	99.6	NS	Habib-Ur-Rehman et al. (2012)
Milk-based formula	ED-XRF	None	Multi-analyte	Milk powder (IAEA 153)	RSD_{Ca}^{c} = 4.9	NS	LOQ[d] = 820	Papachristodoulou et al. (2018)
Milk powder	ED-XRF (handheld)	None	Ca	Milk powder + $CaCO_3$	RSD = 0.6	99.3	NS	Chan and Palmer (2013)
Milk	ED-XRF INAA	None	Multi-analyte	SMP[e] (ERM-BD 150 and ERM-BD 151)	RSD_{multi} = 1–10	$ED\text{-}XRF_{150}$ = 93.5 $INAA_{150}$ = 97.0 $ED\text{-}XRF_{151}$ = 95.0 $INAA_{151}$ = 95.7	NS	Potočnik et al. (2016)
Milk-based formula	ED-XRF	None	Multi-analyte	SMP (ERM-BD 151)	RSD_{Ca} = 2.6	Declared acceptable	11	Rossmann et al. (2016)
Milk and foods	ED-XRF	None	Multi-analyte	SMP (ERM-BD 150)	NS	$z\text{-}score_{Ca}$ = -0.45	LOQ = 118	Fiamegos et al. (2018)
SMP[e]	ED-XRF vs ICP-MS	None	Multi-analyte	SMP samples (n = 27)	NS	104.5	NS	McCarthy et al. (2019)
Milk and dairy products	FAAS	HNO_3, lanthanum	Multi-analyte	SMP (BCR 063R)	CV_r [f] < 5.6; CV_R < 7.5	96.4	12.5	Chekri et al. (2010)
Milk fermented products	FAAS	HNO_3, lanthanum	Multi-analyte	SMP (BCR 063R)	RSD_{Ca} = 4.1	97.9	0.1 mg L^{-1}	Navarro-Alarcón et al. (2011)
MIF[g], SMP	ICP-OES	HNO_3	Ca, K, Mg	SMP (BCR 063R)	RSD_{Ca} = 4.0	108	NS	Lei et al. (2011)
MIF	ICP-OES	HNO_3	Multi-analyte	SMP (BCR 151 and NIST 1549)	RSD_{multi} = < 10	>93	NS	da Silva et al. (2013)
Yoghurt	ICP-OES	HNO_3	Multi-analyte	SMP (NIST 1549)	NS	100.8	0.58 mg L^{-1}	Luis et al. (2015)
Yoghurt	ICP-OES	HNO_3, H_2O_2	Multi-analyte	SMP NIST 1549	RSD_{Ca} = 0.6	116.3	5.2	Souza et al. (2018)
MIF powdered	LIBS vs FAAS	None	Ca	MIF (n = 45)	RSD = 13.6	R^2 [h] = 0.85 RMSE[h] = 680 mg kg^{-1}	NS	Cama-Moncunill et al. (2017)
MIF liquid	LIBS vs FAAS	None	Ca	MIF (n = 45)	RSD = 4.5 to 15 RSD_{FAAS} = 2 to 5	R^2 = 0.91 RMSE = 64.5 mg L^{-1}	NS	Cama-Moncunill et al. (2020)
Milk	MIRS vs ICP-OES	None	Multi-analyte	Milk	NS	R^2 = 0.79 RPD[i] = 2.16 RMSE = 64.5 mg L^{-1}	NS	Franzoi et al. (2019)

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[a]: limit of detection

[b]: not specified

[c]: relative standard deviation

[d]: limit of quantification

[e]: skim milk powder

[f]: CV_r: coefficient of variation of repeatability; CV_R: coefficient of variation of reproducibility

[g]: milk-based infant formula

[h]: partial least squares regression-model performance in terms of predictive R^2 (coefficient of determination) and RMSE (root-mean-square error) values for calibration

[i]: ratio performance deviation in cross-validation

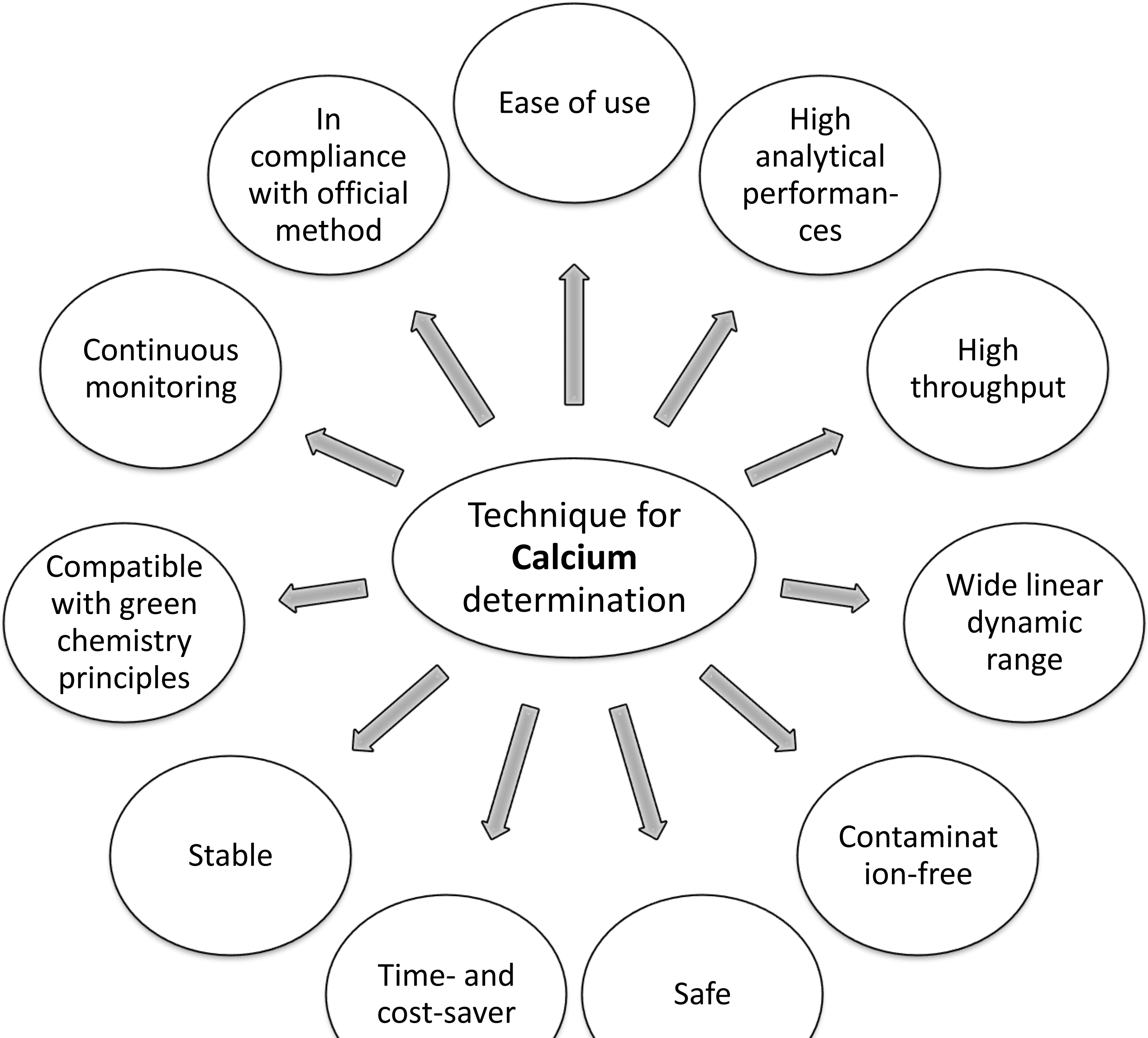
Ease of use
High analytical performan-ces
High throughput
Wide linear dynamic range
Contaminat ion-free
Safe
Time- and cost-saver
Stable
Compatible with green chemistry principles
Continuous monitoring
In compliance with official method
Technique for **Calcium** determination

Fig. 1

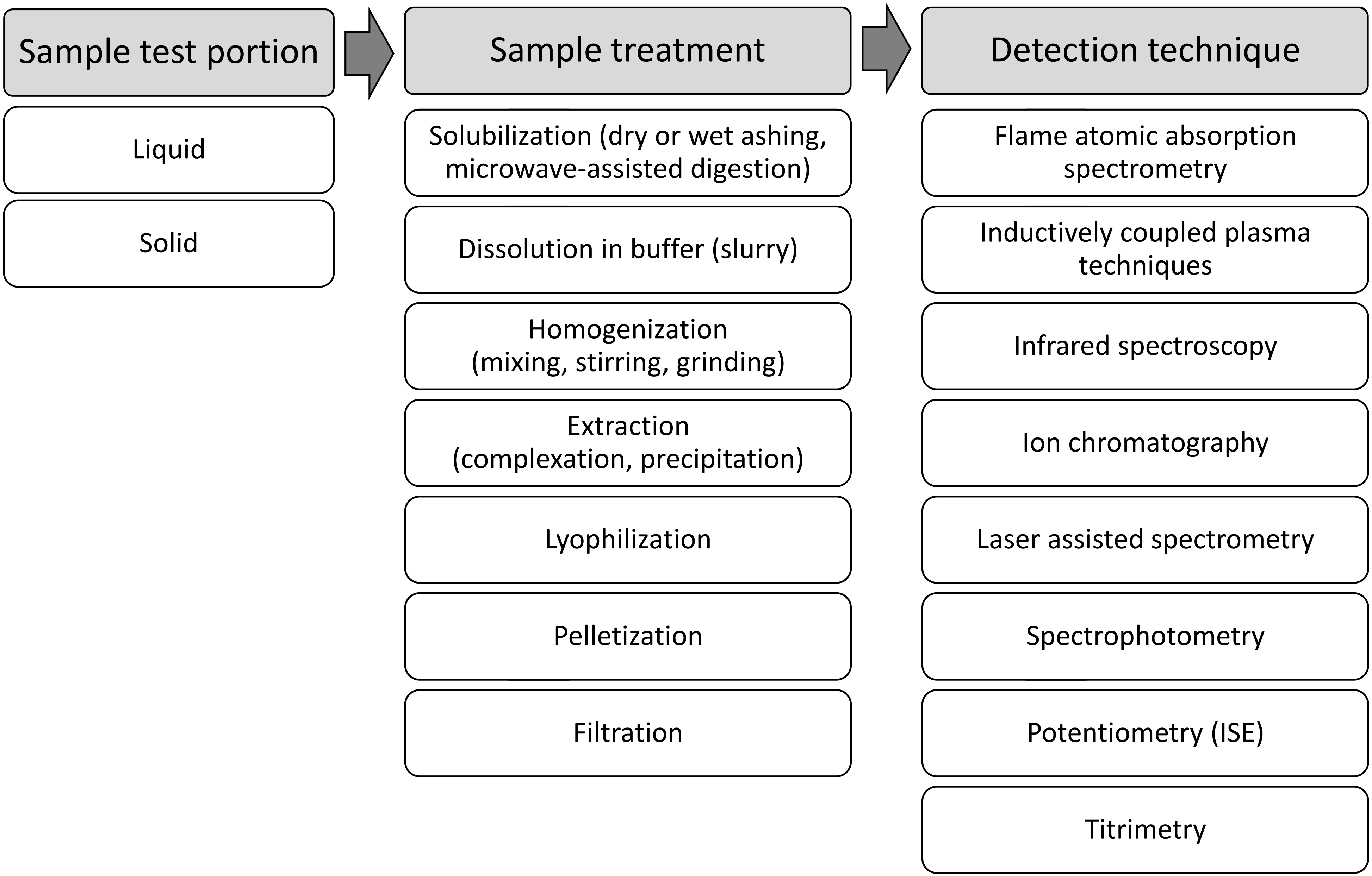
Sample test portion
Liquid
Solid
Sample treatment
Solubilization (dry or wet ashing, microwave-assisted digestion)
Dissolution in buffer (slurry)
Homogenization (mixing, stirring, grinding)
Extraction (complexation, precipitation)
Lyophilization
Pelletization
Filtration
Detection technique
Flame atomic absorption spectrometry
Inductively coupled plasma techniques
Infrared spectroscopy
Ion chromatography
Laser assisted spectrometry
Spectrophotometry
Potentiometry (ISE)
Titrimetry

Fig. 2