WHITE PAPER

Robust multiparameter analysis of infant and follow-on formulas with ion chromatography (IC)

Breast feeding infants is and has always been the most natural and healthy way for their proper nourishment. Nevertheless, there are situations and circumstances where nature needs some help with either a supplement of or complete replacement by artificially produced infant and/or toddler formulas. The special nutritional mixtures must fit the babies' needs and also adhere to very strict quality guidelines which are defined by a variety of local and global regulations.

Most of these regulations lean strongly on the Codex Alimentarius (Codex). As a global alliance, the Codex commission adopts guidelines, standards, and codes of practice to ensure food safety, quality, and fair production lines. In 2020, the European Union (EU) began to mandate that any infant formula produced and sold in the EU must comply with EU regulation 2016/127 (supplementing the active EU regulation 609/2013). Aside from setting minimum and maximum limits for several nutrients (minerals and vitamins), this regulation also gives requirements for contaminants as well as for food labelling.

Ion chromatography (IC) is an economical and robust analytical technique that allows public authorities, analytical laboratories, and manufacturers to assess and fulfill these criteria. This flexible analysis technique offers a large range of applications to ensure a high level of quality and safety for infant food.

Metrohm

BACKGROUND

Infants grow up very fast and consequentially have a high demand for energy and food compared to their body weight. For optimal growth and development, they need an adequate nutritional food source with a high importance on the absorbed nutrients [**1–3**]. Breast milk is the most natural and healthy diet for newborns; nevertheless there is a growing market demand for infant nutritional products. In 2020 the global market for infant formula and baby food was valued at approximately 56 billion USD and is expected to reach nearly 79 billion USD by 2026 [**4**]

This trend towards using baby and toddler formulas is seen especially in Asian countries and in Europe (**Figure 1**). These products must meet strict regulations regarding their nutritional composition and quality [**1,5,6**].

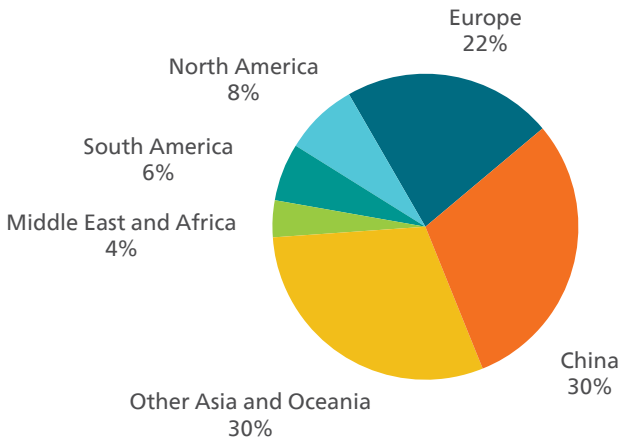


Figure 1. Infant formula consumption for different regions (adapted from [7]).

Quality and nutritional guidelines for infant and follow-on formulas are based on local and global regulation bodies. To fulfill the quality requirements on nutrients and contaminants—on the manufacturer and product development side as well as legislative—robust analytical methods are key.

Metrohm offers powerful, reliable, and flexible ion chromatographic (IC) techniques to ensure simple and effective routine analysis of nutritional products for babies. A wide selection of analytes can be determined with high accuracy (**Annex 1**).

NORMS, STANDARDS, AND REGULATIONS

The Codex Alimentarius (or the «Food Code»), is the major international source of non-binding food standards [**8–10**]. It provides a collection of globally accepted standards, guidelines, and codes of practice to protect consumer health and ensure a fair food trade. The Codex Alimentarius Commission (CAC) is a central part of the Joint Food Standards Program of FAO (Food and Agriculture Organization of the United Nations) and WHO (World Health Organization), and is currently comprised of 189 Codex members from 188 member countries and the European Union [**10**].

A summary of Codex texts guiding the quality and labelling for infant formula (i.e., for babies not older than 12 months) and food products for young children (between one and three years when complementary feeding is introduced, i.e., follow-on or toddler formula) is given in **Annex 2**. In particular, Codex standards CXS 72-1981 and CXS 74-1981 specify the compositional, quality, and safety requirements for infant formulas and cereal-based foods for infants and young children. CXG 10-1979 and CXG 8-1991 give general nutritional advisories for infants and young children (a glossary for related terminologies can be found in directive 2006/141/EC). In the EU, infant formula and follow-on nutritional sources are regulated as special food for particular uses under the regulation (EU) No 609/2013 and the supplementing regulation (EU) 2016/128 (**Annex 2**).

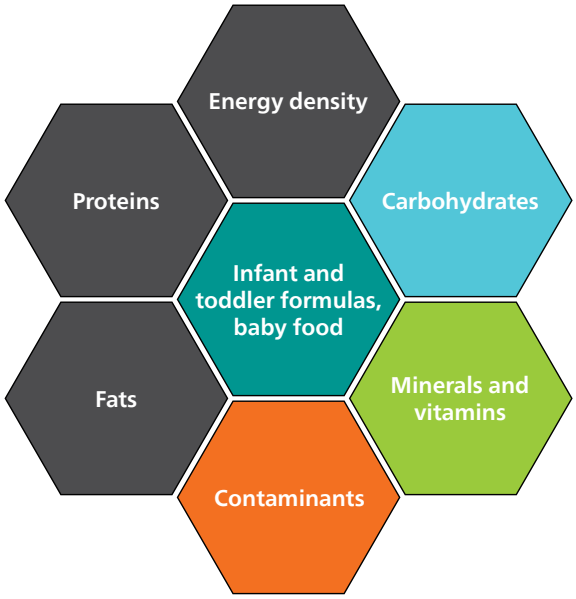
Internationally relevant regulation bodies including FDA/WHO [**10**], AOAC (Association of Official Agricultural Chemists) [**11**], ICC (International Association for Cereal Science and Technology) [**12**], and ISO (International Organization for Standardization) [**13**] provide a wide selection of recommended analytical procedures most often specified by national and local organizations, (e.g., China, Australia – **Annex 2**).

QUALITY AND COMPOSITION OF INFANT AND FOLLOW-ON FORMULAS

Several regulations and guidelines define the ingredients and their allowable quantities that may or must be present in infant formulas and baby food. **Figure 2** gives an overview of recommended intakes based on scientific research and the trends in daily diets of infants and young children [**1**].

Proteins, fat, and carbohydrates are declared as major components with required minimum contents of 1.8, 4.4, and 9.0 g/100 kcal, respectively (CXS 72-1981). Vitamins, minerals, and other supplemental components are also regulated by this standard. For example, vitamins A, D, and B1 (thiamin), should be present at levels above a minimum of 60, 1, and 50 µg/100 kcal, respectively. Essential minerals and trace elements including (but not limited to) iron (Fe, >0.45 mg/100 kcal), chloride (Cl^-, 50–160 mg/100 kcal), calcium (Ca^{2+}, >50 mg/100 kcal), sodium (Na^+, >20 mg/100 kcal), and iodine (I, >10µg/100 kcal), as well as supplements like choline and myo-inositol (>7 and 4 mg/100 kcal, respectively) are also listed in this Codex to guarantee comprehensive nutrition in each serving of formula.

Aside from these, there are also many other optional ingredients and food additives (CXG 10-1979, CXS 1992-1995) in addition to contaminants (CXS 193-1995, EU 1881-2006) which must be monitored in infant formulas such as melamine or nitrite (NO_2^-).



- Lactose
- Sucrose, glucose, fructose, maltose
- Sugar alcohols
- Non-digestible carbohydrates (e.g., fructan, FOS, GOS)
- Maltodextrin, inulin, starch

- K^+, Ca^{2+}, Mg^{2+}, Na^+
- PO_4^{3-}, Cl^-, F^-, I
- Fe, Zn, Cu, Mn, Se, Cr
- Vitamins: C, A, D, E, K, thiamine (B1), B6, B12, riboflavin, niacin, biotin, folate, etc.
- Others: Choline, inositol, etc.

- Melamine, cyanuric acid
- Nitrite (NO_2^-), nitrate (NO_3^-), acrylamide, perchlorate (ClO_4^-)
- Furan, PAHs
- Metal(loid)s: As, Cd, Pb, Hg, etc.
- Pathogens (e.g. bacteria or fungae)
- Veterinary drugs, pesticides (e.g. glyphosate)

Min–max recommended intakes for macro- and micronutrients	Per $kg^{-1} \cdot day^{-1}$
Fluid (mL)	135–200
Energy (kcal)	110–135
Protein, <1 kg body weight (g)	4.0–4.5
Protein, 1–1.8 kg body weight (g)	3.5–4.0
Lipids (g)	4.8–6.6
Carbohydrates (g)	11.6–13.2
Sodium, Na^+ (mg)	69–115
Potassium, K^+ (mg)	66–132
Chloride, Cl^- (mg)	105–177
Calcium (Ca^{2+}) salt (mg)	120–140
Phosphate, PO_4^{3-} (mg)	60–90
Magnesium, Mg^{2+} (mg)	8–15
Iron, Fe (mg)	2–3
Zinc, Zn (mg) (Zn:Cu <20)	1.1–2.0
Copper, Cu (µg)	100–132
Selenium, Se (µg)	5–10
Manganese, Mn (µg)	≤ 27.5
Fluoride, F^- (µg)	1.5–60
Iodine, I (µg)	11–55
Chromium, Cr (ng)	30–1230
Molybdenum, Mo (µg)	0.3–5
Thiamin (µg)	140–300
Riboflavin (µg)	200–400
Niacin (µg)	380–5500
Pantothenic acid (mg)	0.33–2.1
Pyridoxine (µg)	45–300
Cobalamin (µg)	0.1–0.77
Folic acid (µg)	35–100
L-ascorbic acid (mg)	11–46
Biotin (µg)	1.7–16.5
Vitamins, e.g.,vitamin E (mg)	2.2–11
Choline (mg)	8–55
Inositol (mg)	4.4–53

Figure 2. Infant formula composition and scientifically recommended macro- and micronutrient intake values (adapted from [**1**,**2**]). Codex standards with further details for nutritional advisories for infants and young children can be found in **Annex 2**.

ANALYSIS OF INFANT FORMULAS AND BABY FOOD

A selection of analytical methods for the analysis of critical quality parameters in infant formulas and baby food are compiled in CXS 234-1999. This list includes standardized methods from several organizations (AOAC, ISO, ICC, and EN) that differ in complexity, sensitivity, and price.

Ion chromatography (IC) is a state-of-the-art technique that provides straightforward information about samples at an affordable price. This easy-to-use technique offers users high analytical quality and efficiency for a variety of analytes. A wide range of IC applications is available for infant and baby food analysis—in particular, ionic components such as inorganic cations and anions can be resolved, as well as simple and complex carbohydrates (**Annex 1**). With the inclusion of **automation** and Metrohm Inline Sample Preparation Techniques (**MISP**), these analytical methods become even more profitable and accurate as the workload and errors due to manual laboratory work are reduced to a minimum.

In addition to the portfolio of infant product relevant applications presented in **Annex 1**, selected examples of the following components in infant and follow-on formulas will be described in more detail:

- **Carbohydrates** – sugar profiles, lactose, oligosaccharides (GOS, FOS, fructan)
- **Micronutrients** – choline
- **Contaminants** – nitrite, nitrate, melamine

CARBOHYDRATES

As major nutrients, carbohydrates can be divided into two groups: «glycemic carbohydrates» which are digested and absorbed in the small intestine, and «dietary fiber» considered as non-digestible health beneficial substances that influence food pulp viscosity and attenuation of sugar absorption for example, and act as prebiotics for a healthy gut [**1**].

The recommended total carbohydrate value for infants should be around 46–50 E% (energy percent, i.e.,% of total energy intake) with 63–93 g/day [**1**]. Permitted carbohydrates in infant formulas include lactose (a primary sugar found in human breast milk providing approximately 50 E% during the first months of life), maltose, maltodextrins, and glucose, beside pre-cooked and gluten-free starch [**1**]. In general, sucrose (unless needed) and fructose should be avoided in infant formula, due to life-threatening symptoms in case of a fructose intolerance.

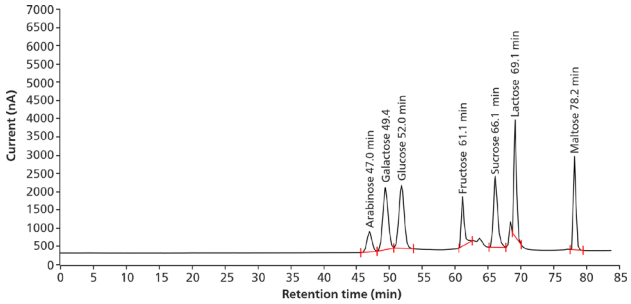
After lactose and fats, the third main component in breast milk that is crucial for optimal growth are the so-called «human milk oligosaccharides» (HMOs), a group of approximately 200 non-digestible compounds that act as prebiotics [**1**]. These HMOs decay into organic acids (e.g., lactic acid) and short-chain fatty acids (e.g., acetic and propionic acid) during digestion, which act as energy sources for colonocytes and stimulate the microbiome [**1,14**]. In general, the artificial addition of oligosaccharides to mimic HMOs is not feasible [**1**]. Nevertheless, prebiotics such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS, not contained in human milk) are added to stimulate the healthy development of infants and were assessed as safe in a mixture of GOS:FOS 90%:10% and a total of ≤ 0.8 g/100 mL in infant and toddler formulas [**1**].

Most carbohydrates are water soluble, weakly acidic, and are thus ideal to be separated and quantified by anion-exchange chromatography [**15–17**]. Almost all applications for carbohydrate analysis can be performed with the **unique sugar column from Metrohm**: the **Metrosep Carb 2** separation column. Several laboratories have already established carbohydrate determination by using **ion chromatography with pulsed amperometric detection (IC-PAD)** and alkaline elution on the Metrosep Carb 2 column as a routine method [**15,16**]. In contrast to enzymatic methods, sensor techniques, or classical high-pressure liquid chromatography (HPLC) methods, **IC-PAD is a sensitive and highly specific method with very low detection levels**. With the high capacity Metrosep Carb 2 anion-exchange column—optimized for complex separations, excellent separation of multiple components like various sugar alcohols, mono-, di-, and oligosaccharides, is possible [**15**]. Adding Metrohm Inline Sample Preparation tools like **Inline Dialysis** for such complex matrices saves time, reduces manual labor steps, and makes this analytical technique even more straightforward and economical for carbohydrate analyses.

– MONO- AND DISACCHARIDES

ISO 22184/IDF 244 describes the quantification of the six most important sugars for food labelling purposes

Standard



NIST 1869 (infant formula, 1:10 dilution)

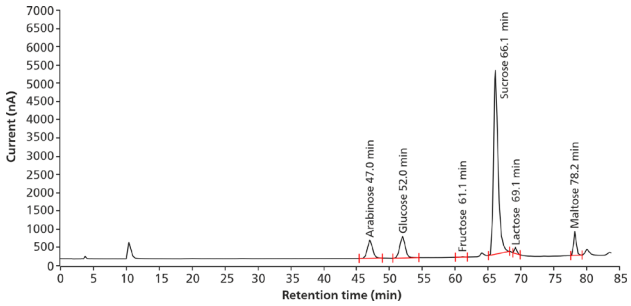


Figure 3. Analysis of six carbohydrates according to ISO 22184/IDF 244 in a standard (0.03 mg/mL arabinose as internal standard, 0.10 mg/mL galactose, glucose, and fructose, and 0.16 mg/mL sucrose, lactose, and maltose) and in a certified infant formula reference (NIST SRM 1869).

(**glucose**, **galactose**, **fructose**, **sucrose**, **lactose**, and **maltose**, **Figure 3**) using high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [**18**]. HPAEC-PAD will be referred to as IC-PAD from here on in this document for simplification, as IC includes high performance anion-exchange chromatography. To improve the precision of the analyses and guarantee full reproducibility of the complex sample preparation, **arabinose** was added as an internal standard (**Figure 3**). The quantification spanned a range of 0.0005–0.4 mg/mL.

A high-pressure gradient (HPG) with post column addition of sodium hydroxide (NaOH) was used for the analysis of a 5 µL injection volume of sample (**Figure 4**). When dealing with highly complex samples and matrices, the gradient elution ensures the appropriate separation—the requirement for high selectivity and high sensitivity of a method. It also functions as a column cleaning step, removing all matrix components properly before the next sample is analyzed. As an alternative to the HPG, the Dose-in gradient has also been approved. The separation of these sugars was achieved on a **Metrosep Carb 2 - 250/4.0** column.

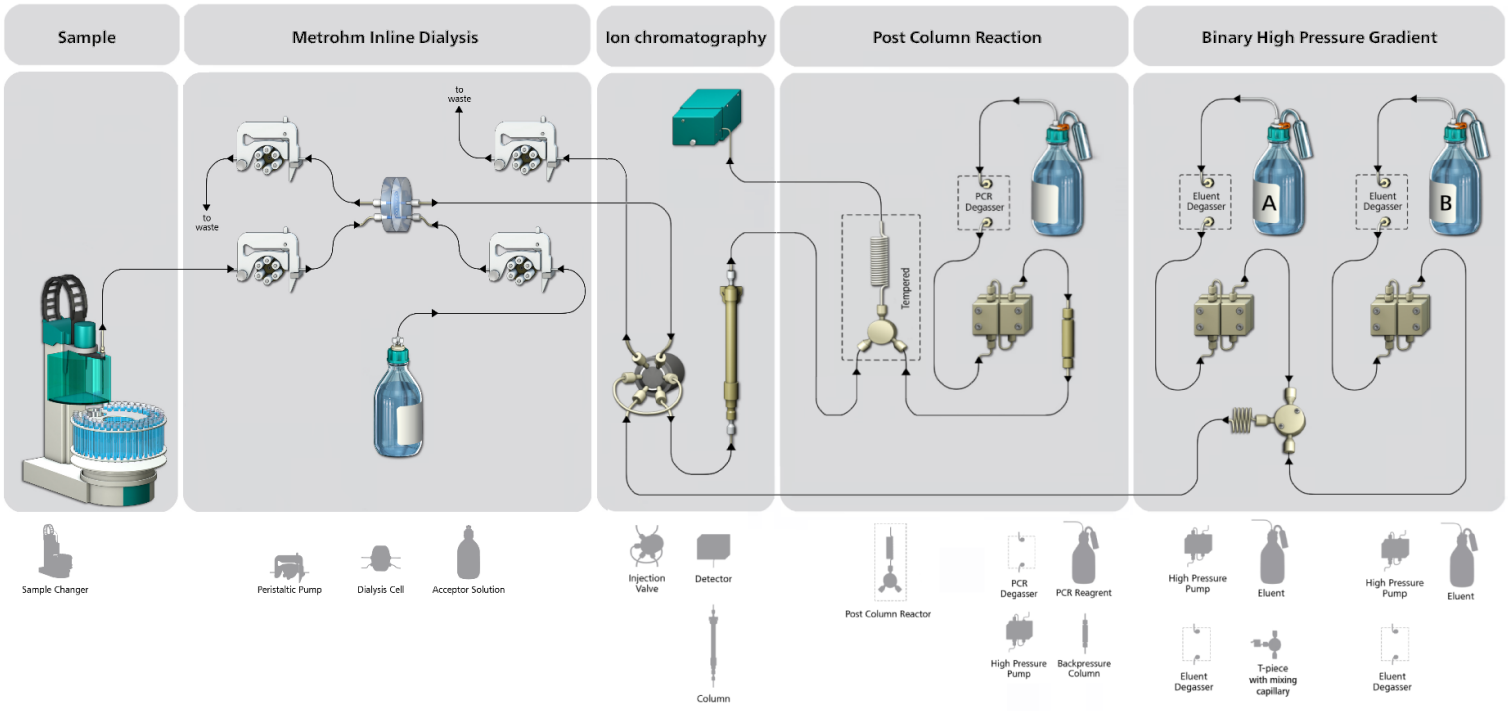


Figure 4. Metrohm IC systems are highly flexible, adapting to completely fulfill any application needs. Requirements for simple but also complex carbohydrate analysis as in ISO 22184/IDF 244 can be fulfilled. The flow path shows the analytical setup for ISO 22184/IDF 244 using a binary HPG for separation and post column reaction (PCR) with NaOH. For Carrez precipitation, the sample is directly injected into the sample loop. Signal recording after separation is performed using PAD. The optional Inline Dialysis option (shown in the second grey box) can be added to any existing instrumentation.

Detection of low concentrations is obligatory for the described norm, and is supported by applying a sweep waveform for optimal sugar oxidation (**Figure 5**). The **Metrohm Thin-Layer cell** with a gold (Au) working electrode and palladium (Pd) reference electrode is the ideal equipment for sugar determination. It is defined by its minimal routine maintenance requirements and long electrode lifetimes.

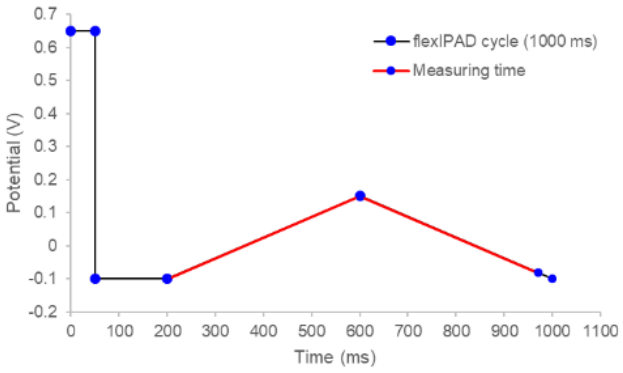


Figure 5. The sweep waveform for carbohydrate oxidation results in the sensitive detection of carbohydrates due to a low noise level.

Sample preparation comprised the described steps for extraction and Carrez precipitation. Metrohm Inline Dialysis is as efficient as Carrez precipitation for sample cleanup, and can be used to replace Carrez precipitation or as an additional column protection step (**Figure 4**). As a fully automated step, no additional time is required, guaranteeing the most straightforward carbohydrate analysis. To prevent the decay of such food matrices, the cooled **Metrohm 889 IC Sample Center** is the best choice for stable samples and more reliable results while maintaining high sample throughput.

The successful participation in a large-scale multilaboratory study (MLT) highlights the applicability, robustness, and accuracy of Metrohm IC-PAD for sugar analysis in infant formulas and other foodstuffs [**18**].

Lactose is the major carbohydrate found in human milk (55–70 g/L) and therefore also a substantial component in infant formulas [**1**]. Analytically, such high concentrations of lactose can be easily determined by the multi-component IC-PAD methods as described in ISO 22184/IDF 244, or shown in **Annex 1**. Nevertheless, infants and young children can also suffer from lactose malabsorption. For this group, a lactose-free diet is crucial, which means formula with a maximum content of <10 mg lactose/100 kcal [**1,19**].

Analyzing such low concentrations of lactose in this matrix is quite challenging. Aside from the correct validity, high analytical sensitivity is mandatory to ensure the claimed accuracy and safety. IC-PAD equipped for **carbohydrate analysis** provides the necessary preconditions for highly sensitive detection—a smooth baseline with a low noise level and a high selectivity with full separation of lactose from any interferences. Details for the validated method to analyze lactose content in a range of 0.2–21,000 mg/100 g are explained in Metrohm Application Note **AN-P-088**.

– OLIGOSACCHARIDES: GOS, FOS, AND FRUCTANS

Quantification of **oligosaccharides** is challenging because their structures are complex and they are often present as mixtures with variable polymerization degrees (DPs), meaning they vary in chain length, linkage, and termination groups, giving characteristic fingerprints [**20**]. IC with PAD is a routine method for the determination of such oligosaccharides—the total concentration as well as their fingerprints. A very detailed description for **total GOS** analysis (galactose units with optional glucose ends, DPs 2–10 [**21**]) in supplements according to AOAC 2001.02 can be found in Ziegler et al. (2021) [**22**]. The method is based on enzymatic hydrolysis of GOS in different matrices and the determination of monosaccharides (i.e., galactose and glucose) as well as lactose before and after the hydrolysis step.

Another approach for this analysis was successfully achieved using a separation column from Hamilton (**Figure 6**). When dealing with milk powders containing a lactose:GOS ratio of >6, the AOAC method becomes imprecise. Quantification using GOS standard materials from the production line of the respective milk powders is one possibility to counter this issue. In a Chinese-sourced milk powder, two peaks were identified as «GOS 1» and «GOS 2» and sufficiently quantified for quality control in the dairy plant (**Figure 6**). Interferences were excluded by spike tests.

A comparable hydrolytic approach to AOAC 2001.02 for total GOS was also performed for **total fructan** analysis (AOAC 2016.14). Fructans are a class of plant-storage carbohydrates comprised of branched and linear chains of fructose monomers with optional glucose ends [**23**].

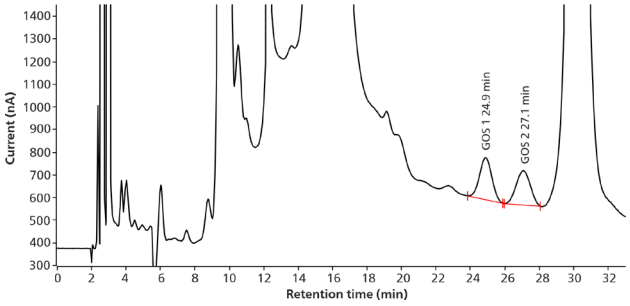


Figure 6. GOS quantification in goat milk powder (GOS 1: 437 ± 2.3 mg/100 g, GOS 2: 548.4 ± 3.5 mg/100 g). Separation was performed with a Hamilton RCX-30 – 250/4.6 column and hydroxide-sodium acetate gradient elution (injection volume 20 µL, flow rate 1.0 mL/min) followed by amperometric detection (IC-PAD).

FOS (short-chain fructans, DPs = 2–9), oligofructose, and inulin, and are of significant economic importance in the food sector as a dietary fiber source for many foods [**23–25**].

While the identification and structural resolution of fructans requires rather complex methods and is hampered by the availability of commercial standards [**26**], several standard methods exist for total fructan quantification (e.g., AOAC 997.08, 999.03, and 997.08). However, these methods lack accuracy due to underestimation from incomplete hydrolysis with colorimetric methods or overestimation from the presence of interfering mono- or disaccharides in complex matrices as infant formulas [**24,27**]. To overcome these issues, an IC-PAD method was developed for total fructan determination in infant and adult/pediatric nutritionals, accepted as the AOAC 2016.14 final action method in 2020 [**27,28**].

The overall capability and robustness of Metrohm IC-PAD to determine total fructan contents in infant formulas was proven extensively during participation in a MLT. The MLT (with over 12 participating laboratories) shows the power and accuracy of IC-PAD for complex carbohydrate analyses even in very challenging matrices.

Fructan concentrations in a range of 0.03–5 g/100 g were determined in different infant formulas (e.g., SRM 1869, ready-to-feed, and powdered formulas) using an enzymatic treatment prior to amperometric detection. According to AOAC 2016.14, the reconstituted and diluted samples were treated with the highly specific sucrase and α-glucanases to hydrolyze the di- and higher order saccharides into monosaccharides. This enzyme mixture does not degrade any fructans. Subsequently, the samples were passed through a graphitized carbon solid phase extraction (SPE) column on which only the fructans were retained while the other carbohydrates passed through. Fructans were released with acetonitrile and hydrolyzed with an inulinase mixture. Finally, the monosaccharides glucose and fructose were analyzed by IC-PAD (**Figure 7**) and the total contents determined as stated in AOAC 2016.14.

Further applications to determine carbohydrates with IC-PAD in infant and follow-on formula, baby food, and milk can be found in **Appendix 1**.

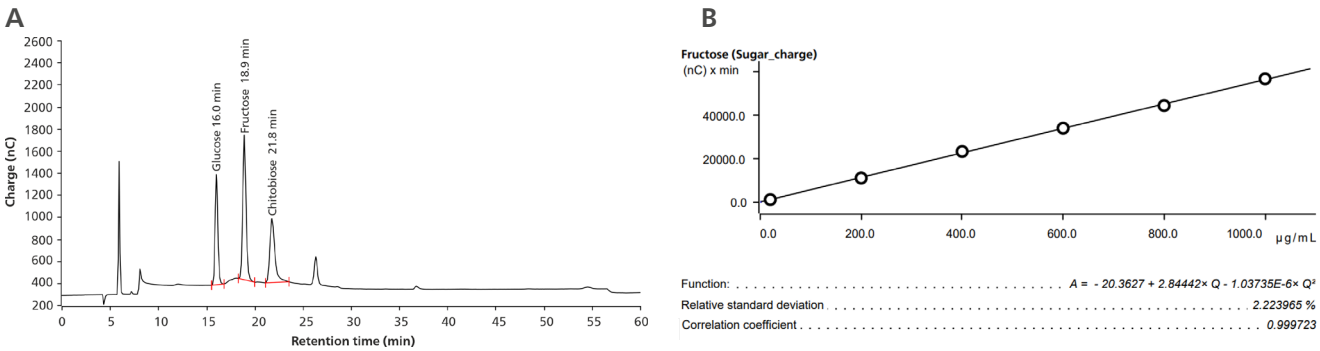


Figure 7. Total fructan determination (injection volume 20 µL) in the certified infant formula powder NIST SRM 1869 (A) within the MLT for AOAC 2016.14 validation [**28**]. The monosaccharide peaks (glucose and fructose) are shown along with the internal standard (chitobiose), after enzymatic treatment of the samples. A Metrosep Carb 2 - 250/4.0 column was used for separation using isocratic elution with NaOH and PAD detection. Quantification was performed following the instructions in AOAC 2016.14—the calibration function is shown for 2–250 µg/mL fructose (B).

MICRONUTRIENTS AND OTHER SPECIFICATIONS

Micronutrients are comprised of vitamins and vitamin-like components (e.g., **choline and inositol**) as well as a range of cationic and anionic minerals, ideal for IC analysis. **Annex 1** gives an overview of IC applications for micronutrients and minerals such as: **calcium** (**Ca^{2+}**), an integral component of the skeleton; phosphorus (determined as **phosphate**, **PO_4^{3-}**), regulating hormones and energy production; **magnesium** (**Mg^{2+}**), a critical cofactor for enzymatic reactions; **sodium** (**Na^{+}**) and **potassium** (**K^{+}**), which control cell membrane potentials for muscle contraction and vascular tones as well as active nutrient transport; **chloride** (**Cl^{-}**), for charge balance in the cells and a major constituent of gastric liquids; iodine (as **iodide**, **I^{-}**, or iodate, **IO_3^{-}**), for normal thyroid gland functioning; and also transition metals and metalloids like **iron** (**Fe**), **zinc** (**Zn**), and **selenium** (**Se**) [**1**]. All of these constituents can be successfully quantified using cation or anion chromatography, either alone or as a combined analysis of cations and anions using a 2-channel Professional IC.

The mineral components are typical IC analytes, while most vitamins are regularly determined by classical HPLC [**29,30**] as shown in a range of norms and standards (e.g., CODEX, AOAC). One vitamin-like mandatory constituent of infant formula that can readily be analyzed with IC is choline [**1**].

Choline is a water-soluble micronutrient vital to cell membrane integrity and is a source of methyl groups for many metabolic and nervous system activities [**31,32**]. It is a phospholipid precursor and is involved in lipid metabolism and transport [**1**]. In human milk, total choline concentration ranges from 160–210 mg/L and is a mandatory ingredient for infant formulas (**Figure 2**) [**1,33**]. Two kinds of choline are present in infant formula powder: choline-containing compounds (e.g., in the forms of lecithin and sphingomyelin) and free choline [**34**].

Analytical determination of choline is performed by IC with conductivity detection, using a **Metrosep C6 - 150/4.0** column (**Figure 8**). Choline elutes within less than 15 minutes with this configuration. In the case that choline determination should occur along with the quantification of other major cations (**Figure 9**), separation can be performed with the **Metrosep C Supp 1 - 150/4.0** column with subsequent conductivity detection after sequential suppression. The baseline noise is reduced to a minimum and detection limits in the lower µg/L range can be achieved. For choline determination with IC, it must be first liberated into its free form as a prerequisite for a proper determination. Therefore, the samples were digested in HCl (5 g sample in 1 mol/L HCl at 70 °C for 3 hours) prior to injection.

Choline analysis with the **Metrosep C 6 - 150/4.0** column was an integral part of the MLT between five laboratories for AOAC 2012.20 and GB 5413.20-2013. The results met the criteria for repeatability, accuracy, and sensitivity as well as robustness required for standardized analytical methods (**Table 1**, **Figure 8**). The validation was performed on nine samples from different manufacturers with choline contents of 50–400 mg/100 g (LOD 0.34 mg/100 g).

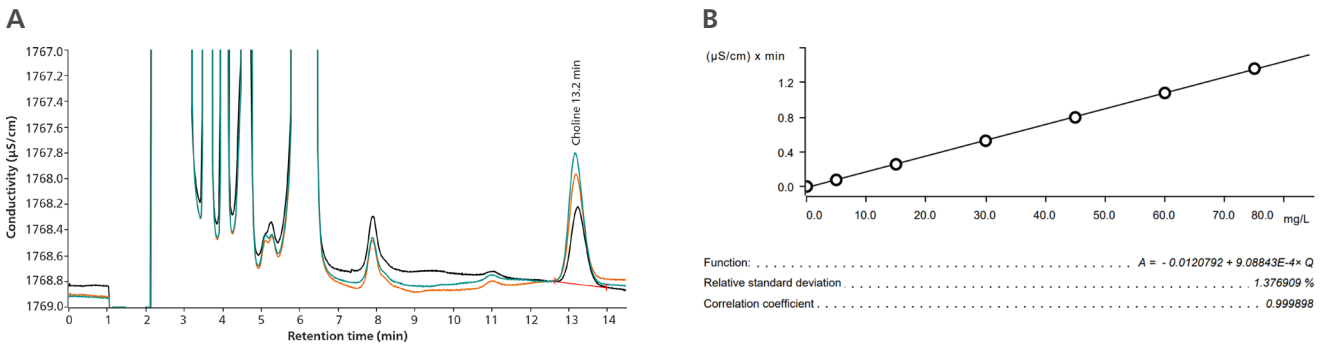
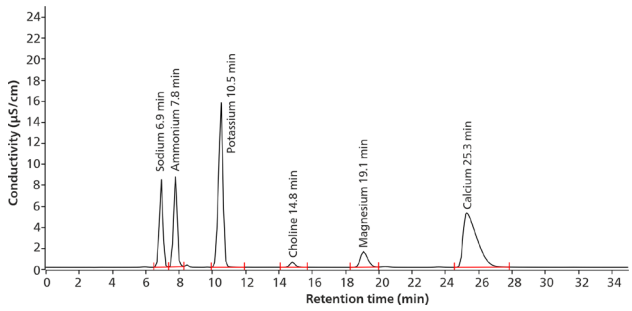


Figure 8. Choline determination in infant formula (Nestle, 73.0 ± 1.7 mg/100 g, n = 6, black) separated on a Metrosep C6 - 150/4.0 column in less than 15 min using a modified nitric acid eluent (flow rate: 0.9 mL/min, column temperature: 30 °C, injection volume: 20 µL) and non-suppressed conductivity detection (A). For validation of AOAC 2012.20 and GB 5413.20-2013, the samples were spiked with 25–100% of the determined concentration (sample: 6.9 mg/L choline (black), spike 1: 9.2 mg/L (orange, recovery 92%), spike 2: 10.7 mg/L, (green, recovery 106%)). Calibration (0–80 mg/L) passed the linearity requirements for the validation (B).



Parameters

Flow rate	1.0 mL/min
P_{max}	15 MPa
Temperature column	40 °C
Recording time	30 min
Injection volume	20 µL
Detection	conductivity
Cell constant	16.7 cm^{-1}
Detector thermostat	40 °C
Temp. coefficient	2.3%/ °C
MSM autostep interval	10 min
MSM Dosino Regeneration*	Volume 5 mL, flow rate 0.5 mL/min
MCS	on

*MSM regeneration with peristaltic pump is also possible.

Figure 9. Choline analysis (infant formula powder: Milupa, 83.3 mg/100 g) next to major cations was resolved within less than 30 min using a Metrosep C Supp 1 - 150/4.0 column (eluent: 4.0 mmol/L HNO_3 and 50 µg/L Rb^+, flow rate: 1.0 mL/min, injection volume: 20 µL) and detected using conductivity after sequential suppression with sodium carbonate/bicarbonate (STREAM mode).

The linear calibration range spanned 1–50 mg/L in solution, allowing dilutions for higher concentrated samples (either done manually or automatically with Metrohm Inline Dilution, which also allows automatic calibration). As the eluent is very stable and the conductivity detection requires no maintenance in general, further automation with the **941 Eluent Production Module** allows 24/7 analysis. A higher sample throughput combined with minimal laboratory costs makes this setup ideal for accurate and precise routine analytics.

Table 1. Validation results for choline determination in infant formula by IC (five laboratories, nine samples) and the AOAC requirements in parentheses.

Experimental results (Requirements AOAC 2012.20)						
Analytical range (mg/100 g)	Range of samples: 50–400 (2–250)					
Limit of detection (LOD) (mg/100 g)	0.3 (0.7)					
Lim.of quantification (LOQ) (mg/100 g)	1.0 (2.0)					
Repeatability (RSD_r) (%)	#1	#2	#3	#4	#5	AOAC
100 mg/100 g	2.29	2.9	0.29	2.26	2.03	≤5
200 mg/100 g	0.77	0.16	1.97	2.95	1.43	≤5
Recovery (%)	92–106 (90–110)					

CONTAMINANTS

Infant formulas are subject to stringent regulations during production regarding the raw materials and final nutritional composition. However, contamination of the raw materials or during the production process can result in the accidental inclusion of harmful chemicals such as pesticide residues, toxic metals, veterinary drugs, or packaging materials [**2,35**].

Contamination pathways can occur through the water used for blending of powdered infant formula or via dairy products—introducing arsenic, lead, or mercury, while aluminum contamination can come from packaging materials [**2**]. Other toxic compounds that can sometimes be found in baby food are nitrogenous chemicals (e.g., **melamine** or **dicyandiamide**), as well as **nitrite** (**NO_2^-**) and **nitrate** (**NO_3^-**). The former is historically associated with contaminated groundwater and the risk of methemoglobinemia (blue-baby syndrome) [**36**]. Nitrite and nitrate can also derive from vegetable intake, such as soy-based infant or pediatric formulas, which have been shown to exceed the WHO acceptable daily intakes (ADI) in some products [**36**]. To guarantee that the ADIs of 0.06 mg/kg body weight (bw) for NO_2^- and 3.7 mg/kg bw for NO_3^- are kept within the legal limits in baby food, IC is the preferred analytical method for reliable quantification (**Annex 1**).

GB 5009.55-2010 describes the procedure for **NO_2^-** and **NO_3^-** determination in milk powder, using a **Metrosep A Supp 5 - 250/4.0** column (combined with the **Metrosep A Supp 10 Guard HC/4.0**). The

peak separation was performed with a carbonate-acetonitrile eluent using a Dose-in gradient with increasing carbonate concentrations (**Figure 10**). The detection was performed using conductivity after sequential suppression.

Nitrite and nitrate were extracted from 1 g sample using UPW (20 mL), acetic acid (1 mL of 3% solution) and ultrasonic extraction, and then made up to a final volume of 50 mL. Centrifugation, filtration, and IC-RP extraction were used to purify the infant formula samples prior injection. Concentrations were determined to be 0.01 mg/kg NO_2^- and 0.37 mg/kg NO_3^- in the tested milk powder samples. Spike recoveries between 90–105% and repeated analysis after 24 hours with relative standard deviations of less than 2.3% reveal that IC is robust and reliable for analysis of the labile NO_2^- and NO_3^- within the recommended 24-hour analysis time.

Additional automation steps like Inline Dialysis or Inline Ultrafiltration could be used to improve the efficiency of the overall method to save time and improve reproducibility. A selection of further applications for nitrate and nitrite determination in several matrices can be found in **Annex 1**.

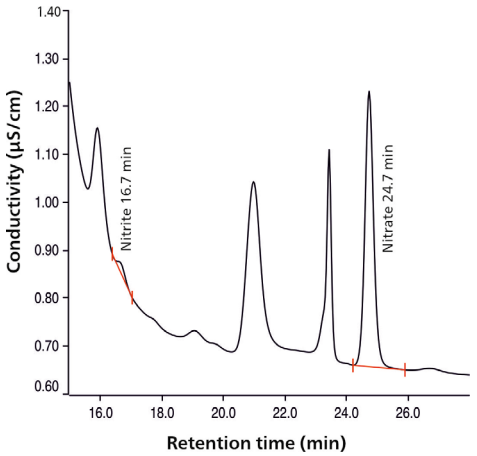
Melamine (1,3,5-triazine-2,4,6-triamine) is an industrially synthesized organic base with the chemical formula $C_3H_6N_6$. It forms a white, crystalline powder, and is only slightly soluble in water [**37**]. Food contaminated with melamine is harmful due to the toxic byproducts found in melamine mixtures (cyanuric acid) as well as through formation of sphere-like crystals that damage the kidneys and can induce kidney failure [**37**]. This poses a serious health risk especially for infants and children.

In 2008, the Chinese food industry was affected by a melamine infant formula scandal. **Melamine** was illegally used to falsely increase the protein content (due to its high nitrogen content) of raw milk [**37**]. Following worldwide concern, legislation and regulatory controls were strengthened. In 2010, ISO published a method for melamine determination with LC-MS/MS (ISO/TS 15495). This method was later overtaken by CODEX and maximum levels of melamine were set at 1 mg/kg product for solid infant formula and 0.15 mg/kg for liquid infant formula (CXS 193-1995, FDA) [**37,38**].

A variety of methods are available to determine melamine as well as structurally related compounds. Hyphenated techniques (e.g., LC-MS/MS) are appealing with low detection limits (LODs 1–10 µg/kg) [**39**], but are rather complex to use. Spectroscopic methods (IR) are simpler and do not require sample pretreatment, but the calibration is matrix-dependent and LODs are around 1 mg/kg [**39**].

IC with UV/VIS detection provides an innovative and sensitive compromise for **melamine** analysis in infant formulas. The overall analytical procedure is straightforward (sample dilution in UPW and acetic acid followed by centrifugation) with a high degree of automation regarding sample injection and inline sample preparation (Metrohm Inline Dialysis) and is sensitive enough to assess minimal melamine residues in infant formulas thanks to intelligent calibration.

The determination of melamine was performed with



Parameters

Column	Metrosep A Supp 5 - 250/4.0
Dose-in gradient	A: 1 mmol/L Na_2CO_3 + 1.0 mmol/L $NaHCO_3$ + 5% acetonitrile B: 40 mmol/L Na_2CO_3 + 5% acetonitrile
Flow rate	0.7 mL/min
Temperature column	30 °C
Recording time	50/35 min (samples/standards)
Injection volume	50 µL
Detection	conductivity
Temperature coefficient	2.3%/ °C
Suppression	MSM + MCS (interval 10 min, STREAM regeneration)

Figure 10. Nitrite and nitrate determination in a milk powder sample extracted in a UPW-acetic acid mix and purified before injection. Chromatograms were recorded using a Metrosep A Supp 5 - 250/4.0 column and suppressed conductivity detection. The calibration range covered 0.005–0.1 mg/L NO_2^- and 0.05–1 mg/L NO_3^-.

a **Metrosep C4 - 150/4.0** column (nitric acid eluent) on a **930 Compact IC Flex** using UV/VIS detection and Metrohm Inline Dialysis to improve the sample preparation (**Figure 11**). Linear calibration showed an excellent fit (R^2 >0.9998), and the spike recoveries for various infant formulas ranged from 95–101%.

Minimal detectable melamine concentrations reached 0.01 mg/L (method detection limit of 0.067 mg/kg)—considerably lower than the maximum allowed contamination levels of 1 mg/kg and 0.15 mg/kg for solid and liquid infant formulas, respectively. The intelligent high-low calibration (low: 0.02–1.00 mg/L, and high: 1–150 mg/L) guarantees high accuracy of the melamine contents determined in samples within a broad concentration range.

Melamine contents of >800 mg/kg were found in two tested Chinese whole milk powders (production date 2007) (**Figure 11**), while no melamine was detected for Similac and Abbott milk powders.

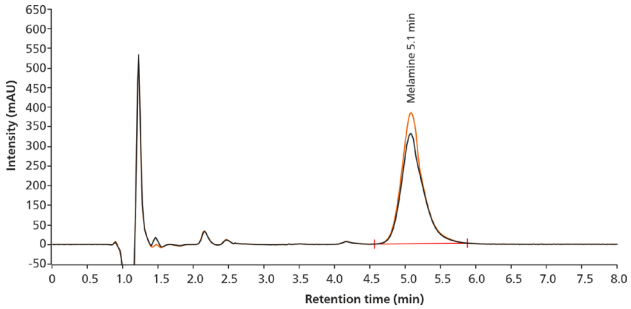


Figure 11. Melamine determination by IC with UV/VIS detection (flow rate: 0.9 mL/min, column temperature: 30 °C, UV/VIS at 240 nm, bunching: 11 nm). The melamine contents for a Chinese brand of whole milk powder (black, 81.4 mg/L and 813.5 mg/kg) exceeded the specified maximum levels for infant formulas by a factor of >800. The spike recovery (spike concentration 10 mg/L, red) yielded 96%.

SUMMARY

Quality control is an essential step during **infant formula production** and is mandatory to guarantee the healthy development of infants and children. Strong regulations and legal requirements are nothing without the adequate analytical techniques necessary to fulfill the legislation.

IC is a reliable, straightforward technique that is highly flexible regarding automation, sample preparation, and detection techniques. Several combinations are possible to fit the exact user needs.

Integration of **automation options** (e.g., autosampler, automatic eluent production, sample cooler, or Metrohm Inline Sample Preparation and intelligent Injection Techniques) supports higher sample throughput, extends sample stability, reduces manual labor and associated errors, and results in higher analytical while keeping the overall price at an acceptable level. This makes IC the ideal choice for contract laboratories with high sample loads, but also for product development laboratories or multifaceted analytics within the product chain.

Validation procedures such as those for norms and standardized methods in accredited laboratories show that the methods meet standardization audits as well as the overall GMP requirements, even if the matrices are as complex as infant and follow-on formulas and other types of baby food.

With the release of one of the newest hyphenated techniques on the market (**TitrIC flex**), these analytics have become even more versatile through the combination of ion chromatography with certain titration parameters which also apply to food laboratories—not only reserved for comprehensive water analytics.

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Annex 1:
Summary of IC applications for quality assurance of infant, child, and toddler formulas

Detection systems: Cond = conductivity detection; UV/VIS = **ultraviolet and visible absorption detection**; PAD = **pulsed amperometric detection**, traditional PAD with 3-step waveform; DC = amperometric detection with direct current; flexiPAD = amperometric detection with flexible potential waveform; PAD sweep = pulsed amperometric detection with sweep waveform

Matrix	IC Column	Detection Method	Analysis range	Sample preparation/MISP
Nutrients and minerals				
Choline (AN-C-100, AN-CS-004, standard: AOAC 2012.20, MLT approved for GB 5413.20-2013)				
Infant formula	Metrosep C Supp 1 - 250/4.0	Cond	0.06–75.0 mg/L	Acid digestion (HCl)
	Metrosep C 6 - 150/4.0		0.5–50 mg/L	
Cations (sodium, ammonium, potassium, calcium, magnesium) (AN-C-028)				
Milk powders, milk	Metrosep C 4 - 150/4.0	Cond	2–14 mg/L Na^+, NH_4^+, K^+, Ca^{2+}, Mg^{2+}	Acid digestion (H_2SO_4), Inline Dialysis
Milk	Metrosep C 4 - 250/4.0		0.5–20 mg/L Na^+, K^+, Ca^{2+} 0.005–0.1 mg/L NH_4^+ 0.1–2 mg/L Mg^{2+}	Inline Dilution, Inline Dialysis
Iodide (AN-S-162)				
Milk powder	Metrosep A Supp 5 - 150/4.0	Cond	100–300 µg/L I^-	Inline Dialysis low volume
Milk	Metrosep A Supp 17 - 150/4.0	DC	2–50 µg/L I^-	
Iodide, thiocyanate, perchlorate (AN-S-297)				
Milk	Metrosep A Supp 17 - 100/4.0	Cond	10–800 µg/L I^-, $[SCN]^-$, ClO_4^-	Manual dilution, Carrez precipitation, Inline Dialysis
Anions (fluoride, chloride, nitrite, nitrate, phosphate, sulfate, and citrate) (TA-008)				
Milk powder	Metrosep A Supp 5 - 250/4.0 Dose-in gradient	Cond	0.05–25 mg/L Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, citrate	Manual dilution, Inline Dialysis
Milk	Metrosep A Supp 5 - 100/4.0		0.5–7 mg/L F^-, NO_2^-, NO_3^-, Br^-, SO_4^{2-} 5–40 mg/L Cl^-, PO_4^{3-}	
Infant formula	Metrosep A Supp 5 - 150/4.0		1–50 mg/L Cl^-, SO_4^{2-} 0.01–0.5 mg/L I^- 0.002–0.1 mg/L Br^-	CIC
Anions (bromide, chlorate)				
Coconut milk	Metrosep A Supp 7 - 250/4.0	Cond	0.5–2 mg/L Br^- 0.05–0.2 mg/L ClO_3^-	Carrez precipitation, Inline Dialysis
Anions (nitrite, nitrate) (standard: GB5009.55-2010)				
Infant formula, milk powder	Metrosep A Supp 5 - 250/4.0, Metrosep A Supp 10 Guard HC/4.0	Cond	0.005–0.1 mg/L NO_2^- 0.05–1.0 mg/L NO_3^-	Acetic acid/acetonitrile extract, (RP column), filtered 0.2 µm
	Metrosep A Supp 16 Guard/4.0 + Metrosep A Supp 17 - 250/4.0, Gradient			
Milk, milk powder	Metrosep A Supp 7 - 250/4.0 Dose-in gradient	UV/VIS	0.02–5 mg/L NO_2^-, NO_3^-	UPW extraction (40 °C), Inline Dialysis
Prebiotics				
Total GOS (AN-P-087, Ziegler et al. 2020 [22], standard: AOAC 2001.02)				
(Goat) milk powder	Metrosep Carb 2 - 250/4.0 or Hamilton RCX-30 - 250/4.6, gradient	PAD	4.46–133.84 mg/L total GOS	UPW extraction in heated water bath
Raw GOS material	Metrosep Carb 2 - 250/4.0	PAD sweep	2–32 mg/L glucose, galactose, lactose	Extraction and hydrolysis according to AOAC 2001.02, Inline Dialysis low volume

Matrix	IC Column	Detection Method	Analysis range	Sample preparation/MISP
Fructan (standard: AOAC 2016.14, MLT approved [30])				
Infant formula	Metrosep Carb 2 - 250/4.0	flexiPAD	2–250 mg/L glucose 20–1000 mg/L fructose	Extraction, hydrolysis, Carrez precipitation according to AOAC 2016.14
Fructosan (standard: GB5009.255-2016)				
Infant formula, milk powder	Hamilton RCX-30 - 250/4.6	PAD	0.8–16 mg/L fructose	Extraction and enzymatic hydrolysis according to GB5009.255-2016
FOS – total FOS and fingerprints				
Infant formula, raw FOS materials	Metrosep Carb 2 - 250/2.0, Gradient	PAD sweep IC-MS	FOS (total FOS, kestose, nystose, kestopentaose)	Extraction and hydrolysis of GOS according to AOAC 2001.02
Carbohydrates				
Lactose (AN-P-055, AN-P-088)				
Infant formula, milk (low lactose/lactose-free)	Metrosep Carb 2 - 150/4.0 or Metrosep Carb 2 - 250/4.0	PAD or PAD sweep	0.05–80 mg/L lactose	Carrez precipitation or Inline Dialysis
Raffinose (standard: GB 5009.258-2016)				
Milk powder	Metrosep Carb 2 - 150/4.0	PAD	0.5–25 mg/L raffinose	Ultrapure water extract (45–50 °C), centrifugation and extraction column
Inositol				
Milk powder	Metrosep Carb 2 - 150/4.0	PAD	0.025–10.0 mg/L inositol	Acid extraction (HCl), filtration (0.22 µm)
Carbohydrates, sugar alcohols				
Galactose, glucose, lactose, lactulose, fructose, sucrose, maltose (Kappes et al. 2016 [15])				
Milk	Metrosep Carb 2 - 150/4.0 and Metrosep Carb 2 - 250/4.0 in series	PAD	0.25–60 mg/L	Manual dilution, Inline Dialysis
Sorbitol, xylitol, mannitol, glucose, fructose, maltitol, lactulose, lactose, sucrose, maltose				
Milk (lactose-free)	Metrosep Carb 2 - 250/4.0	PAD	0.1–100 mg/L	Manual dilution and filtration
Arabinose, galactose, glucose, fructose, sucrose, lactose, maltose, fucose, trehalose, melibiose, lactulose, isomaltulose (standard: ISO/CD 22184/IDF/WD 24, MLT approved [18])				
Infant formula, milk products	Metrosep Carb 2 - 250/4.0	PAD sweep	0.0005–0.26 mg/L (60 min)	Ethanol/methanol extract, Carrez precipitation, manual dilution
Organic acids				
Maleic acid, lactic acid, and acetic acid				
Milk	Hamilton PRP - X300	Cond	5–40 mg/L maleic acid, lactic acid, and acetic acid	Manual dilution, inline matrix elimination, and dialysis
Contaminants				
Melamine (AN-U-042)				
Milk/infant formula powder	Metrosep C 4 - 150/4.0	UV/VIS	0.02–1 mg/L 1–150 mg/L	Acid extraction (acetic acid), centrifugation, Inline Dialysis

Annex 2:
Important regulations and guidelines to assess and guarantee infant and follow-on formula and baby food quality

Reference	Title	Modified
	CODEX	
CXS 234-1999	Recommended Methods of Analysis and Sampling	2019
CXS 1-1985	General Standard for the Labelling of Prepackaged Foods	2018
CXG 2-1985	Guidelines on Nutrition Labelling	2017
CXS 146-1985	General Standard for the Labelling of and Claims for Prepackaged Foods for Special Dietary Uses	2009
CXS 180-1991	Standard for Labelling of and Claims for Foods for Special Medical Purposes	1991
CXA 2-1976	Statement on Infant Feeding	1976
CXC 66-2008	Code of Hygienic Practice for Powdered Formulae for Infants and Young Children	2009
CXG 8-1991	Guidelines on Formulated Complementary Foods for Older Infants and Young Children	2017
CXG 10-1979	Advisory Lists of Nutrient Compounds for Use in Foods for Special Dietary Uses intended for Infants and Young Children	2015
CXS 72-1981	Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants	2020
CXS 73-1981	Standard for Canned Baby Foods	2017
CXS 74-1981	Standard for Processed Cereal-Based Foods for Infants and Young Children	2019
CXS 193-1995	General Standard for Contaminants and Toxins in Food and Feed	2019
	European Union	
1169/2011	Regulation on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004	2011
609/2013	Regulation on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009	2013
2016/127	Commission Delegated Regulation supplementing Regulation (EU) No 609/2013 of the European Parliament and of the Council as regards the specific compositional and information requirements for infant formula and follow-on formula and as regards requirements on information relating to infant and young child feeding	2015
2006/141/EC	Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC	2006
1881/2006	Commission Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs	2006
	FDA (Code of Federal Regulations) U.S. Food and Drug Administration	
21CFR106	Infant formula requirements to current good manufacturing practice, quality control procedures, quality factors, records and reports, and notifications	2020
21CFR107	Infant formula (i.e., labelling, nutrient requirements)	2020
21CFR170.3	Food additives	2020
81 FR 54960	GRAS final rule: Substances Generally Recognized as Safe	2016
	Australia	
2.9.1/2.9.2	Australia New Zealand Food Standards Code: Part 2.9.1 Infant formula products, Part 2.9.2 Food for infants	2021
	China	
GB 13432	National Food Safety Standard – Labeling of Prepackaged Foods for Special Dietary Use	2013
GB 2761	National Food Safety Standard – Maximum Levels of Contaminants in Food	2017
GB 10765	National Food Safety Standard – Infant formula	2021
GB 10767	National Food Safety Standard – Young Children Formula	2021

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