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Marina Saraiva, Petru Jitaru, Jens Sloth. Speciation analysis of Cr(III) and Cr(VI) in bread and breakfast cereals using species-specific isotope dilution and HPLC-ICP-MS. Journal of Food Composition and Analysis, 2021, 102, pp.103991. <10.1016/j.jfca.2021.103991>. <hal-03657291>

HAL Id: hal-03657291

<https://hal.science/hal-03657291v1>

Submitted on 2 May 2022

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Original Research Article

Speciation analysis of Cr(III) and Cr(VI) in bread and breakfast cereals using species-specific isotope dilution and HPLC-ICP-MS

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ARTICLE INFO

Keywords:

Total chromium
Chromium speciation
Species-specific isotope dilution
HPLC-ICP-MS
Bread
Breakfast cereals

ABSTRACT

This study reports the development and validation of a analytical approach for simultaneous Cr(III) and Cr(VI) speciation analysis in bread and breakfast cereals using species specific isotope dilution (SS-ID) and high-performance liquid chromatography (HPLC) coupled to inductively coupled plasma-mass spectrometry (ICP-MS). The species were extracted by sequential complexation of genuine Cr(III) with ethylenediaminetetraacetic acid (EDTA) and subsequently of Cr(III) originating from reduction of Cr(VI) with 1,5-diphenylcarbazone (DPCO) in the same analytical run. Efficient HPLC separation of Cr(III)-EDTA and Cr(III)-DPCO complexes was carried out by anion exchange HPLC in less than 3 min. The method was validated by means of the accuracy profile approach by carrying out 3 measurement series in duplicate on 5 different days over a timespan of one month. The quantification limits were 0.014 $\mu\text{g kg}^{-1}$ for Cr(III) and 0.047 $\mu\text{g kg}^{-1}$ for Cr(VI), respectively. The measurement bias ranged from 0.31 to 0.49 %, whereas the coefficient of variation in terms of repeatability (CV_r) varied from 1.3–4.4 % for Cr(III) and from 0.6–7.9% for Cr(VI). Similarly, the coefficient of variation in terms of intermediate reproducibility (CV_R) ranged from 1.3–4.4% for Cr(III) and from 2.0–8.9% for Cr(VI), respectively. The method was successfully applied to the analysis of a selection of bread and breakfast cereals samples. Cr(VI) was not detected in any of these samples while Cr(III) levels ranged between 5.2 and 176 $\mu\text{g kg}^{-1}$ for bread samples and between 23.8 and 350 $\mu\text{g kg}^{-1}$ for breakfast cereals. These results were comparable with the levels of total Cr analysed in the same samples by ICP-MS. The method developed in this study on the basis of SS-ID and sequential species complexation is a powerful analytical tool for accurate and precise quantification of Cr(III) and Cr(VI) at trace levels and allows for correction of any species interconversion during sample preparation.

1. Introduction

Chromium (Cr) is one of the most abundant metals in the Earth's crust and present in the environment as a result of both natural processes (rocks & soil erosion and volcanic eruptions) and human (industrial) activities. Cr exists primarily in two stable oxidation states as Cr(III) and Cr(VI), which are highly different with respect to their chemical properties and biological activities (Hamilton et al., 2018; Markiewicz et al., 2015).

Cr(VI) has for several decades been recognized as carcinogenic and mutagenic, whereas Cr(III) for a long time was considered as a beneficial element for human health (Pyrzynska, 2016). However, the European Food Safety Authority (EFSA) stated recently that there is no convincing evidence of any beneficial effects of Cr(III) for healthy people (EFSA–European Food Safety Authority, 2014a). Moreover, EFSA reported

that Cr exists in food exclusively as Cr(III), due to the reduction feature of the food matrices. Several other studies showed that in food with a high content of organic matter, Cr(VI) is rapidly reduced to Cr(III) (Nordberg et al., 2007; Novotnik et al., 2013). In contrast, several studies have reported the presence of Cr(VI) in foodstuffs (Lameiras et al., 1998; Soares et al., 2000; Ellis et al., 2009; Ambushe et al., 2009; Elci et al., 2010; Mathebula et al., 2017; Chen et al., 2020) and hence questioning the reducing power of the organic matter and antioxidants in the food matrix. It has been hypothesized that the presence of Cr(VI) in foodstuffs is most likely the result of the formation of analytical artefacts arising from the oxidation of Cr(III) to Cr(VI) during the analytical processes (Hamilton et al., 2018) or the lack in method selectivity, especially when using analytical approaches based on selective Cr(VI) extraction followed by the analysis of the extract fraction without chromatographic separation (Milacich and Šćanar, 2020). It is

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Received 26 February 2021; Received in revised form 10 May 2021; Accepted 23 May 2021

Available online 26 May 2021

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worth to highlight that this controversy can be addressed only by using truly selective analysis methods, which are capable to assess the species interconversion during the analysis itself, such as species specific-isotope dilution (SS-ID) in combination with high performance liquid chromatography (HPLC) in tandem with inductively coupled plasma-mass spectrometry (ICP-MS) (Hamilton et al., 2018; Pyrzynska, 2016; Milačić and Ščančar, 2020; Saraiva et al., 2021a).

The most critical step of Cr speciation analysis is the species extraction, especially due to their chemical behaviour. Cr(VI) is most stable at higher pH and hence generally extracted in alkaline medium (pH \geq 10). On the other hand, Cr(III) is stable at lower (acidic) pH and is easily oxidized under strong alkaline conditions in the absence of organic matter (Kovács et al., 2007; Hosseini and Belador, 2009; Chen et al., 2014). In acidic media, the high redox potential of the Cr(VI)/Cr(III) couple favours Cr(III) stabilization. In contrast, under alkaline conditions the redox potential decreases, which favours stabilization of Cr(VI) under such conditions (Unceta et al., 2010).

In order to stabilize both species during the analytical procedure (e. g. during heating and changing of pH in the extraction process), complexation and/or derivatization reactions are commonly used. For this purpose, ethylenediaminetetraacetic acid (EDTA) is largely being used to complex Cr(III), whereas Cr(VI) cannot be complexed in these conditions (Soares et al., 2009; Sanchez-Hachair and Hofmann, 2018; Lin et al., 2016; Séby and Vacchina, 2018). In turn, Cr(VI) can be selectively reduced to Cr(III), e.g. employing of 1,5 - diphenylcarbazide (DPC), which is further complexed by the oxidized form of DPC, namely the 1,5-diphenylcarbazone (DPCO) in the same analytical hence Cr(VI) is differentiated from the genuine Cr(III). This approach, combined with the SS-ID, separation by HPLC and detection by ICP-MS is currently the state of art analytical approach for accurate speciation analysis of Cr at trace and ultra-trace levels (Saraiva et al., 2021a,2021b).

For this study, samples of bread and breakfast cereals were used, since the time and temperature used in the extraction step of the other food groups, milk and meat samples (Saraiva et al., 2021a) were not applied successfully to other matrices, the need arises to optimize the new extraction conditions and consequently the validation of a new analytical method for bread and breakfast cereals. The aim of this study was the development and validation of a novel analytical approach based on SS-ID HPLC-ICP-MS for Cr(III) and Cr(VI) speciation analysis in bread and breakfast cereals. The method described here is an adaptation and further development of an approach recently reported for simultaneous speciation analysis of Cr(III) and Cr(VI) in milk and bovine meat samples (Saraiva et al., 2021a). The analytical capability of the novel analytical method is illustrated by its application to ultra-trace speciation analysis of Cr(III) and Cr(VI) in a selection of bread and breakfast cereals samples.

2. Materials and methods

2.1. Instrumentation

A closed microwave digestion system (Anton Paar, Multiwave 3000, Graz, Austria) was used for total Cr determination, while a water bath (ED JULABO GmbH, Seelbach, Germany) was used for heating of the samples during the complexation of Cr(III) and Cr(VI) with their respective complexing/derivatization agents.

The total Cr measurements were conducted both at ANSES (Laboratory for Food Safety, Maisons-Alfort, France) and at DTU Food (National Food Institute, Technical University of Denmark, Kemitovet, Lyngby, Denmark). At ANSES, total Cr was measured by using an ICP-MS Agilent 7700 (Agilent, Les Ulis Cedex, France) while at DTU Food, total Cr was measured by using an iCAP TQ ICP-MS (ThermoFischer Scientific, Bremen, Germany). The working conditions for total Cr determination are summarized in Table 1. The ICP-MS iCAP TQ was used for the speciation studies and was equipped with a PFA standard nebulizer and a cyclonic quartz spray chamber and optimized daily in both standard

Table 1

Optimum ICP-MS and HPLC parameters for total Cr determination and Cr speciation analysis in bread and breakfast cereals by SS-ID HPLC-ICP-MS.

HPLC parameters (ICS-5000 ⁺ and ICS-6000 system)		
Column	Dionex IonPac™ AG7 (2 mm × 50 mm, 10 μm)	
Mobile phase	10 mM HNO ₃ + 2.5% MeOH +0.30 mM EDTA (pH = 2.1)	
Injection volume	5 μL	
Flow rate	0.20 mL min ⁻¹	
Elution mode	Isocratic	
Injector temperature	5 ± 3 °C	
Column temperature	30 ± 1 °C	
ICP-MS parameters		
	Thermo iCAP-TQ	Agilent 7700
Plasma gas flow (Ar)	15 L min ⁻¹	15 L min ⁻¹
Auxiliary gas flow (Ar)	≈ 0.80 L min ⁻¹	≈ 0.99 L min ⁻¹
Nebuliser gas flow (Ar)	≈ 0.92 L min ⁻¹	≈ 0.95–1.00 L min ⁻¹
Sampling cone orifice (Ni)	1.1 mm orifice	1.0 mm orifice
Skimmer cone (high matrix) orifice (Ni)	0.5 mm orifice	0.8 mm orifice
Collision gas flow (He)	≈ 5.0 mL min ⁻¹	≈ 4.3 mL min ⁻¹
Plasma power	1550 W	1550 W
Dwell time	250 ms	100 ms
Monitored isotopes	⁵⁰ Cr, ⁵² Cr, ⁵³ Cr, ⁴⁵ Sc	⁵⁰ Cr, ⁵² Cr, ⁵³ Cr, ⁴⁵ Sc

and KED (kinetic energy discrimination) modes.

The HPLC separation of Cr(III) and Cr(VI) species was carried out at ANSES by using a Dionex ICS-5000⁺ system (ThermoFisher Scientific, France) while at DTU Food by using a Dionex ICS-6000 system (ThermoFisher Scientific, Waltham, MA, USA), both equipped with a Dionex IonPac™ AG7 column (2 mm x 50 mm, 10 μm) (ThermoFisher Scientific, Waltham, MA USA). The outlet of the HPLC column was directly connected to the sample introduction system of ICP-MS TQ via a 0.18 mm i. d PEEK tubing. The HPLC operating conditions are included in Table 1.

2.2. Reagents and standard solutions

Ultrapure water (18.2 MΩ cm), prepared by Milli-Q™ Integral 5 Elix Technology (Merck Millipore, Darmstadt, Germany) was used throughout the experiment. A solution of ethylene diamine tetraacetic acid (EDTA, 99.995 %, Sigma-Aldrich, Darmstadt, Germany) was used to chelate Cr(III) to [Cr(III)-EDTA]-complex while 1,5 - diphenylcarbazide (DPC, Sigma-Aldrich) was used for Cr(VI) derivatization. This approach requires that both complexed species (of Cr(III) and Cr(VI)) can be subsequently separated by HPLC. In this study, Cr(III) was complexed selectively using EDTA while Cr(VI) (represented as chromate anion, CrO₄²⁻) was derivatized by reaction with DPC. Is important to note that although both EDTA and DPC are well known for their selective affinity towards Cr(III) and Cr(VI) (Sanchez-Hachair and Hofmann, 2018; Mathebula et al., 2017), this approach was not previously used for simultaneous speciation analysis of Cr in foodstuffs.

EDTA solution was prepared by dissolving 0.45 g EDTA to a final volume of 500 mL in ultrapure water using ultrasounds for 15 min at the maximum power at 25 °C. DPC solution was prepared by dissolving 0.05 g of DPC in 10 mL of methanol (\geq 99.8 %, AnalaR NORMAPUR® ACS, VWR, Fontenay-sous-Bois, France) and subsequently adding (while stirring) approximately 50 mL of ultrapure water containing 2.8 mL of sulfuric acid (95–97 %, Merck KGaA, Darmstadt, Germany). Finally, the volume was made up to 100 mL with ultrapure water in a volumetric flask.

HNO₃ (67 % m/m) used as an eluent was purchased from Merck KGaA, Darmstadt, Germany.

Certified standard stock solutions containing Cr(III) and Cr(VI) at 1000 mg L⁻¹ (SCP Science, Quebec, Canada) were used for the external calibration.

Isotopically enriched ⁵⁰Cr(III) (97.4 %) and ⁵³Cr(VI) (92.4 %) spike

solutions at 100 mg L⁻¹ (ISC Science, Oviedo, Spain) were used throughout for SS-ID. The natural abundances of the Cr isotopes used for SS-ID are those reported by commission on isotopic abundances and atomic weights (Meija et al., 2016) (⁵⁰Cr(III): 4.35 %, ⁵²Cr(III): 83.79 %, ⁵³Cr(III): 9.50 %, ⁵⁴Cr(III): 2.37 %).

Intermediate solutions of Cr(III) and Cr(VI) (natural and spikes) were prepared daily by dilution of the stock solutions with ultrapure water.

2.3. Food samples

Twenty-two samples of various types of bread and breakfast cereals were purchased from local supermarkets in Maisons-Alfort, France and Birkerød, Denmark (see Table 2). It is worth to note that the bread samples were produced with different types of flour such as rye, spelt, wheat as well as toasted and untoasted bread whereas the breakfast cereals differed in their composition in terms of cereals, cacao, fruits and nuts levels. A wheat flour certified reference material (NIST 8436, Durum) from NIST (Gaithersburg, USA) was also analyzed using the same procedure as food analysis for the internal quality control.

2.4. Determination of total Cr

Cr_{total} levels in 11 different breads and 11 different brands of breakfast cereals were determined by ICP-MS following the digestion using concentrated HNO₃ (67 % m/m) in a closed microwave digestion system using an accredited method reported elsewhere (Chevallier et al., 2015).

In brief, approximately 0.3 g of bread and breakfast cereals were accurately weighed into 80 mL quartz vessels and then thoroughly mixed with 3 mL of concentrated HNO₃. Before the microwave treatment, 3 mL of ultra-pure water was added. The digests were quantitatively transferred to 50 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) and then diluted to 50 mL with ultrapure water after the addition of the internal standards (⁴⁵Sc). The total Cr analysis were performed in duplicate for each independent samples. After dilution with ultrapure water, the digests were analysed by ICP-MS using external calibration in the range 1.0–10.0 µg L⁻¹ according to the operating conditions given in Table 1.

2.5. Cr speciation analysis

For the determination of total Cr, 11 types of bread made with

Table 2
Bread and breakfast cereals purchased and used in this study.

Sample (flour type)	Place of purchase – retail shop
Bread	
Danish rugbrød (Rye)	Birkerød (Denmark)
Malt flour bread (malted wheat)	–
“Burger” buns (wheat)	–
Spelt bread (spelt)	–
“Pita” bread (wheat)	–
Baguette (wheat)	Maisons-Alfort (France)
Sandwich (wheat)	–
Wheat bread (wheat)	–
Bread toasted (wheat)	–
Bread toasted complete (wheat)	–
French bread (wheat)	–
Breakfast cereals	
Cocoa (20.1 %) cereals (brand I)	Birkerød, Denmark
All bran	–
Bran flakes bio	–
Corn flakes	–
Whole-grain wheat	–
Whole-grain wheat + Fruit + Nuts	–
Cocoa (16.5 %) cereals (brand II)	–
Granola (rolled oats) bio	–
Granola (rolled oats) gluten free	–
Fruity muesli	–
Toasted muesli	–

various types of flour and 11 different types of breakfast cereals were analyzed in duplicate. Briefly, a subsample of 0.3 g was thoroughly mixed with the optimum amounts of ⁵⁰Cr(III) and ⁵³Cr(VI) spikes directly into a 50 mL polypropylene tube. Subsequently, 16 mL of EDTA solution was added to reach a final concentration of 2.4 mM and the mixture was heated in the water bath at 90 °C for 60 min. The solution was then cooled down (≈ 15 min) and 200 µL of DPC solution was added to reach a final concentration of 0.02 mM and the final volume was made up to 20 mL with ultrapure water. After addition of the DPC solution, the mixture was heated again at 90 °C for 25 min. The extract was cooled down to room temperature (≈ 30 °C for 15 min) and then filtered using 0.45 µm polyvinylidene fluoride syringe filters prior to the analysis (the same day). A standard mixture of Cr(III) (5.0 ng L⁻¹) and Cr (VI) (2.5 ng L⁻¹) was analyzed daily (before the real samples) in order to assess the overall instrumental performance and the stability of the ⁵⁰Cr (III) and ⁵³Cr(VI) spikes used for SS-ID.

As it was reported in detail in a previous work (Saraiva et al., 2021a), to carry out SS-ID, the samples were spiked with known amounts of ⁵⁰Cr (III) and ⁵³Cr(VI) spikes to achieve isotope ratios (⁵⁰Cr/⁵²Cr and ⁵³Cr/⁵²Cr) in the blend samples ~1. For this purpose, the mass of each spike solution added to each sample was calculated based on Eq. 1 (example provided here for Cr(III) spike solely).

$$M_s^{50} = \frac{(C^{III} \times m_x) / F^{50/52}}{C_s^{50}} \quad (1)$$

where:

M_s^{50} , mass of ⁵⁰Cr(III) spike solution (g)

C^{III} , expected concentration of Cr(III) in the sample (µg kg⁻¹)

m_x , sample mass (g)

$F^{50/52}$, ratio of the abundance of ⁵⁰Cr(III) spike and the natural abundance of ⁵²Cr(III)

C_s^{50} , concentration of the ⁵⁰Cr(III) spike solution (µg kg⁻¹)

2.6. Method validation

One of the main challenges related to Cr speciation analysis is the assessment of the accuracy of the analytical methodology, taking into account that food based CRMs are currently lacking from the market, most probably because of the Cr species instability in such matrices (Oliveira, 2012). Therefore, the common approach used to assess the method accuracy is *via* the determination of samples spike recoveries.

In this study, the method for Cr(III) and Cr(VI) speciation analysis was validated based on the accuracy profile approach according to guidelines described in detail elsewhere (Thompson et al., 2002; AFNOR, 2010). This approach takes into account the simultaneous assessment of the method accuracy and precision. Basically, the accuracy profile is an expression of the combination of the systematic (trueness) and the random error (repeatability and/or intermediate precision) for a series of analyte's levels in various matrices within a range of concentrations called the validity domain. Trueness is expressed either through bias (in case of the analysis of CRM) or in this case by means of the recovery factor for the analysis of spiked samples.

To construct the accuracy profile, the measurement accuracy is assessed for each analyte at different levels corresponding to natural or spiked matrices. For this purpose, three bread were spiked with Cr(VI) at three levels (5, 10 and 15 µg kg⁻¹) and each blend was analyzed in duplicate on 5 different days during one month (analysis performed once a week) to assess the accuracy, the repeatability and the intermediate reproducibility. LOQs was estimated based on the IUPAC criterion (10×σ₀), where σ₀ is the standard deviation determined from the measurements of 21 blank samples in repeatability conditions. The validity domain was between the estimated method limit of quantification (LOQ) and the upper tested concentration (15 µg kg⁻¹).

2.7. Risk assessment related to Cr(III) in bread and breakfast cereals

Cr species are considered a chemical hazard in the context of risk assessment and therefore their identification and characterization have to be carried out.

The global dietary exposure to a chemical contaminant can be expressed by summing the contributions from all the different types of foods consumed daily, each contribution being calculated as the product of the level of the contaminant in the given food multiplied with the amount of that specific food consumed, normalized to body weight (Eq.1).

$$E_i = \sum_{k=1}^n \frac{C_{i,k} \times L_k}{W_i}$$

where:

E_i daily exposure of a household normalized by consumption units or adult male equivalents ($\mu\text{g kg}^{-1}$ of total Cr and/or Cr species/capita/day).

$C_{i,k}$ daily consumption of food (kg/capita/day).

L_k level of the chemical in the food (total Cr and/or Cr species - $\mu\text{g kg}^{-1}$).

W_i body weight of the individual (kg).

According to Institute of Medicine, the dietary reference intakes (DRI) for Cr ($\mu\text{g day}^{-1}$) were between 30–35 for adult males and 20–25 for adult females (Institute of Medicine (IOM), 2001).

3. Results and discussion

3.1. Optimization of the extraction of Cr(III) and Cr(VI)

The principles of the procedure for the extraction of Cr(III) and Cr(VI) was based on a previous study reported on Cr speciation in milk and meat samples (Saraiva et al., 2021a). It is worth to note that the conditions for extraction of Cr(III) and Cr(VI) from milk and meat (first step: 70 °C during 25 min. and in the second step again 70 °C during 25 min.) showed not to be optimal for bread and cereal samples. This observation is in agreement with other studies which emphasized the need for matrix-specific optimization of the extraction conditions (Milačić and Ščančar, 2020; Hernandez et al., 2017). Therefore, in this study, the extraction temperature and time was specifically optimized for bread analysis. When the extraction protocol developed for milk and meat was applied to bread analysis, most of the Cr was erroneously identified as Cr(VI) (Table 3). This is most probably because Cr(III) was not released

Table 3

Levels of Cr_{total} (by ICP-MS) and Cr species, including their sum (SS-ID HPLC-ICP-MS) in French bread using different extraction temperature and time.

EDTA step	DPC step	Mass fraction + SD ^a ($\mu\text{g kg}^{-1}$, n = 2)				
		Cr _{total}	Cr(III)	Cr(VI)	Cr(III)+Cr(VI)	Cr(III)+Cr(VI)/Cr _{total} (%)
70 °C-25 min	70 °C-25 min		1.8 ± 0.3	28.5 ± 2.8	30.3 ± 2.4	114 ± 5
70 °C-60 min	70 °C-60 min		15.5 ± 0.8	6.6 ± 0.9	22.4 ± 1.9	84 ± 2
90 °C-25 min	90 °C-25 min	26.6 ± 3.8	19.6 ± 0.3	< 0.05 (LOQ)	19.6 ± 1.8	74 ± 3
90 °C-60 min	90 °C-60 min		25.8 ± 0.4	< 0.05 (LOQ)	25.8 ± 1.5	97 ± 1
90 °C-80 min	90 °C-80 min		27.7 ± 0.4	< 0.05 (LOQ)	27.7 ± 2.0	104 ± 2
90 °C-120 min	90 °C-120 min		28.9 ± 0.1	< 0.05 (LOQ)	28.9 ± 2.1	109 ± 6

^a standard deviation.

during the first step of the extraction (EDTA chelation), hence the genuine Cr(III) was actually not determined. Cr(III) was then released during the second step of the extraction (DPC derivatization) and consequently erroneously identified as Cr(VI). Increased temperature and duration during the first extraction step was therefore necessary to quantitatively release the genuine Cr(III) from the matrix and complex it with EDTA. In such conditions, a decrease in the apparent Cr(VI) level was also observed (see Table 3).

The experiments showed that a temperature of 90 °C and an extraction during at least 60 min was necessary for the first step to ensure quantitative extraction of Cr(III). Hence, these conditions were used in the subsequent work of the present study.

In order to assess the optimum duration of the second extraction step (DPC derivatization), a set of extraction experiments using a spiked bread sample subjected to extraction at different times (5–30 min) at fixed optimum temperature (90 °C) was carried out and the results are reported in Table 4.

Quantitative recovery ($102 \pm 3\%$) of the spiked Cr(VI) was obtained while performing the 2nd extraction step at 90 °C for 25 min. Hence, these conditions were chosen for the subsequent part in this study.

In order to verify that the optimized extraction conditions provided quantitative extraction also for Cr(III), two different types of bread with different levels of Cr(III) were spiked with Cr(III) at different levels from 5 to 15 $\mu\text{g kg}^{-1}$ and analyzed with the optimized procedure (Table 5).

In all cases, good recoveries (97–113 %) were obtained for the different levels of spiking, which confirmed that the extraction conditions were optimal for the determination of both Cr(III) and Cr(VI) species from bread.

3.2. Analytical performance characteristics

The main analytical performance characteristics are summarised in Table 6. As can be seen, the trueness, expressed here in terms of bias (%) was in all cases <1 %, hence showing the excellent capability of the method for the simultaneous (single run) and accurate quantification of Cr(III) and Cr(VI) in bread samples.

Excellent repeatability (expressed in terms of relative standard deviation, CV_r, %) was also obtained; CV_r for Cr(III) ranged between 1.3 and 4.4 % and 0.6 and 7.9 % for Cr(VI) determination. Similarly, the intermediate reproducibility (CV_R, %) was between 1.3–4.4 % for Cr(III) between 2.0–8.9 % for Cr(VI) determination, which is similar to other studies (Vacchina et al., 2015).

The LOQs obtained were 14 ng kg⁻¹ and 47 ng kg⁻¹ for Cr(III) and Cr(VI), respectively. Noteworthy that the LOQs obtained in the present study were considerably lower compared to other studies on dealing with Cr(VI) determination in food using on-line speciation-based analytical protocols (Hernandez et al., 2017, 2018; Mathebula et al., 2017).

3.3. Total Cr and Cr speciation results in bread and breakfast cereals

The results obtained from the determination of total Cr and Cr

Table 4

Levels of Cr(VI) and the corresponding recovery factors obtained by the analysis of a bread sample spiked with Cr(VI) at different extraction times.

Extraction time (DPC step, 90 °C)	Cr(VI) level ($\mu\text{g kg}^{-1}$) ^a (mean ± SD ^b , n = 2)	Recovery (%)
5 min.	2.14 ± 0.01	214 ± 9
10 min.	1.94 ± 0.08	194 ± 6
15 min.	1.48 ± 0.14	148 ± 8
20 min.	1.23 ± 0.04	123 ± 4
25 min.	1.02 ± 0.03	102 ± 3
30 min.	1.06 ± 0.04	106 ± 4

^a spiking level = 1.0 $\mu\text{g kg}^{-1}$; ^b standard deviation.

Table 5

Accuracy (expressed as recovery factor, %) and intermediate precision (relative standard deviation, %) obtained for the analysis of two bread samples spiked with Cr(III) at different levels (the genuine Cr(III) levels in these samples are also reported).

Spiking level of Cr(III) ($\mu\text{g kg}^{-1}$)	0	5	10	15
Bread 1				
Genuine Cr(III) level ($\mu\text{g kg}^{-1}$) ^a	130 ± 2	137 ± 4	153 ± 1	158 ± 3
Recovery factor (%) ^{a,b}	97 ± 1 ^a	99 ± 3	106 ± 1	105 ± 1
RSD ^c (%)	1.2	2.7	0.9	1.6
Bread 2				
Cr(III) level ($\mu\text{g kg}^{-1}$) ^a	27 ± 2	32 ± 1	41 ± 2	45 ± 1
Recovery factor (%) ^{a,b}	101 ± 2 ^a	101 ± 3	113 ± 6	107 ± 2
RSD ^c (%)	2.2	2.9	5.1	1.6

^a mean ± standard deviation (n = 2).

^b recovery factor for Cr(III) determination by SS-ID compared to Cr_{total} (ICP-MS).

^c relative standard deviation.

Table 6

Analytical performance characteristics for Cr(III) and Cr(VI) speciation analysis in bread samples by SS-ID HPLC-ICP-MS.

Sample	Mass fraction ^a ($\mu\text{g kg}^{-1}$)	Bias (%)	CV _r (%)	CV _R (%)	LOD ^b (ng kg ⁻¹)	LOQ ^b (ng kg ⁻¹)
Cr(III)						
French bread (wheat)	26.6	0.30	4.4	4.4		
Spelt bread (spelt)	173	-0.02	3.3	3.3		
Breakfast cereals (fruit + nuts)	61.8	-0.31	2.5	3.4	4	14
Breakfast cereals cacao (16.5 %)	219	0.01	1.3	1.3		
Cr(VI)						
Bread + 5 $\mu\text{g kg}^{-1}$	5.1	0.03	0.6	3.3		
Bread + 10 $\mu\text{g kg}^{-1}$	9.9	0.14	7.9	8.9	14	47
Bread + 15 $\mu\text{g kg}^{-1}$	15.1	0.49	2.0	2.0		

^a genuine level for Cr(III) and spiking level for Cr(VI).

^b test portion size of 0.3 g.

species in the bread and breakfast cereal samples are presented in Table 7.

The values for Cr_{total} varied between LOQ (10 $\mu\text{g kg}^{-1}$) and 202 $\mu\text{g kg}^{-1}$ for bread samples and 24.4 and 360 $\mu\text{g kg}^{-1}$ for breakfast cereals samples. The results are in agreement with other reports on Cr_{total} content in bread samples obtained by Roussel et al., 2007 (5.82–225 $\mu\text{g kg}^{-1}$). For breakfast cereals, the levels found are agreement with reported by Farre and Lagarda, 1986 (118–162 $\mu\text{g kg}^{-1}$) and 316 $\mu\text{g kg}^{-1}$ reported by Roussel et al. (2007) in breakfast cereals with cacao.

The levels for Cr(III) varied between 5.2 and 176 $\mu\text{g kg}^{-1}$ for bread samples and 23.8 and 350 $\mu\text{g kg}^{-1}$ for breakfast cereals. In all samples Cr(VI) was not detected and the Cr(III) results were in good agreement with Cr_{total} results (87–102% in bread samples and 90–109% in breakfast cereals). Based on the speciation analysis, the results clearly indicate that all Cr present in these sample types is in the form of Cr(III). This finding is in agreement with a range other studies (Kovács et al., 2007; Novotnik et al., 2013; Vacchina et al., 2015; Hernandez et al., 2017; Miličević and Ščančar, 2020), where also only Cr(III) and not Cr(VI) was detected in cereal samples.

Table 7

Mean levels of total Cr and Cr species in bread and breakfast cereals obtained by ICP-MS and SS-ID HPLC-ICP-MS, respectively.

	Mass fraction ± SD ($\mu\text{g kg}^{-1}$, n = 2)		Cr(III) (SS-ID)/ Cr _{total} ^a (%)
	Cr _{total} (ICP-MS)	Cr(III) (SS-ID)	Cr(VI) (SS-ID)
Bread sample			
Danish rugbrød (Rye)	86 ± 5	88 ± 5	102
Malt flour bread (malted wheat)	67 ± 2	67 ± 3	101
“Burger” buns (wheat)	170 ± 2	170 ± 6	100
Spelt bread (spelt)	202 ± 3	176 ± 6	87
“Pita” bread (wheat)	76 ± 5	77 ± 2	101
Baguette (wheat)	135 ± 19	131 ± 8	97
Sandwich (wheat)	45 ± 6	44 ± 3	98
Wheat bread (wheat)	< 10	6 ± 1	–
Bread toasted (wheat)	< 10	5 ± 1	–
Bread toasted complete (wheat)	< 10	10 ± 1	–
French bread (wheat)	27 ± 4	27 ± 1	101
Breakfast cereal			
Cocoa (20.1 %) cereals (brand I)	360 ± 51	350 ± 6	97
All bran	67 ± 3	63 ± 5	94
Bran flakes bio	26 ± 1	25 ± 1	96
Corn flakes	24 ± 1	26 ± 1	106
Whole-grain wheat	25 ± 1	25 ± 2	100
Whole-grain wheat + Fruit + Nuts	62 ± 2	63 ± 2	102
Cocoa (16.5 %) cereals (brand II)	219 ± 7	238 ± 1	109
Granola (rolled oats) bio	42 ± 2	40 ± 3	96
Granola (rolled oats) gluten free	26 ± 0	27 ± 1	105
Fruity muesli	26 ± 1	24 ± 2	90
Toasted muesli	41 ± 1	40 ± 1	97

^a Cr_{total} level measured by ICP-MS.

A wheat flour CRM (NIST 8436 – Durum) with a certified value for total Cr was used as quality control for the quantification of the sum of Cr(III) and Cr(VI). This was analyzed in 5 different days during 1 month and the percentage of recovery ranged from 96 to 103%.

3.4. Conflicting reports on the potential presence of Cr(VI) in cereals

Several studies dealing with Cr speciation in foodstuffs reported the presence of Cr(VI) in bread (Soares et al., 2010; Mathebula et al., 2017). One particular example, reported by Soares et al. (2010), is the presence of Cr(VI) in bread representing 12 % of the total Cr level, namely $5.7 \pm 5.4 \mu\text{g kg}^{-1}$ for white bread and $6.8 \pm 4.9 \mu\text{g kg}^{-1}$ for whole grain bread. More recently, Mathebula et al. (2017) reported Cr(VI) in bread samples from South Africa. This study showed that 33–73 % of total Cr (58.2 ± 5.1 – $156.1 \pm 6.7 \mu\text{g kg}^{-1}$) in bread exist as Cr(VI). In both of these cases, selective extraction of Cr(VI) followed by the determination of total Cr in the extract fraction by atomic absorption spectrometry was employed. It is therefore highly plausible that the application of such off-line analytical methods, without prior species separation by HPLC, are not truly selective for Cr(VI) hence producing erroneous results in terms of (falsely positive) Cr(VI) presence in bread samples (Miličević and Ščančar, 2020). Additionally, such off-line approaches are not able to tackle the potential species-interconversions (Miličević and Ščančar, 2020). It is nowadays largely accepted that on-line analytical procedures relying on coupling of HPLC and ICP-MS should be employed in combination with the use of SS-ID. Such approaches allow the accurate species quantification and correct for any species transformation during the analytical procedure leading to reliable speciation results, as reported also by other

authors (Novotnik et al., 2013).

In order to further investigate Cr(VI) stability in bread samples, a series of spiking experiments were carried out. For this purpose, French bread (wheat) was first spiked with Cr(III) at different levels and subsequently the experiment was repeated by spiking the same sample also with Cr(VI) (also at different levels). The outcome of these experiments is shown in Fig. 1.

In the case of Cr(III), the spiked amounts were quantitatively recovered ($104 \pm 5\%$, $n = 4$) and in all cases Cr(VI) was not detected. When spiking with Cr(VI), the spikes were quantitatively recovered as Cr(III) for all spiking levels ($98 \pm 7\%$, $n = 4$) and no Cr(VI) was detected. The results support the hypothesis that Cr(VI) is not stable in a bread matrix and all Cr is present in the Cr(III) form.

The experimental work carried out in this study supports therefore the findings of Novotnik et al. (2013) and confirms that Cr(VI) is not present in bread and breakfast cereals. Hence, the previously reported findings of Cr(VI) are most probably erroneous and caused by analytical artefacts.

3.5. Risk assessment: Consumption data of cereals made with different flour types in France and Denmark

For this study, the consumption data provided by FAO, 2018 in Denmark and France were used (Table 8).

The data are related to total Cr levels, since only these data are available via FAO (2018), but these data were extrapolated to Cr(III) levels because the present study demonstrated that in cereal samples the levels of total Cr are similar to Cr(III) levels. The chemical exposure related to Cr (Eq.1) was assessed using the consumption data of cereals products, Cr(III) levels found and average body weight of 70 kg, as presented in Table 8.

Comparing the values obtained in the exposure assessment with dietary reference intakes (DRI) for Cr ($\mu\text{g day}^{-1}$), between 30 and 35 for adult males and 20–25 for adult females (Institute of Medicine (IOM), 2001), the values are very low both in Denmark and France, with values between 0.001 and $1.59 \mu\text{g kg}^{-1}$ body weight per day. Taking also into account the European tolerable daily intake (TDI) of Cr ($300 \mu\text{g kg}^{-1}$ body weight per day (EFSA–European Food Safety Authority, 2014b), it is confirmed that the exposure of French and Danish populations to Cr(III) via cereals is indeed of no concern.

4. Conclusion

This study provides a novel analytical method for Cr speciation analysis in bread and breakfast cereals by species-specific isotope-dilution (SS-ID) and HPLC-ICP-MS. Actually, the extraction procedure optimized in a previous study for milk and meat samples turned out not to be efficient for bread and breakfast cereals. It was therefore necessary to optimize the extraction step by using more energised conditions (higher temperature and duration) for quantitative extraction of Cr(III).

Table 8

“Dairy” exposure assessment of Cr by FAOSTAT to cereals products consumption by the Danish and French population.

Flour type	Consumption (kg/capita/day)		Cr(III) levels ($\mu\text{g kg}^{-1}$)	Exposure assessment of Cr ($\mu\text{g kg}^{-1}$ /capita/day) ^a	
	Denmark	France		Denmark	France
Wheat	0.233	0.318	5.2 – 350	0.017 – 1.168	0.024 – 1.593
Maize	0.016	0.037	25.8	0.006	0.014
Rye	0.035	0.001	87.7	0.044	0.001
Oats	0.010	0.002	27.4 – 39.8	0.004 – 0.006	0.001 – 0.001
Malt	0.000	0.001	67.2	0.000	0.001

^a body weight (W_i) = 70 kg.

This confirms that speciation analysis of Cr in foodstuffs is matrix dependant.

Based on the results obtained in the validation of the method, this novel procedure was successfully applied to the analysis of a selection of bread (made with different types of flour) and breakfast cereals (with different flour compositions, with and without: fruits, nuts and cacao) samples (which were also analyzed for their total Cr content). Cr(VI) was not quantified in any of these samples hence supporting the EFSA statement related to the absence of Cr(VI) in foods, notably bread, due to the reducing power of such matrices. Our study based on the use of a primary analysis method (SS-ID) responded also to the actual controversy related to the presence of Cr(VI) in bread samples, indicating that most probably, the reports on the quantification of Cr(VI) in such samples is due to analytical artefacts.

Finally, a pilot risk assessment related to the consumption of cereals products in France and Denmark showed extremely low exposure to Cr in both countries. The study can be pursued by enlarging the panel of the cereal based samples as well as the impact of their (heating based) cooking on the fate of Cr species and the associated health risk.

Author statement

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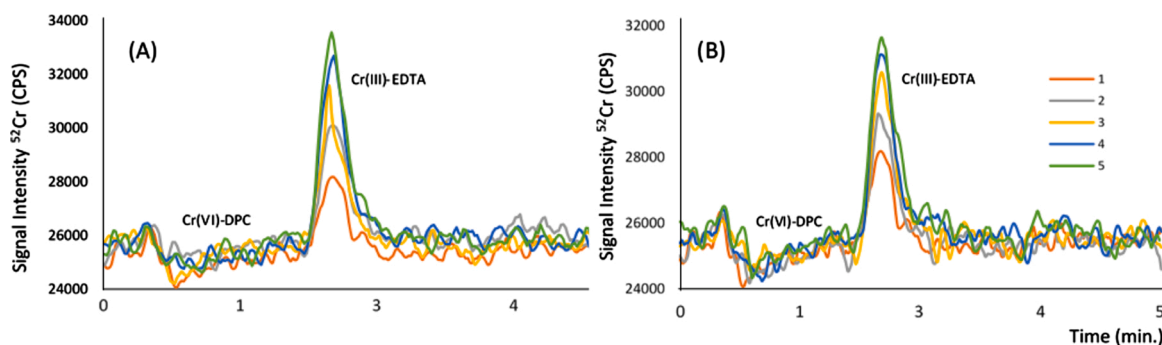


Fig. 1. Chromatograms obtained by the analysis of a bread sample spiked with Cr(III) (A) and Cr(VI) (B), at different levels, such as: (1) $0 \mu\text{g kg}^{-1}$ (no spiking), (2) $5 \mu\text{g kg}^{-1}$; (3) $10 \mu\text{g kg}^{-1}$; (4) $15 \mu\text{g kg}^{-1}$ and (5) $20 \mu\text{g kg}^{-1}$.

Declaration of Competing Interest

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

Acknowledgements

This Ph.D. research project was funded by French Agency for Food, Environmental and Occupational Health & Safety (ANSES, France) and National Food Institute (DTU Food, Denmark).

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