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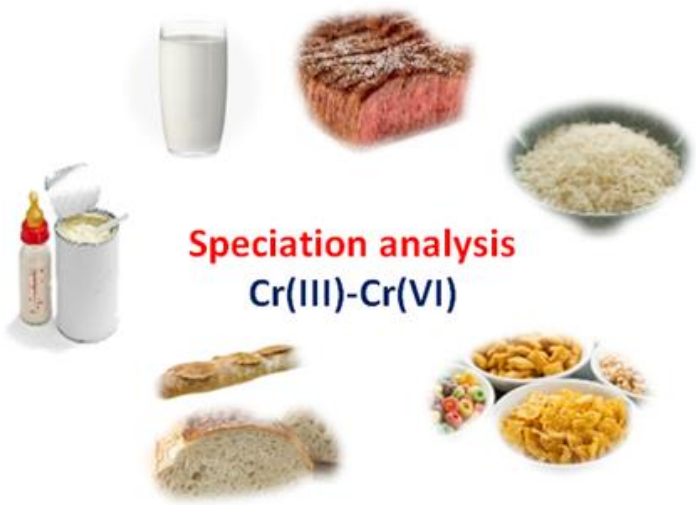

Marina Amaral Saraiva

PhD Thesis

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PhD thesis

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2021

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Preface

This manuscript presents the results from three years of research that was carried out within the framework of the PhD project entitled: "Ultra-trace speciation analysis of chromium in foodstuff by high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry using species specific isotope dilution". The research was carried out at the Laboratory for Food Safety-French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Maisons-Alfort in France from 1st of February 2018 until 16th August 2019 and at National Food Institute, Technical University of Denmark (DTU Food), from 19th August 2019 until 31 January 2021.

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January 2021

Kongens Lyngby, Denmark

Marina Amaral Saraiva

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Summary

It is nowadays largely accepted that the determination of the total content of potentially toxic elements (PTEs) in foodstuffs does not always provide sufficient information to support a correct risk assessment, especially when the PTE can be present in different chemical forms (i.e. chemical species). In such a case, information on each chemical species is necessary and hence speciation analysis is required.
Chromium speciation analysis is a very relevant issue in food chemistry, on one hand due to the toxicity of Cr(VI) species and furthermore due to the analytical challenge to accurately determine Cr(III) and Cr(VI) in foodstuffs. The challenge is greater when the simultaneous determination of these two species is desired, as they are unstable with variations in temperature and pH. Actually, a polemic exists in the scientific community related to the possible presence of Cr(VI) (the most toxic Cr species) in foods.
EFSA stated (2014) that in such sample Cr can be present only as Cr(III), because the foodstuffs matrices are reductive media and hence Cr(VI) incorporated into a diet is immediately reduced to C(III) (less toxic). The polemic in terms of Cr status in food was sustained by several authors who found Cr(VI) in several foodstuffs (bread, tea, etc.) but assessed also the formation of Cr(VI) by oxidation of Cr(III) during various culinary processing (e.g. bread toasting, etc.). Actually, it is reasonably assumed that the "discovery" of Cr(VI) in foodstuffs is attributed to analytical artefacts. Indeed, both Cr(III) and Cr(VI) are stable in different conditions, hence making their accurate determination very difficult. The clarification of this controversy using the species-specific isotope dilution (SS-ID), the only method that allows correcting the interconversions of species that may occur during the analytical procedure, is one of the main achievements of this thesis.

The present PhD project aims at the development, validation and application of novel analytical approaches for the simultaneous speciation analysis of Cr(III) and Cr(VI) in foodstuffs such as dairy products, meat and meat products, bread, breakfast cereals and rice by high-performance liquid chromatography (HPLC) coupled to inductively coupled plasma-mass spectrometry (ICP-MS). Species specific-isotope dilution (SS-ID) was thoroughly employed for species quantification and for the correction of their potential mutual inter-conversion.
The species extraction was carried out by complexation of Cr(III) with ethylenediaminetetraacetic acid (EDTA) and by derivatisation of Cr(VI) with 1,5-diphenylcarbazide (DPC) in the same analytical run. By this procedure Cr(VI) is not directly complexed with DPC, but is first reduced to Cr(III) and then subsequently Cr(III) is complexed by DPC. The HPLC separation of complexed species was carried out using a short microbore anion-exchange HPLC column, which allowed baseline separation of the two species in less than 3 minutes. The method was validated by means of the accuracy profile approach by carrying out six measurement series in duplicate on (six) different days over a timespan of two months. The quantification limit was 0.013 μg kg^{-1} for Cr(III) and 0.049 μg kg^{-1} for Cr(VI), respectively.

The measurement bias corresponding to the validity domain ranged from 0.01 to 0.11%, whereas the coefficient of variation in terms of repeatability (CV_r) varied from 2.9 to 11.6 % (depending on the analyte level) for Cr(III) and from 6.7 to 11.8% for Cr(VI). Similarly, the coefficient of variation in terms of intermediate reproducibility (CV_R) ranged from 6.8 to 13.0% for Cr(III) and from 6.8 to 25.9% for Cr(VI), respectively.

The method was successfully applied to the analysis of a selection of different types of food samples including dairy products, meat and cereals. Cr(VI) was not quantified in any of these samples while Cr(III) levels ranged between 0.22 µg kg^{-1} (infant formula milk) and 350 µg kg^{-1} (breakfast cereals with cacao). Cr(III) levels in all samples were comparable with the levels of total Cr determined in the same samples by ICP-MS. The results obtained in the various food samples analysed support the hypothesis that all Cr present in foods is in the form of Cr(III), clearly rejecting reports on the presence of Cr(VI) in foods. The results obtained with this work contributed positively to the current dispute over the speciation of Cr in foods.

The methodology was furthermore used to evaluate the potential impact of different culinary processes on the fate of Cr species in infant formula milk, semi-skimmed milk and bovine meat samples. The cooking procedures consisted of boiling the milk samples at 70°C and 100°C and frying the bovine meat without (95°C) and with oil and (120°C). The results showed no significant differences between the content of the Cr species in raw and cooked samples, hence indicating that no inter-species transformation happened during the processing. A risk analysis was performed taking into account the levels of Cr(III) found in cooked bovine meat and milk and showed an extremely low risk for consumers of these products.

This work contributed to obtain more knowledge in terms of the Cr speciation in foodstuffs with the development of an important tool for assessing the risk of Cr in foods with total control over the interconversion of species that occur during the analytical procedure. It also contributed to resolve the current dispute over the presence of the toxic specie of Cr in food. For future work, the developed and validated method can be applied to other types of matrices, such as environmental or pharmaceutical.

Resumé

Det er i dag stort set accepteret, at bestemmelsen af det totale indhold af potentielt toksiske grundstoffer (PTE'er) i fødevarer ikke altid giver tilstrækkelig information til at understøtte en korrekt risikovurdering, især når grundstoffet kan være til stede i forskellige kemiske former (dvs. kemiske specier). I disse tilfælde er information om hver kemisk form nødvendig, og der kræves derfor specieringanalyse.

Kromspecieringsanalyse er et meget relevant emne inden for fødevarekemi. På den ene side på grund af toksiciteten af Cr(VI)-s og desuden på grund af den analytiske udfordring i at opnå nøjagtig bestemmelse af indholdet af Cr(III) og Cr(VI) i fødevarer. Udfordringen bliver endda endnu større, når samtidig bestemmelse af disse to arter ønskes, da de er ustabile ved variation i temperatur og pH. Faktisk findes der en kontrovers i det videnskabelige samfund relateret til om tilstedeværelsen af Cr(VI) i fødevarer er biologisk mulig eller ej.

EFSA erklærede i 2014, at Cr i fødevarer kun kan være til stede som Cr(III), fordi fødevarematricerne er reduktive medier, og derfor reduceres Cr(VI) i en fødevare straks til Cr(III). Kontroversen med hensyn til Cr(VI)'s mulige tilstedeværelse- i fødevarer er dog blevet opretholdt af flere studier, der har rapporteret fund af Cr(VI) i flere fødevarer (brød, te osv.), og man vurderede også om dannelse af Cr(VI) kunne ske ved oxidation af Cr(III) under forskellige kulinariske tilberedninger af fødevarene (f.eks. brødristning osv.). Det er dog efterfølgende blevet postuleret, at "opdagelsen" af Cr(VI) i fødevarer kan tilskrives analytiske artefakter. Afklaring af denne kontrovers kan foretages ved hjælp af den species-specifik isotopfortynding (SS-ID), som er den eneste metode, der muliggør korrigering af interkonversioner af specier, der dannes under den analytiske procedure. Anvendelse af SS-ID til afklaring af denne kontrovers er et af de vigtigste formål med arbejdet i denne afhandling.

Dette ph.d.-projekt sigter mod udvikling, validering og anvendelse af en ny analytisk metode til simultan specieanalyse af Cr(III) og Cr(VI) i fødevarer så som mejeriprodukter, kød og kødprodukter, brød, morgenmadsprodukter og ris. Til dette formål anvendes højtydende væskekromatografi (HPLC) koblet til induktivt koblet plasma massespektrometri (ICP-MS). Speciespecifik isotopfortynding (SS-ID) blev anvendt til speciekvantificering og til korrektion af deres potentielle gensidige inter-konvertering.

Ekstraktion af Cr specier blev udført ved kompleksdannelse af Cr(III) med ethylendiamintetraeddikesyre (EDTA) og ved derivatisering af Cr(VI) med 1,5-diphenylcarbazid (DPC) i samme analytiske forløb. Ved denne procedure er Cr(VI) ikke direkte kompleksbundet med DPC, men reduceres først til Cr(III), og derefter komplekseres Cr(III) med DPC. HPLC-adskillelsen af kompleksene blev udført under anvendelse af en kort mikrobore anion-bytter HPLC-kolonne, som separerede de to specier på mindre end 3 minutter. Metoden blev valideret ved hjælp af en såkaldt "nøjagtighedsprofil metode" (accuracy

profile) ved at udføre seks måleserier i to replikater på (seks) forskellige dage over en tidsperiode på to måneder. Kvantificeringsgrænsen var henholdsvis 0,013 μg kg^{-1} for Cr(III) og 0,049 μg kg^{-1} for Cr(VI). Den systematiske fejl (bias) i måleområdet varierede fra 0,01 til 0,11%, hvorimod variationskoefficienten med hensyn til repeterbarhed (CVr) varierede fra 2,9 til 11,6% (afhængigt af analytniveauet) for Cr(III) og fra 6,7 til 11,8 % for Cr(VI). Tilsvarende varierede variationskoefficienten med hensyn til reproducerbarhed (CVR) fra henholdsvis 6,8 til 13,0% for Cr(III) og fra 6,8 til 25,9% for Cr(VI).

Metoden blev med succes anvendt til analyse af et udvalg af forskellige typer fødevarer, herunder mejeriprodukter, kød og korn. Cr(VI) blev ikke kvantificeret i nogen af disse prøver, mens Cr(III) niveauerne varierede mellem 0,22 μg kg^{-1} (modermælkserstatning) og 350 μg kg^{-1} (morgenmadsprodukter med kakao). Cr(III) niveauerne i alle prøver var sammenlignelige med niveauerne af total Cr bestemt i de samme prøver ved brug af ICP-MS. Resultaterne opnået i de forskellige analyserede fødevareprøver understøtter hypotesen om, at al Cr, der er til stede i fødevarer, er i form af Cr(III), hvilket klart afviser rapporterne om tilstedeværelsen af Cr(VI) i fødevarer. De opnåede resultater med dette arbejde bidrager derfor afklaring af den aktuelle tvist om speciering af Cr i fødevarer og den mulige tilstedeværelse af Cr(VI) i fødevarer.

Metoden blev desuden anvendt til at evaluere den potentielle indvirkning af forskellige kulinariske processer på skæbnen af Cr-specier i modermælkserstatning mælk, skummetmælk og kødprøver. Tilberedningsprocedurerne bestod af kogning af mælkeprøverne ved 70°C og 100°C og stegning af kød uden (95°C) og med olie (120°C). Resultaterne viste ingen signifikante forskelle mellem indholdet af Cr-specierne i rå og kogte prøver, hvilket tydeligt indikerer, at der ikke skete nogen transformation mellem specierne under behandlingen.

En risikoanalyse blev udført under hensyntagen til niveauerne af Cr(III) fundet i tilberedt oksekød og mælk og viste en meget lav risiko for forbrugerne ved konsum af disse produkter.

Dette arbejde har bidraget til at opnå mere viden om Cr-speciering i fødevarer og har udviklet af et vigtigt analytisk værktøj til vurdering af risikoen fra Cr i fødevarer. Den udviklede metode har kontrol over potentiel interkonvertering af specier, der måtte dannes under den analytiske procedure. Det bidrog også til at løse den nuværende kontrovers om tilstedeværelsen af Cr(VI) i fødevarer. Til fremtidigt arbejde kan den udviklede og validerede metode anvendes på andre typer matricer, hvor det er interessant at kunne afklare Cr specieringen, f.eks. i forbindelse medmiljøprøver eller farmaceutiske prøver.

List of publications

The PhD thesis is based on the publications listed below.

Paper I: **Saraiva M.**, Chekri R., Leufroy A., Guérin T., Sloth J.J., Jitaru P. Development and validation of a single run method based on species specific isotope dilution and HPLC-ICP-MS for simultaneous species interconversion correction and speciation analysis of Cr(III)/Cr(VI) in meat and dairy products, Talanta 222 (2021) 121538. DOI: 10.1016/j.talanta.2020.121538

Paper II: **Saraiva M.**, Chekri R., Guérin T., Sloth J.J., Jitaru P. Chromium speciation analysis in milk and meat samples by species-specific isotope dilution and HPLC- ICP-MS and their fate after various types of cooking, Food Addit. Contam. A (2021). DOI: 10.1080/19440049.2020.1859144

Paper III: **Saraiva, M.**, Jitaru, P., Sloth J.J. Speciation analysis of Cr(III) and Cr(VI) in bread and breakfast cereals using a novel analytical approach based on species-specific isotope dilution and HPLC-ICP-MS (2021) (to be submitted to Journal of Food Composition and Analysis).

Paper IV: **Saraiva, M.**, Jitaru, P., Sloth J.J. Speciation analysis of Cr(III) and Cr(VI) in rice using species-specific isotope dilution and HPLC-ICP-MS (2021) (to be submitted to Journal of Agricultural and Food Chemistry).

Contributions to international conferences

Saraiva M., Leufroy A., Sloth J J., Jitaru P. Development of a method for ultra-trace speciation analysis of chromium in foodstuff by HPLC-ICP-MS using species specific isotope dilution. Poster presentation at European Winter Conference on Plasma Spectrochemistry 2019, Pau, France

Saraiva M., Jitaru P., Sloth J.J., Development and application of a novel analytical methodology for simultaneous speciation analysis of Cr(III) and Cr(VI) in foodstuffs by HPLC-ICP-MS using species-specific isotope dilution. Oral presentation at 4th IMEKOFOODS 2019, Brussels, Belgium

Saraiva M., Leufroy A., Chekri, R., Guérin, T., Jitaru P., Sloth J.J. Development and validation of a method for ultra-trace speciation analysis of chromium in foodstuffs by HPLC-ICP-MS using species-specific isotope dilution. Poster presentation at 9th International Symposium on Recent Advances in food analysis 2019, Prague, Czech Republic

Jitaru P., **Saraiva M.**, Leufroy A., Chekri, R., Sloth J.J. Development and application of a primary method for speciation analysis of chromium in foodstuffs by HPLC coupled to ICP-MS using SS-ID. Oral presentation at 13th International Food Data Conference 2019, Lisbon, Portugal

List of abbreviations and acronyms

AAS	Atomic absorption spectrometry
AES	Atomic emission spectrometry
ATSDR	Agency for Toxic Substances and Disease Registry
CIAAW	Commission on isotopic abundances and atomic weights
COFRAC	French accreditation committee
CRI	Collision-reaction interface
CRM	Certified reference material
DPC	1,5-Diphenylcarbazide
DRC	Dynamic reaction cell
DR-FTIR	Diffuse reflectance - Fourier transform infrared
EC	European commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ETA	Electrothermal atomization
ETAAS	Electrothermal atomic absorption spectrometry
FAAS	Flame atomic absorption spectrometry
FDA	Food and Drug Administration (USA)
GFAAS	Graphite furnace atomic absorption spectroscopy
HNO_3	Nitric acid
HPLC	High performance liquid chromatography
HR-CS	High-Resolution Continuum Source
IARC	International Agency for Research on Cancer
IC	Ion chromatography
ICP-AES	Inductively coupled plasma-atomic emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of detection
LOQ	Limit of quantification
MilliQ	Ultra-pure water - Pure water system
m/z	Mass to charge ratio
NAA	Neutron activation analysis
NIST	National Institute of Standards and Technology
OES	Optical emission spectrometry
RP	Reversed phase
SEC	Size-exclusion chromatography
SSID-MS	Species specific isotope dilution mass spectrometry
SPE	Solid phase extraction
TDI	Tolerable Daily Intake
USEPA	United States Environmental Protection Agency
USGS	United States Geological Survey
WHO	World Health Organization

Chemical formulas and denomination of chromium compounds

Cr	Chromium	Na_2CrO_4	Sodium chromate
CrIII or Cr(III)	Trivalent chromium	H_2CrO_4	Chromic acid
CrVI or Cr(VI)	Hexavalent chromium	FeCr	Ferrochromium
$Cr_2O_7^{2-}$	Dichromate	K_2CrO_4	Potassium chromate
$FeCr_2O_4$	Chromite	$SrCrO_4$	Strontium chromate
Cr_2O_3	Chromium sesquioxide	$ZnCrO_4$	Zinc chromate
$CrCl_2$	Chromium chloride	$PbCrO_4$	Lead chromate
CrO_2	Chromium dioxide	$Na_2Cr_2O_7$	Sodium dichromate
CrO_3	Chromium trioxide	$(NH_4)_2Cr_2O_7$	Ammonium dichromate
CrO_4^{2-}	Chromate	$K_2Cr_2O_7$	Potassium dichromate

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Introduction and Aim

Chromium (Cr) is a chemical element with different physico-chemical and toxicological characteristics, depending on its species. It is used extensively in various industrial processes such as electroplating, metal finishing, leather tanning and pigments production. Cr industries produce waste that is discharged into the surrounding air, water and terrestrial environments and, therefore, having adverse effects on the ecosystem and, ultimately, on human health through the food chain (Kotaś and Stasicka, 2000; Yu-Ling et al., 2001; Séby et al., 2003).

The most stable species of Cr are Cr(III) and Cr(VI), having very different toxicity and mobility in the environment and also in the human body. Cr(III) is poorly soluble and relatively immobile in aqueous environments compared to Cr(VI), which is highly soluble and consequently, more bioavailable (Hamilton et al., 2018; Markiewicz et al., 2015). Cr(VI) has been recognized for several decades as being mutagenic and carcinogenic (IARC, 1990), whereas for Cr(III) the European Food Safety Authority (EFSA) stated relatively recently that there is no convincing evidence of beneficial effects of Cr(III) for healthy people (EFSA, 2014a). Hence the interest in Cr speciation, which was focused almost entirely on Cr(VI), shifted to the determination of both Cr species, Cr(III) and Cr(VI), especially in foodstuffs.

Speciation analysis is an important tool to study the effects of different species, and the data obtained can be used to define the maximum permitted concentration for Cr species in food (Heena and Malika, 2020). However, the simultaneous speciation analysis of Cr(III) and Cr(VI) tends to be more challenging than the total concentration of Cr mainly due to its high chemical instability. In addition, Cr species are generally present at trace levels in food samples, which requires the use of very sensitive and highly selective analytical tools for their determination.

The main sources of Cr in the human diet are meat, dairy products, potatoes, bread, rice, tea, spices and chocolate (Alberti-Fidanza et al., 2002; Karak and Bhagat, 2010; Lendinez et al., 2002; Novotnik et al., 2013, Tiwari et al., 2017, Chen et al., 2020).

In 2014, EFSA stated that Cr(VI) species is not present in foodstuffs due to the specific features (reductive potential) of the organic (food) matrices that reduces Cr(VI) to Cr(III), and hence total Cr in foodstuffs consists of Cr(III) solely. However, several recent studies reported the presence of Cr(VI) in various foodstuffs such as milk, bread, cereals, meat, tea and rice (Soares et al., 2000/2010; Ambushe et al., 2009; Chen et al., 2014; Umesh et al., 2015; Pyrzynska, 2017; Shittu et al., 2020; Chen et al., 2020). The quantification of Cr(VI) in these samples has been disputed and proposed as being most likely due to an analytical artefact formed during the extraction or conversion of Cr(III) to Cr(VI) during the extraction and/or the HPLC separation, given that these processes cannot be assessed by conventional speciation analysis methods (Novotnik et al., 2013; Soares et al., 2010; Milačič and Ščančar, 2020).

The determination of Cr(III) and Cr(VI) is conventionally based on the selective extraction of Cr(III) and/or Cr(VI) followed by the determination of each species by measuring the total Cr content in the extract without prior chromatographic separation (*off-line* approaches) (Kovács et al., 2007; Uluozlu et al., 2009; Mandiwana et al., 2011; Chen et al., 2014). These approaches are prone to the formation of analytical artefacts, because interconversions of the species may occur during the analytical process (mainly during the sample preparation) without any possibility of evaluating these side effects when the total Cr is measured in the extract fraction (Novotnik et al., 2013; Vacchina et al., 2015; Milačič et al., 2018; Milačič and Ščančar, 2020).

Cr speciation analysis shifted relatively recently towards carrying out species separation by using high performance liquid chromatography (HPLC) before the on-line detection by atomic spectrometry, especially inductively coupled plasma-mass spectrometry (ICP-MS) (*on-line* approaches) (Pyrzynska, 2016; Marcinkowska et al., 2016; Hernandez et al., 2017,2018; Shittu et al., 2020; Milačič and Ščančar, 2020). Nevertheless, these (on-line) approaches are not *per se* capable to assess species interconversion during the analytical process. In fact, SS-ID in combination with HPLC-ICP-MS technique has proven to be the only analytical approach suitable for simultaneous speciation analysis of Cr(III) and Cr(VI) in foodstuffs due to its capability to quantify the species and to correct for their potential interconversion during the same analytical run (USEPA, 2014; Martone et al., 2013; Milačič and Ščančar, 2020).

SS-ID, which is based on the measurement of isotope ratios in a sample whose analyte isotopic composition is altered by the addition of a known amount of an isotopically modified analyte (spike) (Rodriguez-Gonzalez and Alonso, 2019), allows the species quantification and interconversion correction in the same analytical run (Kingston et al., 1998; Ma et al., 2008; Guidotti et al., 2015; Drinčić et al., 2018; Caporale et al., 2019; Saraiva et al., 2021). Another advantage of SS-ID is that once the isotopic equilibrium between the sample and the spike(s) is achieved, the matrix effects, the species loss (e.g. incomplete extraction) or transformation (e.g. oxidation and/or reduction) during the analytical process do not influence the accuracy. This is due to the measurement of isotopic ratios rather than the absolute or relative signals as in the case of conventional analytical methods (Rodriguez-Gonzalez and Alonso, 2019). It is in this context also worth to highlight that SS-ID is considered a primary analytical method, hence, it counteracts the lack of certified reference materials (CRM) in terms of Cr speciation analysis (Tirez et al., 2003; Novotnik et al., 2012; Zuliani et al., 2013; Wolle et al., 2014).

The main aim of the present study was to develop and validate new analytical approaches for simultaneous (single run) and accurate speciation analysis of Cr(III) and Cr(VI) in foodstuffs by HPLC-ICP-MS and SS-ID using selective species complexation/derivatisation with full capacity to correct for any species interconversion and accurate quantification of the contents of Cr(III) and Cr(VI).

The PhD project was divided into the following objectives:

Objective 1: *Development and validation of method(s) by HPLC-ICP-MS and using SS-ID for Cr(III) and Cr(VI) quantification in foodstuffs*

Objective 2: *Investigation of Cr species stability during processing of milk and meat samples*

Objective 3: *Clarification of the formation of Cr(VI) artefacts during the analysis of bread, breakfast cereals and rice.*

An overview of the thesis content is also provided hereafter.

Chapter 1 describes the work done in papers I-IV, while Chapter 2 addresses the toxicity and sources of Cr as well as the main analytical aspects in terms of Cr speciation analysis in food. Chapter 3 presents the data related to the quantification of total Cr in milk, meat, bread, breakfast cereals and rice, including the analytical approaches employed for this purpose (Paper II, III and IV). Chapter 4 presents the development and validation of the analytical procedure for Cr speciation analysis in food samples (papers I, III and IV). Chapter 5 deals with the study of the stability of Cr(III) and Cr(VI) in milk and meat during their cooking (paper II) and also with the risk assessment for the Danish and French population related to the consumption of these foods. Chapter 6 deals with the general conclusions in terms of the scientific output of the PhD project and future perspectives.

Finally, the training courses that I attended to acquire the doctoral credits, but most importantly, the complex PhD work that was carried out at high scientific levels at the Anses (Trace Metals and Minerals unit) and DTU Food (Applied Analytical Food Chemistry) have contributed to my growing as an analytical chemist notably in the Food chemistry field.

CHAPTER 1

SUMMARY OF THE PAPERS INCLUDED IN THE PhD THESIS

This PhD thesis is based on four research papers dealing with the quantification of Cr(III) and Cr(VI) in various foodstuffs and their interconversion either during the analytical procedure or during different heat (culinary) treatments. A short summary of the papers is given in the paragraphs below.

Paper I: The aim of this study was to develop and validate a new analytical approach for simultaneous (single run) and accurate speciation analysis of Cr(III) and Cr(VI) in foodstuffs by HPLC-ICP-MS and SS-ID with full capacity to correct for any species interconversion. The species extraction was achieved by sequential complexation of Cr(III) with ethylenediaminetetraacetic acid (EDTA) and derivatisation of Cr(VI) with 1,5-diphenylcarbazide (DPC). The method was validated by using the accuracy profile approach; for this purpose, spiked samples were analysed in duplicate on six different days over a timespan of two months. The quantification limit was 0.013 μg kg^{-1} for Cr(III) and 0.049 μg kg^{-1} for Cr(VI), respectively. The measurement bias corresponding to the validity domain ranged from 0.01 to 0.11%, whereas the coefficient of variation in terms of repeatability (CV_r) varied from 2.9 to 11.6 % (depending on the analyte level) for Cr(III) and from 6.7 to 11.8% for Cr(VI). Similarly, the coefficient of variation in terms of intermediate reproducibility (CV_R) ranged from 6.8 to 13% for Cr(III) and from 6.8 to 25.9% for Cr(VI), respectively. The method was successfully applied to the analysis of infant formula milk, semi-skimmed milk and meat. Cr(VI) was not quantified in any of these samples while Cr(III) levels ranged between 2.7 and 4.7 μg kg^{-1}, which were comparable with the levels of total Cr analysed in the same samples by ICP-MS.

Paper II: The aim of this study was to assess potential interconversions of Cr(III) and Cr(VI) in milk and bovine meat during two cooking procedures consisted of boiling the milk samples at 70 °C and 100 °C and frying the bovine meat without (95 °C) and with oil and (120 °C). The analytical procedure used for this purpose is described in Paper I. ANOVA was used to compare the mean Cr species levels and showed no significant differences between raw and cooked samples. The results show that oxidation of Cr(III) to Cr(VI) did not occur during thermal cooking of milk and bovine meat samples, as reported by Kovács et al., 2007 and Lin et al., 2016 in similar heating treatment. A selection of 10 samples of each type were analyzed in terms of total Cr as well as Cr speciation (Cr(III) and Cr(VI)). Cr(VI) was not quantified in any of these samples, whereas Cr(III) levels ranged from 0.22 (infant formula milk) up to 80 μg kg^{-1} (chorizo sausage). Additionally, Cr(III) and total Cr levels were comparable, hence demonstrating that in the samples analyzed in this study, Cr was found exclusively as Cr(III), as stated by EFSA, 2014a.

Paper III: This study aims to clarify the production of Cr(VI) during the analysis of breads and breakfast cereals, as this side effect was reported by several authors (Soares et al., 2010, Umesh et al., 2015, Mathebula et al., 2017) The analytical approach (SS-ID HPLC-ICP-MS) used described in Paper I. The method was validated for this matrix through the accuracy profile approach, making 3 series of measurements in duplicate on (five) different days (one month). The limit of quantification was 0.014 µg kg^{-1} for Cr(III) and 0.047 µg kg^{-1} for Cr(VI), respectively. The measurement bias corresponding varied from -0.31 to 0.49%, while the coefficient of variation in terms of repeatability (CV_r) varied from 1.3 to 4.4% for Cr(III) and for 0.6 to 7.9% for Cr(VI). Likewise, the coefficient of variation in terms of intermediate reproducibility (CV_R) varied from 1.3 to 4.4% for Cr(III) and from 2.0 to 8.9% for Cr(VI), respectively. The method was successfully applied to the analysis of a selection of breads and breakfast cereals. Cr(VI) was not quantified in any of these samples, while Cr(III) levels ranged between 5.2 and 176.3 µg kg^{-1} for bread and 23.8 and 350.3 µg kg^{-1} for breakfast cereals, which were comparable to the total Cr levels analyzed in the same samples by ICP-MS. The results are in line with those reported in breads by Roussel et al., 2007 and breakfast cereals by Farre and Lagarda, 1986 and Roussel et al., 2007.

Paper IV: This study addresses the measurement of Cr(III) and Cr(VI) in rice samples using a novel analytical approach based on SS-ID HPLC-ICP-MS and hence investigate the presence of Cr(VI) in rice samples, as reported by Chen et al. (2020). Cr(VI) was not detected in any of the rice samples while Cr(III) levels ranged between 0.59 and 103.87 µg kg^{-1}, which, again, are highly compatible with the levels of total Cr analysed in the same samples by using ICP-MS.

Cr(VI) was added to a type of rice (Basmati) and with the use of SS-ID it was possible to verify that Cr(VI) was converted to Cr(III) during the analytical procedure due to the reducing power of foodstuffs (Hamilton et al., 2018; Milačič and Ščančar, 2020). SS-ID approach with sequential species complexation/derivatisation is a powerful analytical tool for accurate and precise quantification of Cr(III) and Cr(VI) at trace levels , which also allows for correction of any species interconversion during the analytical process (Hamilton, et al., 2018; Milačič and Ščančar, 2018).

CHAPTER 2
PROPERTIES, USE, SOURCES AND TOXICITY OF CHROMIUM

Abstract

Chromium (Cr) is an element widely present in the earth's crust, while in the environment it originates from both natural (volcanic activity, rocks erosion, etc.) and anthropogenic sources (industries of chromium production, etc.). Most of the Cr in the air will eventually settle and end up in the surface waters and/or the soil. Cr binds strongly to soil particles, making it difficult to move to groundwater. In the water (environmental) systems, most of the Cr found is retained in the sediments, hence becoming immobile. Only a small part of the Cr that ends up in the water will eventually dissolve. Cr in food results primarily from its presence in the environment (water, soil and plants) due to natural or anthropogenic sources.

Cr has different levels of toxicity depending on its chemical species. Cr(III) was for a very long time considered as beneficial to human health but since then its benefit in healthy people has not been verified (EFSA 2014), while Cr(VI) was considered cancerinogenic and mutagenic by inhalation.

The levels of total Cr in food differ greatly between foodstuffs, with the highest values found in meat products and seafood, followed by oils, bread and cereals. The lowest levels were found in milk, fruits and vegetables. Despite the improbable presence of Cr(VI) in foodstuffs because its reducing features, some authors reported its presence in dairy & meat products, cereals, mushrooms, tea, beer and wine. However, these results are highly questionable, as shown throughout this work.

2.1. Physical and chemical properties of chromium

Chromium (Cr, atomic number=24) is a chemical element whose name is derived from the Greek word χρῶμα, *chrōma*, meaning *colour*, because it exhibits various colours including black, gray, green, blue, violet, orange, yellow and red, depending on its chemical species. Cr metal is white, hard, lustrous, and brittle and is extremely resistant to ordinary corrosive reagents; this resistance accounts for its extensive use as an electroplated protective coating.

Cr belongs to the VIB group of the periodic table; it has four stable isotopes with different abundance in nature, as follows: ^{52}Cr (83.79%), ^{53}Cr (9.50%), ^{50}Cr (4.35%) and ^{54}Cr (2.36%) (CIAAW, 2019) (none of its natural isotopes are radioactive). Cr is present in several oxidation states varying from 0 to VI, Cr(III) and Cr(VI) being the two most stable and widespread in the environment but very different in relation to their mobility and bioavailability. In general, Cr(III) compounds are relatively immobile and poorly soluble compared to highly mobile, soluble and, consequently, more bioavailable Cr(VI) compounds (Cornelis et al., 2005; Hamilton et al., 2018; Markiewicz et al., 2015). The intermediate Cr

oxidation states of such as Cr(II), Cr(IV), Cr(V) are unstable and they are easily converted to trivalent and/or hexavalent Cr, respectively (Metze et al., 2005).

The predominant forms of Cr can undergo a series of transformations, changing from one to another under the influence of various physical-chemical processes, especially pH changes that lead to chemical imbalance. In addition, the redox reactions, precipitation/dissolution and adsorption/desorption processes can cause Cr conversions (Unceta et al., 2010; Wolf et al., 2007) in environmental (atmosphere, soil, water) compartments and/or in food samples. The main physical and chemical properties of Cr are provided in Table 2.1.

Table 2.1. Physical and chemical properties of Cr (Lenntech, 2019)

Element	CAS Nº	Atomic Nº	Molar mass (g/mol)	Density (g/cm^3)	Instable oxidation state	Stable oxidation state	Water solubility	Natural state
Chromium (Cr)	7440-47-3	24	51.996	7.19	+1, +2, +4, +5	+3, +6	Insoluble	Solid

2.2. Uses of chromium

The uses of Cr are linked to its chemistry, its reactivity and its properties. Cr is a relatively abundant element in Earth's crust, with chromite ($FeCr_2O_4$) being the most abundant and most stable form. Chromite is often reduced with carbon in a furnace, producing the alloy ferrochromium, which contains iron and chromium in an atom ratio of approximately 1:2. To obtain pure Cr, chromite is first treated with molten alkali and oxygen, converting all of the chromium to the alkali chromate, and the latter is dissolved in water and eventually precipitated as sodium dichromate, $Na_2Cr_2O_7$. The dichromate is then reduced with carbon to Cr sesquioxide (Cr_2O_3), and that oxide in turn is reduced with aluminium to give the Cr metal. Cr is also added to iron and nickel in the form of ferrochromium (FeCr) to produce alloys specially that are highly resistant to corrosion. Cr alloys are used to fabricate a large panel of products such as oil tubing, automobile trim, and cutlery. Chromite is also used as a refractory and as a raw material for the production of Cr chemicals.

As mentioned above, the most abundant mineral is chromite, $FeCr_2O_4$, and it is the only one to be exploited for commercial purposes (Köleli and Demir, 2016). In 2019, the worldwide production of $FeCr_2O_4$ was 44,000 tons, with a contribution of 39% from South Africa, 22.7% from Turkey, 15% from Kazakhstan, 9.2% from India, 5% from Finland and 9.1% from the other countries (U.S. Geological Survey, 2019). Other uses of Cr include metallurgy (production of stainless steel, ferrous and nonferrous alloys), refractory industry (high temperature-resistant (melting point) materials used as linings for furnaces) and chemical manufacturing (textile dyes, production of pigments, electroplating, leather tanning ($K_2Cr_2O_7$) and wood preservatives). The magnetic properties of Cr make it also perfect for magnetic tapes, and Cr dioxide (CrO_2) is used in recording tapes.

2.3. Chromium sources

Cr compounds are discharged into air, water and soil through natural processes (rocks & soil erosion and volcanic eruptions) and human (industrial) activities in their more stable oxidation states such as Cr(III) and Cr(VI) (EFSA, 2014a). Information on the estimates of in the atmosphere emission of Cr by anthropometric sources and estimated Cr(VI) fractions as well as their characteristics and the degree of oxidation are described in Table A1 and Table A2. Additionally, more information concerning Cr sources in the environment (with focus on atmosphere, soil and water system) and foodstuffs is provided in the next sections.

2.3.1. Environment

Large quantities of Cr compounds are released into the environment, which has an adverse effect on the ecosystem (Kotaś and Stasicka, 2000; Ellis et al., 2002; Séby et al., 2003; Jyothi et al., 2013; Namiesnik et al., 2012; Dotaniya et al., 2014; Coetzee et al., 2020). The levels of Cr found in environment, including atmosphere, soil and water are summarised in Table 2.2.

Table 2.2. Cr levels in environmental samples (Shanker and Venkateswarlu, 2011)

Soil	**Concentration (mg kg^{-1})**	**Water**	**Concentration (mg L^{-1})**
Natural	5-3 000	Fresh water	0-117
Serpentine	634-125 000	Seawater	0-0.5
World	100-300	**Atmosphere**	**Concentration (ng m^{-3})**
USA	25-85	Air	1-545 000
Canadian	100-5 000	**Plants and animals**	**Concentration (mg kg^{-1})**
Japanese	87 (mean)	Plants	0.006-18
Swedish	74 (mean)	Animals	0.03-1.6
Sediments	0-31 000		

The results published in 2011 by Shanker show a great variation in Cr concentrations in soils, depending on its location and its mineral base. Equally, for the sediments, water and air analyzed, there was a great variation for total Cr concentrations, while for plants and animals, the variation is not so noticeable. Environment pollution by Cr is due, among other things, to the spreading of Cr effluents, to chromium dust emitted into the atmosphere, to the sludge resulting from the treatment of effluents or to solid waste from tanneries. Of the 82 minerals reported in the environment, 23 are Cr(VI)-containing ones. As it was previously mentioned, the main Cr mineral is chromite (Fe, Mg, Al)Cr_2O_4, that contains 45–80% Cr as Cr(III) and Cr(VI) (Coetzee et al., 2020).

(a) Chromium in the atmosphere

Due to the extremely high boiling point of chromium (2676°C), gaseous Cr is not encountered in the environment (atmosphere). The atmospheric transformation and Cr transport largely occurs in the liquid and solids phases (i.e., droplets and particles) or, more generally, as aerosols (Seigneur and Constantinou, 1995). The origin of the Cr aerosols in the atmosphere is 60 to 70% anthropogenic and 30-40% natural sources, such as volcanic eruptions or rock/soil erosion (Seigneur and Constantinou, 1995). The anthropogenic sources that contribute to Cr in the atmosphere are metallurgical industries, production of refractory bricks, electroplating, fuel combustion and Cr chemical production, mainly chromate and dichromate pigments, as previously mentioned.

The transport and deposition of the Cr particles present in the atmosphere depends largely on the size of the particles and density (Duce, 2001; Gheisari et al., 2010). The deposition rates of these particles tend to be higher in urban areas, since atmospheric Cr levels are higher when compared to rural areas (Azimi et al., 2003). Cr chemistry in the atmosphere is similar to that of aqueous systems with reactions of precipitation, complexation and oxidation, which influence the abundance and proportion of Cr forms (Kotaś and Stasicka, 2000).

Cr(VI) reduction is far more likely than oxidation of Cr(III) in the atmosphere due the presence of reducing agents (such as V^{2+}, Fe^{2+}, H_2S, HSO_3^-, NO_2^-, and organic materials) as well as the atmosphere acidity (Kimbrough et al., 1999). In the presence of ozone, few compounds are capable of oxidizing Cr(III) to Cr(VI), but they occur in too low concentrations to produce measurable conversions in the atmosphere. Chromate may also react with other metallic species, precipitating as lead or zinc chromate (Seigneur and Constantinou, 1995). The present Cr species depends on the medium pH value, when the values are lower, the favoured species of Cr(III) are: $Cr(H_2O)_6^{3+}$ and $CrOH^{2+}$, whereas for Cr(VI) they are mainly $HCrO_4^-$ and $Cr_2O_7^{2-}$. The load of atmospheric Cr(VI) can be alleviated by the formation of very low solubility of chromates due to the loss of metal ions such as Pb^{2+}, Cu^{2+} and Zn^{2+} (Kotaś and Stasicka, 2000). At high pH values, $Cr(OH)_3$.aq droplets precipitate in the presence of iron compounds, resulting in mixed hydroxides such as (Cr, Fe)$(OH)_3$. In the presence of chloride or fluoride, mixed chlorine and fluorine complexes are formed, which increase their solubility (Seigneur and Constantinou, 1995). Approximately one-third of the atmospheric chromium releases are believed to be in the hexavalent form (Johnson et al. 2006).

In the Netherlands, the Cr levels recorded were 2 to 5 ng m^{-3}; in Continental Europe of 1-140 ng m^{-3}; in European industrial areas, the values found were 5-200 ng m^{-3}, while in Japan and Hawaii they ranged between 20-70 ng m^{-3} (WHO, 2000). The levels of Cr in the air range from 0.07 to 1.1 ng m^{-3} in remote areas, such as the Arctic and Antarctic poles (WHO (2003).

(b) Chromium in the soil

Cr concentration in the soil is typically associated with the precipitation and washing of atmospheric Cr-containing particles, industrial activity and ultramafic and metamorphic rocks, such as peridotites and serpentinites, and their derived soils (Chrysochoou, 2016).

Cr(VI) is the form of chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) is commonly found in contaminated soils because these forms precipitate readily in the presence of metal cations (especially Ba^{2+}, Pb^{2+}, and Ag^{+}). Chromium can also occur in the +III oxidation state, depending on pH and redox conditions (Wuana and Okieimen, 2011). The reduction of Cr(VI) to Cr(III) occurs in soil rich in organic matter, S^{2-} and Fe^{2+} ions under anaerobic conditions (Wuana and Okieimen, 2011). The most mobile forms of Cr(VI) in soil are CrO_4^{2-} and $HCrO_4^{-}$ ions, but insoluble species such as $BaCrO_4$ and $PbCrO_4$ may also be present (Kotaś and Stasicka 2000). Cr(III) in soil is present mostly as insoluble $Cr(OH)_3$.aq or adsorbed to soil components, which prevents Cr leaching into the ground water or its uptake by plants. Crops contain systems that arrange the Cr-uptake to be low enough not to cause any harm.

Cr species present in the soil depend on the soil properties, such as pH, particle size, cation-exchange capacity, content of organic matter, content and type of clay minerals and Al, Fe and Mn oxides, redox potential, microbiological activity and proximity of industrial activity (Dhal et al. 2013; Leśniewska et al., 2017; Wu et al., 2020). Coupling the oxidation, reduction and complexing processes into the Cr cycle in soil are presented in Fig. 2.1.

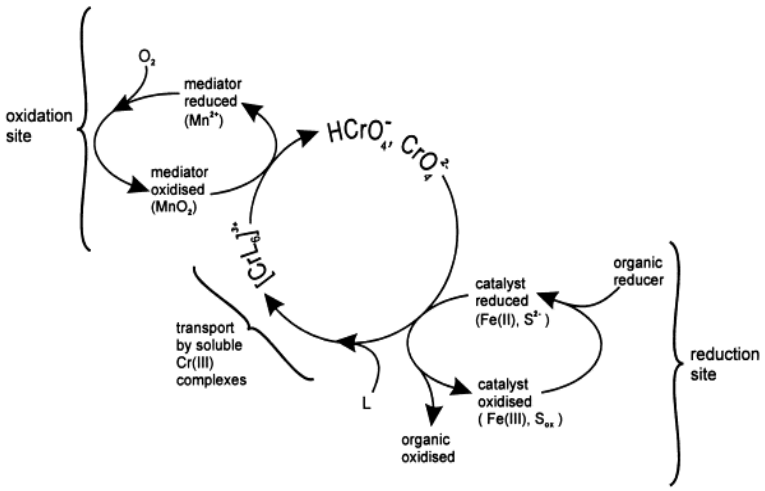

Figure 2.1. The cycle of Cr oxidation–reduction in soil (Kotaś and Stasicka, 2000)

Cr levels in soil vary according to area and the degree of contamination from anthropogenic Cr sources. Cr levels in soil ranging from 1 to 1000 mg kg^{-1} with an average concentration ranging from 14 to about 70 mg kg^{-1} (WHO, 2000).

Same authors reported values of Cr(VI) in uncontaminated soils between 0.01-40 mg L^{-1} (Cathum et al., 2002), 10-100 mg kg^{-1} (Tirez et al., 2003), 141-341 µg g^{-1} (D'Amore et al., 2005) and 0.8-3.4 mg kg^{-1} (Oliveira et al., 2013).

(c) Chromium in the water environment

Cr is present in ground and surface water due to both natural and anthropogenic sources. Natural sources include rocks erosion, evapotranspiration, depositions due to wind, leaching from soil, run-off due to hydrological factors, and biological processes in the aquatic environment. Anthropogenic input is due to agriculture, use of fertilizers, manures and pesticides, animal husbandry activities, inefficient irrigation practices, wood deforestation, aquaculture, pollution due to industrial effluents and domestic sewage, mining, and recreational activities (Khatri and Tyagi, 2014). In aqueous systems, Cr moves via surface runoff and through groundwater. In rivers and lakes, transport mechanisms are associated with suspended particles although in the latter case, the large amount of organic matter further favours the reduction of Cr(VI) to Cr(III) leading to its immobilisation. In both aqueous systems, the precipitated and dissolved forms exist in equilibrium (Chiffoleau, 1994; Rusiniak et al., 2020).

The distribution of Cr species in the aquatic environment will depend on its origin. Cr(VI) will dominate in the metallurgical waste water, metal finishing and refractory industry and production or application of pigments (chromate colour pigments and corrosion inhibiting pigments). Cr(III) is found mainly in effluents from tannery, textile (printing, dyeing) and decorative coating industry (Nriagu, 1988).

The factors that are responsible for the occurrence of high concentrations of Cr(III) in surface waters are the pH, oxygen concentration, nature and concentration of reducing agents and the presence of complexing agents (Janssen et al., 2020). It is worth to note that at intermediate pH values, the Cr(III)/Cr(VI) ratio is dependent on O_2 concentration (Kotaś and Stasicka, 2000).

The behaviour of Cr(III) and Cr(VI) in the aquatic environment is complex and can be summarized according to the biogeochemical cycle shown in Fig. 2.2.

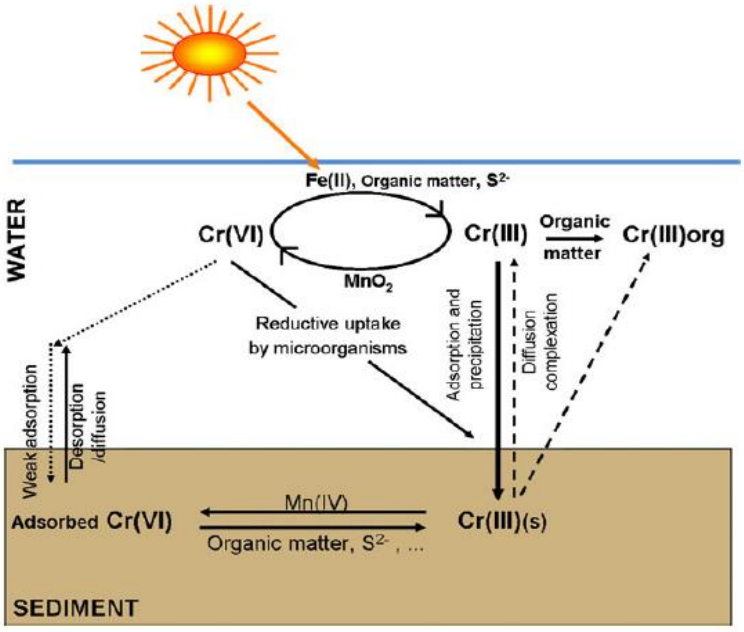

Figure 2.2. Biogeochemical processes affecting Cr species distribution in the water-sediment system (Unceta, 2010 adapted from Kotaś and Stasicka, 2000 and Richard and Bourg, 1991)

The chemical form of Cr conditions its mobility and, therefore, bioavailability. The reducing agents naturally present in the water, Fe(II), S^{2-} and organic substances are capable to reduce Cr(VI) to Cr(III), while oxidizing agents, such as MnO_2 and Mn^{+3} in sufficiently high concentrations can produce measurable yields of Cr(VI) (Kimbrough et al., 1999; Unceta et al., 2010).

In surface waters in which Cr(III) is dominant, the maximum allowable Cr concentration has been settled as 100 µg L^{-1} while the average concentration of Cr found was 0.2-1 µg L^{-1} in rainwater and 0.04-0.5 µg L^{-1} in seawater (WHO, 2017). Cr(III) and Cr(VI) levels in contaminated effluents and waters were 0.5-30 mg L^{-1} and 0.005-0.30 mg L^{-1}, respectively (Soares et al., 2009), in wastewater 0.215-0.245 mg L^{-1} for Cr(III) and 0.206-0.212 mg L^{-1} for Cr(VI) (Muhammad et al., 2005). In natural waters the concentration for Cr(III) and Cr(VI) found were 0.72 µg L^{-1} and 1.14 µg L^{-1}, respectively (Jia et al., 2017). In tap water Cr(III) found was 150 µg L^{-1} and for Cr(VI) was 48 µg L^{-1} (Hosseini et al., 2007).

2.3.2. Chromium in foodstuffs

Due to its toxicity, especially in its Cr(VI) form, Cr determination in foods is important to assess its impact on humans health via foodstuffs consumption. . According to the Scientific committee in food (EC, 2003), the highest levels of Cr are found in meat products (≈230 µg kg^{-1}) and seafood (≈120-470 µg kg^{-1}), followed by oils and fats (≈170 µg kg^{-1}), bread and cereals (≈150-220 µg kg^{-1}), sugar and preserves (≈130 µg kg^{-1}). The lowest levels are found in milk (≈10 µg kg^{-1}) and fresh fruits and vegetables (≈20 µg kg^{-1}) while Cr levels in uncontaminated drinking water are generally below 1 µg L^{-1} (EGVM, 2003). More recent values reported by EFSA show higher levels in seafood, such as mussels, oysters and shrimp with values of 128, 57 and 26 µg 100 g, respectively, followed by Brazil nuts, dates (dehydrated) and hazelnut with values of 100, 29 and 12 µg of Cr per 100 g. Fruits and vegetables, such as tomatoes, mushrooms and broccoli appear in third place with Cr values close to 20 µg 100 g. Meat, fish and eggs with values between 10 and 20 µg Cr per 100 g (EFSA, 2010 and 2014).

Several authors reported Cr levels in various foodstuffs are these results are present in Table 2.3. In dairy products, the Cr levels varied between 0.43 and 47.5 ng mL^{-1}, in meat and fish samples the values were 18.3-2403 and 14.7-39.5 ng mL^{-1}, respectively. For bread and cereals, the values found varied between 5.82-224.8 ng mL^{-1}, 0.40-72.3 ng mL^{-1} in fruits and vegetables. For marmalades and chocolates bars, these values were 6-1428 ng mL^{-1} and in beverages, the Cr values found varied between 0.03 to 19.6 ng mL^{-1} (Roussel et al., 2007). An overview of the literature data on total Cr (Cr_{total}) in foodstuffs is provided in Table 2.3.

Table 2.3. Overview of the literature data on total Cr in foodstuffs

Group food	Cr_{total} (mg kg^{-1})	Reference
Dairy products:		
	Milk: 17-43 [a] ; 0.030 [b]	Farre and Lagarda, 1986
	Milk: 0.43-47.5 [c]	Roussel et al., 2007
	Strained yoghurt: 1.23; Salted yoghurt: 0.43	Güler, Z., 2007
	Dairy-based desserts: 95-232[b]	Chekri, et al., 2019
Meat and meat products:		
	Meat: 0.144; Fish: 0.141;	Farre and Lagarda, 1986
	Abdominal muscle: 0.018-0.088; Hepatopancreas: 0.042-0.18	Jorhem, L. et al., 1994
	Meat: 11.8-66.7	Alberti-Fidanza et al., 2002
	Meat: 2.93-4553	Roussel et al., 2007
	White meat: 0.12-0.15; Brown meat: 0.38-0.62	Perugini et al., 2014
	Beef: 0.004-1.540 [f]	Wang and Farid, 2015
	Infant food meat-based: 68.9	Chekri, et al., 2019
Fish and seafood:		
	Fish: 44.0-87.9	Alberti-Fidanza et al., 2002
	Sea bass fillets: 0.112-0.18 [b]	Beyza, et al., 2006
	Fish: 14.71-39.53	Roussel et al., 2007
	Mediterranean finfish and shellfish: 0.05-0.52 [b]	Kalogeropoulos et al., 2012
	Seafood: prawns, halibut and salmon: 0.12-0.15	Rasmussen et al., 2017
	Whitefish: 0.0020-0.0028	Mehdipour et al., 2018
	Seafood: 0.15-0.25	Barbosa et al., 2018
Cereals:		
	Cereals: 0.135;	Jorhem, L. et al., 1994
	Pasta: 54.9-107.7	Alberti-Fidanza et al., 2002
	Bread and Cereals: 5.82-224.8	Roussel et al., 2007
	Foodstuffs: 78.6 - 810.6 [b]	Lee et al., 2013
	Rice: 0.27-0.41	Rittirong et al., 2018
	Rice: 40.6-408 [a]	Chen et al., 2020
Fruits, vegetables and pulses:		
	Fruits: 0.037; Vegetables: 0.050	Farre and Lagarda, 1986
	Aromatic herbs: 0.01-1.11 [b]	Garcia, E., et al., 2000
	Vegetables: 41.9-62.6; Pulses: 81.8-89.4 [g]	Alberti-Fidanza et al., 2002
	Fruits: 0.40-72.3 ; Vegetables: 3.6-52 [g]	Roussel et al., 2007
	Raw and Processing Vegetables: 6.61-18.66 [d]	Kmiecik, et al., 2007
	Broad bean: 0.006-0.007; Broad pea: 0.011-0.016; French-bean: 0.014-0.017 [e]	Lisiewska et al., 2008
	Pumpkin and Amaranthus viridis: 0.1-0.9	Azi et al., 2018
	Infant food vegetable-based: 50.4	Chekri, et al., 2019
Drinks and Beverages:		
	Beverages: 0.03-19.60	Roussel et al., 2007
Sugar:		
	Marmalades and chocolates: 6.00-1428	Roussel et al., 2007

[a] $ng.g^{-1}$; [b] $\mu g.kg^{-1}$; [c] ng.mL; [d] g.100 g dry matter; [e] mg.100g fresh matter; [f] mg.120g fresh matter; [g] mg.100g dry matter

2.3.3. Chromium speciation analysis in foodstuffs

Speciation analysis of Cr in foods, drinking water and other edible materials is vital for a better comprehension of global Cr status and its impact on human health (Pyrzynska, 2017; Ambushe et al., 2009; Yu and He, 2017; Arnich et al., 2012; EFSA, 2014a).

The first study carried out for Cr speciation in food samples was in 1998 by Lameiras et al. in milk samples and the interest of this type of analysis continue to this day in relation to a larger panel of foodstuffs such as bread and breakfast cereals, dairy products, tea, meat, fish and rice.

Most of the studies so far showed that Cr(VI) is absent in foodstuffs due to the reduction potential of the organic matter hence leading to conversion of Cr(VI) to Cr(III) (Novotnik et al., 2013, 2015; EFSA, 2014a; Vacchina et al., 2015, Milačič and Ščančar, 2020). Nevertheless, several authors reported the presence of Cr(VI) in foodstuffs such as milk, meat, bread and cereals, tea, mushrooms and rice or in cooked food (Figueiredo et al., 2007; Ambrushe et al., 2009; Mandiwana et al., 2011; Soares et al., 2010; Chen et al., 2020; Shittu et al., 2020) and notably in toasted bread (Mathebula et al., 2017), as can be seen in Table 2.4.

Table 2.4. Overview of the literature data on levels of Cr_{total} and Cr(VI) in foodstuffs

Food group	Mass fraction (µg kg^{-1})		Reference
	Cr_{total}	Cr(VI)	
Dairy products			
UHT Milk	0.95-2.70	< 0.15-1.20	Lameiras et al., 1998
Powdered Milk formula	-	10-75	Soares et al., 2000
Cow milk	33.2-57.1	0.61-1.44	Ambushe et al., 2009
Cheese, yoghurts and milk	<13-255	< LOD	Hernandez et al., 2018
Meat and meat products			
Meat	-	6.3-14.3a	Shittu et al., 2020
Cereals			
Bread	8.9-92.2	< LOD	Kovács et al., 2007
Bread	47.3-50.9	5.65-6.82	Soares et al., 2010
Bread	0.041-7.6	< LOD	Novotnik et al., 2013
Bread and cereals	5.8-1857	< LOD	Vacchina et al., 2015
Cereals	30-437	< LOD	Hernandez et al., 2017
Bread and breakfast cereals	58.17-156	20.4-470	Mathebula et al., 2017
Rice	180-250	< LOD	Dokpikul et al., 2018
Rice	40.6-408	5.93-142	Chen et al., 2020
Fruits, vegetables and pulses			
Mushrooms	1.11-1.14[a]	0.103-0.143[a]	Figueiredo et al., 2007
Red lentils	14.8	< LOD	Narin et al., 2008
Apple, marrow, quince	< LOD (1.11)	< LOD	Çimen et al., 2013
Drinks and Beverages			
Tea	1.4-8.0[a]	0.12-0.48[a]	Panichev et al., 2005
Drink water	5.11-21.14	0.91-21.75	Hosseini et al., 2009
Tea	0.74-21.95[a]	0.46-8.07[a]	Elci et al., 2010
Herbal tea	0.22-14[a]	0.03-3.15[a]	Mandiwana et al., 2011
Tea leaves and infusion	3.39-4.41	< LOD	Chen et al., 2014
Lager beer	1.13-4.32	0.94-2.51	Vieira et al., 2014
Tea leaves and tea infusion	0.56-5.63[a]	0.12[a]	Chen et al., 2014
Wine, beer	0.01-0.026	0.51-0.135	López-Garcia et al., 2015
Tea infusions	0.095-1.9	< LOD	Novotnik et al., 2015
Oils			
Edible animal oils	131-2500	< LOD	Lin et al., 2016

[a] µg.g^{-1}; [b] µg.mL^{-1}

These findings have led recently to a controversy regarding the presence of Cr(VI) in raw or cooked food (Milačič and Ščančar, 2018). Most probably, the presence of Cr(VI) in foodstuffs is due to analytical artefacts due to the easiness of species interconversion during the analytical process (Milačič and Ščančar, 2020).

2.4. Chromium toxicity

The differences in the chemical and biochemical reactivity of Cr(III) and Cr(VI) lead to opposite behaviours from the toxicological point of view, with Cr(III) being considered non-toxic while Cr(VI) being considered carcinogenic by inhalation (IARC, 2012; Pyrzynska, 2016). The toxicity, bioavailability and transport properties depend on the specific form in which Cr is present in the environment or foodstuffs. Consequently, knowledge in terms of its speciation is required rather than its total content to assess its physiology and toxicological effects, as well as its chemical transformations in environment, atmosphere, soil, water, and food, and distribution, transport and elimination.

The U.S. Environmental Protection Agency (USEPA), the International Agency for Research on Cancer (IARC, 1990), the World Health Organization (WHO) and the Agency for Toxic Substances and Disease Registry (ATSDR, 2012) have classified Cr(VI) as a human carcinogen (IARC 1990; USEPA 1998).

Cr(VI) is toxic because of its high oxidation potential and ability to penetrate biological membranes. The ability of Cr(VI) (in CrO_4^{2-} form) to diffuse freely through cell membranes is possible due to its structural similarity to anions such as SO_4^{2-} or PO_4^{3-}, which are transported by the anion exchange channels. The reduced form of Cr (Cr(III)) is practically unable to enter the cell by the same ion channels, thus Cr(VI) compounds are recognized as being 1000 times more cytotoxic.

Cr(III) is considered essential to humans due to the maintenance of glucose tolerance factor and lipid/protein metabolism. The dietary deficiency of Cr(III) has been believed to contribute to the development of type 2 diabetes mellitus (IOM, 2001). Nevertheless, the European Food Safety Authority (EFSA) stated relatively recently that there is no convincing evidence of beneficial effects of Cr(III) for healthy people (EFSA, 2014b). Additionally, intracellular Cr(III) as a product of the Cr(VI) reduction, can also exhibit toxic effects e.g., by suppressing the activity of some metallo-enzymes or reaction with macromolecules (including DNA) (Ray, 2016).

The Institute of Medicine (IOM) of the National Research Council (NRC) (United States, Washington, DC) determined an adequate intake, a level typically consumed by healthy individuals of 20-45 μg Cr(III)/day for adolescents and adults. IOM reported average plasma Cr concentrations of 0.10-0.16 μg L^{-1} and an average urinary Cr excretion of 0.22 μg L^{-1} or 0.2 μg per day. Currently, the biological target for the essential effects of Cr(III) is unknown (Wilbur et al., 2012).

The first epidemiological study of CrO_4^{2-} production workers in USA, that demonstrated an association with lung cancer, was conducted with 1445 workers in seven plants engaged in the extraction of chromates from ore from 1930 to 1947. The percentage death due to cancer of the respiratory system was 21.8% (Machle and Gregorius, 1948).

Severe exposures to Cr(VI) compounds are usually accidental or intentional (suicide), and are rarely occupational or environmental. Oral intake of Cr(VI) compound may cause intense gastrointestinal irritation or ulceration and corrosion, epigastric pain, nausea, vomiting, diarrhea, vertigo, fever, muscle cramps, haemorrhagic diathesis, toxic nephritis, renal failure, intravascular hemolysis, circulatory collapse, liver damage, acute multisystem organ failure, lung cancer, coma, and even death, depending the exposition dose and if the exposure is acute or chronic (WHO, 2000).

In both animals and humans, the primary route of Cr elimination is urinary excretion, which accounts for a little over 50% while the fecal excretion accounts for only 5%. The remaining Cr is deposited into deep body compartments, such as bone and soft tissue. Elimination from these tissues proceeds very slowly: the estimated half-time for whole-body chromium elimination is 22 days for Cr(VI) and 92 days for Cr(III) following intravenous administration (USEPA, 1984).

The Cr intake in Europe is estimate to be between 57.3 and 83.8 µg per day for adults (Regulation (EU) No 1169/2011; EFSA, 2014b), 30.1-42.9 µg per day in young children (12 months to < 36 months), 54.3-71.2 µg per day in children (36 months to < 10 years) and 63.5-83.4 µg per day in adolescents (10 years to < 18 years).

It is worth to note that no tolerable upper intake level has been established yet for Cr, but EFSA CONTAM Panel in 2014 suggested a tolerable daily intake (TDI) of Cr at 300 µg kg^{-1} per body weight per day (EFSA, 2014).

According to the U.S. EPA (1998), the maximum acceptable concentration (MAC) of Cr(VI) that should be consumed in food is 0.003 mg kg^{-1} day^{-1} and therefore, MAC is limited by the body weight of an individual (USEPA, 1998).

WHO has recommended that the concentration of Cr in drinking water should be ≤ 0.05 mg L^{-1} (WHO, 2017). However, the recommended values of Cr concentration in drinking water do not take into consideration particular species of this element. They provide just a temporary solution implemented because no routine or reliable method exists for the determination of low concentrations of Cr in water (Markiewicz et al., 2015).

CHAPTER 3
DETERMINATION OF TOTAL Cr IN FOOD SAMPLES BY INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY

Abstract

This chapter presents the analytical approach and its application to the total Cr quantification in dairy products, meat and meat products and cereals (bread, breakfast cereals and rice). The procedure is based on the sample preparation by using acidic (HNO_3) digestion in a closed microwave digestion and further quantification by inductively coupled plasma-mass spectrometry (ICP-MS, accredited method).

This method was validated previously in the Anses laboratory using the accuracy profile approach for the determination of 31 trace elements including Cr. The measurement bias corresponding to the validity domain ranged from -26% to 11%, whereas the coefficient of variation in terms of repeatability (CV_r) varied from 5% to 10% (depending on the analyte level). The coefficient of variation in terms of intermediate reproducibility (CV_R) ranged from 10% to 20%. The limit of quantification for Cr was 0.010 mg kg^{-1} (wet weight).

Several internal quality controls (IQC) were put in place for the analytical quality assurance and the results were valid when all quality control criteria were met. When the acceptability criteria were not satisfactory, the sample(s) were re-analysed. The method was successfully applied to the analysis of a selection of food samples mentioned above.

For semi-skimmed and infant formula, total Cr ranged between 1-7 µg kg^{-1} while in meat and meat products it was measured at 5-82 µg kg^{-1}. For bread and cereals samples (breakfast cereals and rice) Cr_{total} was between 10-202 µg kg^{-1}, 24-360 µg kg^{-1} and 3-102 µg kg^{-1}, respectively.

3.1. Introduction

Cr and other trace metals can occur as residues in food due to their transfer from the environment to raw materials (cereals, meat, milk, etc.) or as a result of food contamination during its processing and storage.

One of the first modern instrumental techniques for total Cr determination is atomic absorption spectrometry (AAS) either using graphite furnace (GF) or electrothermal (ETA) atomization (Farre and Lagarda, 1986, Alberti-Fidanza et al., 2002, Azi et al., 2018; Jorhem, L. et al., 1994, Garcia et al., 2000).

Although AAS is a robust technique, its use nowadays is rather limited, mostly because of relatively high limits of detection (LOD); additionally, AAS is a mono-elemental technique, which makes its use rather limited nowadays because of low sample throughput.

Inductively coupled plasma-mass spectrometry (ICP-MS) is nowadays the technique of choice for the quantification of total Cr in foodstuffs because of its low limits of detection, multi-element capability, and high sensitivity and selectivity (Beyza, et al., 2006, Roussel et al., 2007, Güler, Z., 2007, Kmiecik, et al., 2007, Lisiewska et al., 2008, Wang and Farid, 2015; Chekri, et al., 2019; Perugini et al., 2014; Rasmussen et al., 2017; Rittirong et al., 2018). Nevertheless, total Cr quantification by conventional ICP-MS is hampered by the polyatomic interferences from chloride (Cl): $^{35}Cl^{16}O^{1}H^{+}$, $^{35}Cl^{17}O^{+}$ and carbon (C): $^{36}Ar^{16}C^{+}$, $^{40}Ar^{12}C^{+}$, present in foodstuffs. The use of a collision/reaction cell (CRC) reduces the interferences caused by the presence of Cl^{+} and C^{+} leading to more reliable results in terms of Cr isotopes detection.

As can be seen in the Table 3.1, the mostly used digestion technique for Cr determination in food is microwave assisted acidic (HNO_3) approach, which seems to be the state of the art technique (Alberti-Fidanza et al., 2002, Roussel et al., 2007, Chekri, et al., 2019). Other studies reported mineralization with mixture of HNO_3 and perchloric acids ($HClO_4$) (Lisiewska et al. in 2008) or a mixture of methyl isobutyl ketone (MIBK) and hydrochloric acid (HCl) (Farre and Lagarda, 1986).

The results in terms of total Cr were compared with those of Cr(III) and Cr(VI) obtained by speciation, as will be presented in Chapter 4.

Table 3.1. Overview of the literature on Cr_{total} determination in different types of samples

Samples types	Extraction procedure	Detection	Cr_{total} (mg kg^{-1})	Reference
Dairy products				
UHT Milk	Nitric acid and a liquid-liquid extraction with HCl/MIBK at 0 °C	AAS	17-43 [a]	Farre and Lagarda, 1986
Spanish milk	Nitric acid at 0 °C with MIBK after addition of HCl	AAS	0.030 [b]	Farre and Lagarda, 1986
Margarine, milk, cheeses and yogurts	Microwave digestion with 5 mL HNO_3 and 5 mL distilled water	ICP-MS	0.43-47.5 [c]	Roussel et al., 2007
Strained and salted yoghurt	Hot plate heat: 95 °C for 20 min. and left for cooling to 40-45 °C	ICP–OES	1.23 and 0.43	Güler, Z., 2007
Dairy-based desserts	Microwave digestion with 3mL HNO_3 and 3mL MilliQ water	ICP-MS	95-232 [b]	Chekri, et al., 2019
Cr content range – Dairy products			*0.030-232* [b]	
Meat and meat products				
Meat	Nitric acid and a liquid-liquid extraction with HCl/MIBK at 0 °C	AAS	66-275	Farre and Lagarda, 1986
Spanish meat	Nitric acid at 0 °C with methyl isobutyl ketone after addition of HCL	AAS	0.144	Farre and Lagarda, 1986
Meat	Microware digestion with 5 mL HNO_3	AAS	11.8-66.7	Alberti-Fidanza et al., 2002
Chicken, pork, veal and minced beef	Microwave digestion with 5 mL HNO_3 and 5mL distilled water	ICP-MS	2.93-4553 [c]	Roussel et al., 2007
Beef meet	Ash was dissolved in 15 mL HNO_3 (20%) and filtered through ashless filter paper	ICP-MS	0.004-1.540 [f]	Wang and Farid, 2015
Infant food meat-based	Microwave digestion with 3 mL HNO_3 and 3 mL MilliQ water	ICP-MS	68.9	Chekri, et al., 2019
Cr content range – Meat and meat products			0.004[f]-275	
Fish and seafood				
Fish	Nitric acid and a liquid-liquid extraction with HCL/MIBK at 0 °C	AAS	105-188	Farre and Lagarda, 1986
Spanish Fish	Nitric acid at 0 °C with MIBK after addition of HCL	AAS	0.141	Farre and Lagarda, 1986
Crayfish: muscle and hepatopancreas	Samples were dry at 450 °C in platinum crucibles and the ash was dissolved in 0.1 M HNO_3	GF-AAS	0.018-0.088 and 0.042-0.18	Jorhem, L. et al., 1994
Fish	Microware digestion with 5 mL HNO_3	AAS	44.0-87.9	Alberti-Fidanza et al., 2002
Sea bass fillets	Microware digestion with 5 mL HNO_3	ICP-AES	0.112-0.18 [b]	Beyza, et al., 2006
Coalfish, cod, sea bass and sole	Microwave digestion with 5 mL HNO_3 and 5mL distilled water	ICP-MS	14.71-39.53 [c]	Roussel et al., 2007
Mediterranean finfish and shellfish	Microwave digestion with 10 mL HNO_3 (65%)	GF-AAS	0.05-0.52 [b]	Kalogeropoulos et al., 2012
Norway lobster	Microwave digestion with 7 mL HNO_3 (68%), 1.5 mL of H_2O_2 (30%) and 0.05 mL of HF (37%)	ICP-MS	White: 0.12-0.15; Brown: 0.38-0.62	Perugini et al., 2014
Prawns, halibut and salmon	Digestion with 60 g anhydrous sodium sulphate; with 150 mL acetone:hexane (1:1)	ICP-MS	0.12-0.15	Rasmussen et al., 2017
Whitefish	Digestion by hot plate with 4.5 mL HNO_3 and 1.5 mL of $HClO_4$ (72%) for 6 hours at 150 °C	AAS	0.0020-0.0028	Mehdipour et al., 2018
Seafood	Microwave digestion with 4 mL HNO_3 and 2 mL MilliQ water.	ICP-MS	0.15-0.25	Barbosa et al., 2018
Cr content range – Fish and seafood			0.0020-188	
Cereals				
Breakfast cereals	Nitric acid and a liquid-liquid extraction with HCl/MIBK at 0 °C	AAS	118-162	Farre and Lagarda, 1986
Breakfast cereals	Nitric acid at 0 °C with methyl isobutyl ketone after addition of HCL	AAS	0.135	Farre and Lagarda, 1986
Fresh and toasted bread, six-cereal bread	Microwave digestion with 5 mL HNO_3 and 5mL distilled water	ICP-MS	5.82-225 [c]	Roussel et al., 2007
Rice	Samples heated by hot plate 95 °C for 2 hr with 5 mL of HNO_3	ICP-MS	0.27-0.41	Rittirong et al., 2018
Pasta	Microware digestion with 5 mL HNO_3	AAS	54.9-108	Alberti-Fidanza et al., 2002
Rice	Microwave digestion with 3 mL HNO_3	DRC ICP-MS	40.6-408 [a]	Chen et al., 2020
Cereals with chocolate	Microwave digestion with 5 mL HNO_3 and 5 mL distilled water	ICP-MS	316 [c]	Roussel et al., 2007
Cr content range – Cereals			0.135-108	

Table 3.1. Overview of the literature on Cr_{total} determination in different types of samples *(continued)*

Samples types	Extraction procedure	Detection	Cr_{total} (mg kg^{-1})	Reference
Fruits, vegetables and pulses				
Fruits and Vegetables	Nitric acid and a liquid-liquid extraction with HCL/MIBK at 0 °C	AAS	9.6-89 and 8.5-65	Farre and Lagarda, 1986
Spanish Fruits and vegetables	Nitric acid at 0 °C with methyl isobutyl ketone after addition of HCL	AAS	0.037 and 0.050	Farre and Lagarda, 1986
Vegetables and Pulses	Microware digestion with 5 mL HNO_3	AAS	41.9-62.6 and 81.8-89.4 [g]	Alberti-Fidanza et al., 2002
Nuts, fruits and vegetables	Microwave digestion with 5 mL HNO_3 and 5 mL distilled water	ICP-MS-CRI	0.40-72.3 [c]	Roussel et al., 2007
Raw and Processing Vegetables	Microwave digestion with 3:1 mixture of HNO_3:perchloric acids	ICP-AES	6.61-18.66 [d]	Kmiecik, et al., 2007
Broad bean/pea and French-bean	Mineralization in a 3:1 mixture of nitric and perchloric acids.	ICP-AES	0.006-0.017 [e]	Lisiewska et al., 2008
Pumpkin and Amaranthus viridis	Samples dilution with distilled water containing 1.5 ml concentrated HNO_3/L	AAS	0.1-0.9	Azi et al., 2018
Infant food vegetable-based	Microwave digestion with 3 mL HNO_3 and 3 mL MilliQ water	ICP-MS	50.4	Chekri, et al., 2019
Cr content range – Fruits, vegetables and pulses			0.037-89	
Drinks and beverages				
Water	Microwave digestion with 5 mL HNO_3 and 5 mL distilled water	ICP-MS	0.03-0.33 [c]	Roussel et al., 2007
Tea (mint and chicory)			0.45-1.20 [c]	
Coffee			0.74-7.32 [c]	
Fruit juice			0.82-19.60 [c]	
Red wine, beer and whisky			0.78-16.44 [c]	
Cr content range – Drinks and beverages			0.03-19.60 [c]	
Sugar and others				
Spices and aromatic herbs	Microwave digestion with HNO_3 and V_2O_5	ETA-AAS	0.01-1.11 [b]	Garcia, E., et al., 2000
Marmalades, desserts and bonbons	Microwave digestion with 5 mL HNO_3 and 5 mL distilled water	ICP-MS	6.00-131.30 [c]	Roussel et al., 2007
Chocolate powder	Microwave digestion with 3 mL of HNO_3, 1.5 mL H_2O_2 and 3.5 mL MilliQ water	GF-AAS	81-4790 [b]	Peixoto et al., 2018
Cr content range – Sugar and others			0.01-4790 [b]	

[a] $ng.g^{-1}$; [b] $\mu g.kg^{-1}$; [c] $ng.mL^{-1}$; [d] $g.100g^{-1}$ dry matter; [e] $mg.100g^{-1}$ fresh matter; [f] $mg.120g^{-1}$ fresh matter; [g] $mg.100g^{-1}$ dry matter

3.2. Analytical procedures

3.2.1. Samples

For total Cr quantification, ten different samples of infant formula milk, semi-skimmed milk and eleven meat and meat products were purchased in retail shops in Maisons-Alfort (France). The bread, breakfast cereals and rice were purchased in retail shops in Maisons-Alfort (France) and Birkerød (Denmark). Photos of the various rice samples analyzed here are shown in appendix Fig. A1 and an overview of the samples is presented in Table 3.2.

Table 3.2. Food samples analyzed for total Cr by ICP-MS

Food sample	Physical state	Quantity purchased	Place of purchase	Papers results
Dairy products				
Infant formula milk	Powder	10 brands (1 kg each)	Maisons-Alfort (France)	I and II
Semi-skimmed milk	Liquid	10 brands (1 L each)	Maisons-Alfort (France)	I and II
Meat and meat products				
Beef steak	Solid fresh	1 kg		I and II
Beef Grilled Pavé		2 kg		I
Chicken cutlet		500 g		
Turkey cutlet		500 g		
Pork rib		500 g		
Duck liver		500 g	Maisons-Alfort (France)	II
Ham slice		300 g		
Spanish chorizo sausage		250 g		
Bacon strips		200 g		
Pork sausage (100%)		4 units – 150g		
Cutlet veal		500 g		
Bread				
Danish Rye		500 g	Birkerød (Denmark)	
Malt flour		450 g		
“Burger” bread		300 g		
Spelt flour		350 g		
“Pita” bread		400 g		
Baguette	Solid	220 g	Maisons-Alfort (France)	III
Sandwich		500 g		
Wheat flour		350 g		
Bread toasted		500 g		
Bread toasted complete		500 g		
“French” bread		400 g		
Breakfast cereals				
Cacao cereals		625 g		
All bran		375 g		
Bran flakes bio		375 g		
Corn flakes		375 g		
Weetabix 100% whole		450 g		
Weetabix Fruit + Nuts	Solid	450 g	Birkerød (Denmark)	III
Cacao cereals		625 g		
Granola bio		500 g		
Granola gluten free		500 g		
Fruity muesli		500 g		
Toasted muesli		450 g		

Table 3.2. Food samples analyzed for total Cr by ICP-MS *(continued)*

Food sample	Physical state	Quantity purchased	Place of purchase	Papers results
Rice				
Risotto Sushi Basmati Black Red Brown Jasmin Arborio Parboiled Whole grains	Solid	1 kg	Birkerød (Denmark)	IV

3.2.2. Sample preparation and analysis

All solid samples (meat, bread, breakfast cereals and rice) were homogenized using a stainless steel free automatic mixer (Retsch Grindomix GM 200, Haan - Germany) with titanium blades. The powdered infant formula milk were prepared as described on the packaging with the addition of the appropriate amount of ultra-pure water (MilliQ). The liquid samples (infant formula milk and semi-skimmed milk) were homogenized for 4 h using a rotary shaker (Heidolph reax 2, Merck KGaA, Darmstadt, Germany).

For total Cr determination, the samples were digested in a closed microwave system (Multiwave 3000, Anton Paar, Courtaboeuf, France) with nitric acid (HNO_3). For this purpose, homogenized sub-samples of 0.5 g of meat, 2.0 g of milk and 0.3 g of bread, breakfast cereals and rice, were directly weighed into 80 mL quartz vessels, thoroughly mixed and then left overnight with 3 mL of concentrated HNO_3 (67% v/v); then 3 mL of MilliQ (ultra-pure water) was added and the mixture was microwave digested using the conditions presented in Table 3.3.

Table 3.3. Food samples digestion program of the Multiwave 3000 system

Step	Power (W)	Ramp (min)	Hold (min)
1	1400	25	15
2	0		15

p-rate: 0.5 bar/s; IR: 200°C; p: 80bar

The digests were then quantitatively transferred to polypropylene tubes (50 mL) and then filled up with MilliQ after the addition of the internal standard.

At ANSES (France), total Cr was measured by using an ICP-MS Agilent 7700 (France) and the working conditions are summarised in Table 3.4.

At DTU Food (Denmark), total Cr was measured by using an iCAP TQ ICP-MS (Denmark) and the working conditions are summarised also in Table 3.4.

Table 3.4. Optimum ICP-MS conditions for total Cr determination

Parameter	Agilent 7700	Thermo iCAP-TQ
Plasma gas flow (Ar)	15 L min^{-1}	15 L min^{-1}
Auxiliary gas flow (Ar)	≅ 0.99 L min^{-1}	≅ 0.80 L min^{-1}
Nebuliser gas flow (Ar)	≅ 0.95-1.00 L min^{-1}	≅ 0.92 L min^{-1}
Sampling cone orifice (Ni)	1.0 mm	1.1 mm
Skimmer cone orifice (Ni)	0.77 mm	0.50 mm
KED gas flow (He)	≅ 4.3 mL min^{-1}	≅ 5.0 mL min^{-1}
Plasma power	1500 W	1550 W
Dwell time	100 ms	250 ms
Monitored isotopes	^{52}Cr	^{52}Cr

These approaches were used in the papers I and II for milk and meat samples, paper III for bread and breakfast cereals and paper IV for rice samples.

3.2.3. Internal quality control

In this work, several internal quality controls (IQC) were put in place for the analytical quality assurance and the results were valid when all quality control criteria were met. If the criteria were not met, the sample(s) were re-analysed.

Each sample digestion was carried out in duplicate and at least a digestion blank was included in each sample preparation series (by microwave). Also, in parallel with the samples, a certified reference materials (CRM) was digested and analysed in the same conditions. For the analysis of milk and meat samples, NIST® SRM® 1849a -Infant/adult nutritional formula (milk-based) CRM (Merck KGaA, Darmstadt, Germany) was used for the IQC. The certified mass faction of total Cr in this material is 1.072 ± 0.032 mg kg^{-1}. The average value obtained after 18 analyses (part of IQC) in different days was 1.132 ± 0.125 mg kg^{-1} (overall recovery factor of 106%), which is in agreement with the certified value. For bread and breakfast cereals analysis, durum wheat flour SRM® 8436 ((Merck KGaA, Darmstadt, Germany) CRM was used. The certified value is 0.023 ± 0.009 mg kg^{-1} and the average measured in 3 days (6 replicates) was 0.022 ± 0.006 mg kg^{-1} (96% of overall recovery), which is in agreement with the certified value.

A mid-range calibration standard was analysed every 8 samples and at the end of the sequence in order to assess the stability of the instrumental response and the calibration drift; the analyses were validated when the deviation of this standard compared to that of the calibration curve was ≤ 20%.

Finally, the quantification was carried out by using external calibration at six levels (0, 1, 2.5, 5, 7.5, 10 µg L^{-1}) and using ^{45}Sc (scandium) as internal standard (IS); a measurement was validated when the variation of the IS was < 30%. The calibration was valid when the determination coefficient (r^2) was ≥ 0.995.

3.3. Assessment of total Cr in foodstuffs

The data in terms of total Cr obtained for the analysis of semi-skimmed milk, meat and meat products, bread, breakfast cereals and rice are presented in Figs. 3.1 to 3.5. It is important to note that the total Cr values for each sample are shown as the mean ± SD (n=2).

3.3.1. Total Cr levels in dairy products

For dairy products, the samples used were semi-skimmed milk and infant formula milk.
Infant milk (in powder) were reconstituted with ultra-pure water as described on the packaging. The total Cr levels in the samples are presented in Fig. 3.1.

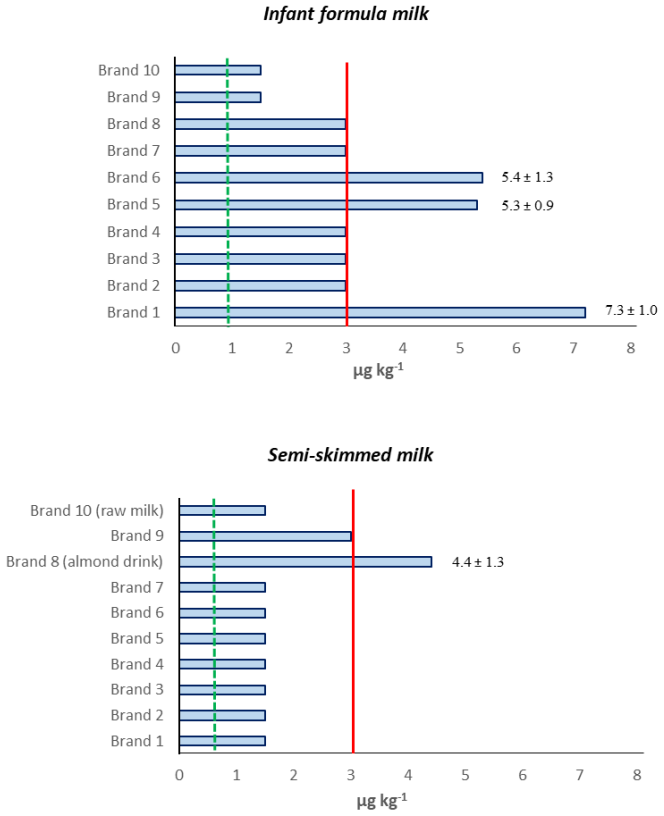

Figure 3.1. Levels of total Cr in infant formula and semi-skimmed milk (µg kg^{-1}). The LOD is presented by the green line (0.9 µg kg^{-1}) and LOQ is presented by the red line (3 µg kg^{-1}). Note: * an almond drink and a raw milk was also analysed in the group of semi-skimmed milk

Most samples of infant formula milk showed levels of total Cr below LOD (0.9 µg kg^{-1}), except for 3 samples were the levels varied between ≅ 5.0 and 7.0 µg kg^{-1}.

The levels of Cr_{total} in semi-skimmed milk varied between the LOD (0.9 µg kg^{-1}) and 4.4 µg kg^{-1} (the almond drink). It is worth to note that this drink, although of vegetable origin, contains the same percentage of fat (≈ 1.4-1.6 %) as the conventional semi-skimmed milk, so it was included in this food group.

3.3.2. Total Cr levels in meat and meat products

Fresh meat (six samples) and meat products (four samples) were analysed and the results are present in Fig. 3.2.

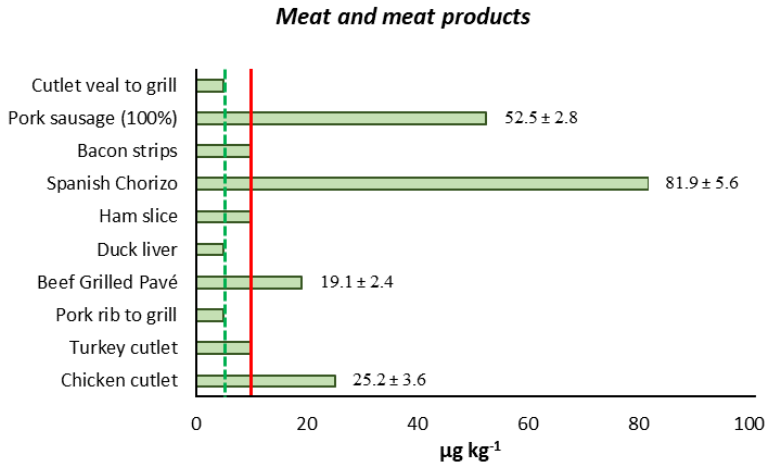

Figure 3.2. Levels of total Cr in meat and meat products (µg kg^{-1}). The detection limit are present by vertical green line (3 µg kg^{-1}) and quantification limit are present by vertical red line (10 µg kg^{-1})

Total Cr levels varied between the LOD (3 µg kg^{-1}) and 81.9 µg kg^{-1}. In terms of total Cr in meat is that the lowest levels were found in fresh meat (e.g. duck liver, cutlet veal to grill, etc.) while the highest levels were measured in processed meat (pork sausage (52.5 µg kg^{-1}) and Spanish chorizo sausage (82 µg kg^{-1}), see also Fig. 3.2.

3.3.3. Total Cr levels in bread, breakfast cereals and rice

The bread samples analysed show a large variation in total Cr levels, as can be seen in Fig. 3.3. The results are present also in the papers III and IV.

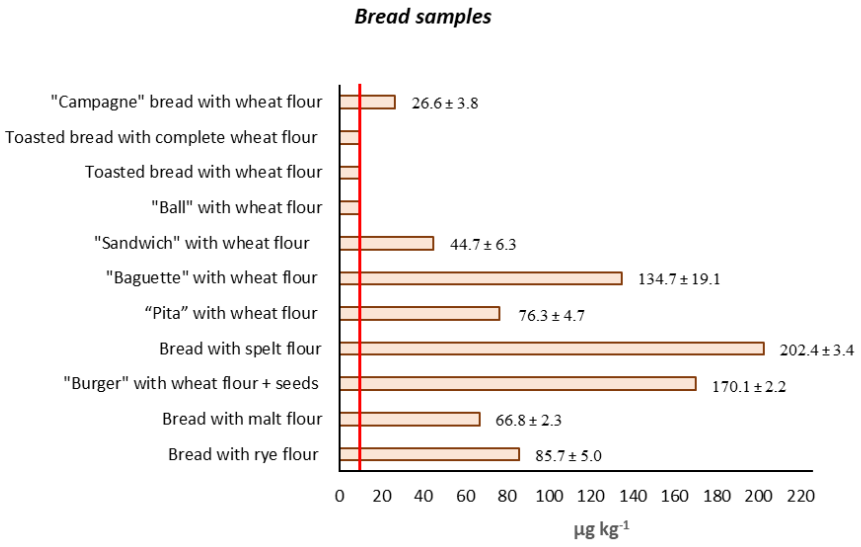

Figure 3.3. Levels of total Cr in bread samples. Note: The LOQ is represented by the red line (10 µg kg^{-1})

In the bread samples, the total Cr levels were between the LOQ (10 µg kg^{-1}) and 202.4 µg kg^{-1} in bread made with spelt flour (gluten free) followed by the typical bread for hamburgers with seeds (170.1 µg kg^{-1}). Comparing the type of bread consumed in France ("campagne" – wheat flour) with that consumed in Denmark ("Rug brød" – rye flour), the latter contains more than three times as much total Cr than the first (85.7 vs 26.6 µg kg^{-1}).

For the various types of bread used: "Sandwich", "Baguette" and "Pita", the levels of total Cr were 44.7, 134.7 and 76.3 µg kg^{-1}, respectively. For bread made with malt flour, the total Cr level was 66.8 µg kg^{-1}. The data on Cr in the French bread are comparable with those reported by Vacchina et al., 2015 in different flours commercialized in France, namely 16 µg kg^{-1} for wheat flour, 28 µg kg^{-1} for corn flour, 21 µg kg^{-1} for rye flour, 63 µg kg^{-1} for spelt flour and 67 µg kg^{-1} for malt flour. It is worth to note that total Cr levels in the toasted bread samples (both with wheat flour) were < LOQ.

The levels of total Cr ranged between 24.4 and 360.2 µg kg^{-1}, showing a large variation between all samples. The highest concentrations were found in cacao-based cereals. It appears that the higher the level of cacao, the higher level of total Cr. For instance, brand 1 of cacao based cereal having 16.5% of cacao contained 219.3 µg kg^{-1}, whereas brand 2 with 20.1% cacao showed a level of 360.2 µg kg^{-1} total Cr. A relatively high level of total Cr was also found in the Weetabix containing fruit and nuts (61.8 µg kg^{-1}) compared to Weetabix 100% whole grains (25.1 µg kg^{-1}), most probably with total Cr coming from the fruit and nuts.

Total Cr was quantified in all in the breakfast cereals and the levels found are present in Fig. 3.4.

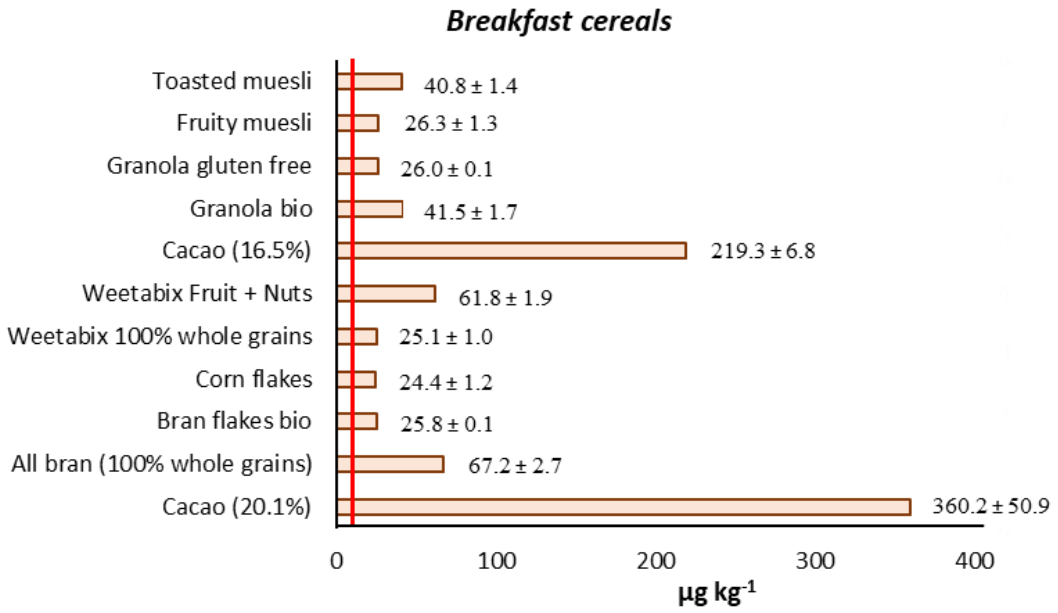

Figure 3.4. Levels of total Cr in breakfast cereals. The LOQ are present by vertical red line (10 µg kg^{-1})

Apart from the cacao-based breakfast cereals, the sample with 100% whole grains showed the highest total Cr level (67.2 µg kg^{-1}) compared to the other samples. The lowest level of total Cr was measured in the regular cornflakes (24.4 µg kg^{-1}), but the level is highly comparable with corn flakes bio (25.8 µg kg^{-1}), granola gluten free (26.0 µg kg^{-1}) and fruity muesli (26.3 µg kg^{-1}).

Ten different rice samples were analysed in total Cr and all results are present in Fig. 3.5.

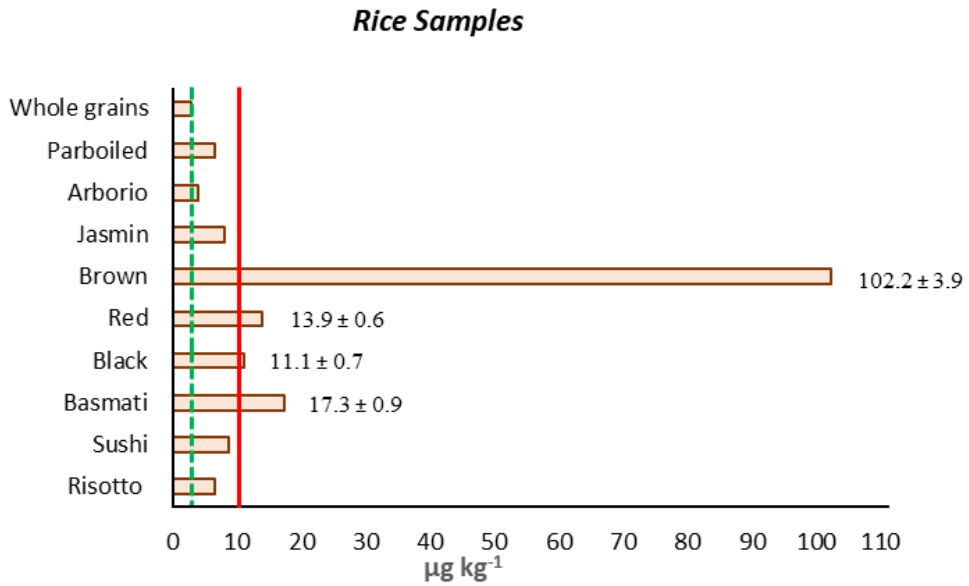

Figure 3.5. Concentration of total Cr in rice samples. The LOD are present by vertical green line (3 µg kg^{-1}**)**, while the LOQ are present by vertical red line (10 µg kg^{-1}**)**

Most of the rice sample analyzed contained total Cr ranging between < LOD (3 µg kg^{-1}) and 17.3 µg kg^{-1} (basmati rice). The highest level (102.2 µg kg^{-1}) was measured in brown (complete) rice. This difference in total Cr in brown rice compared to other types of (white) rice analyzed may be the consequence of the total use of bran in brown rice. It is worth to note that the bran is removed for the production of the white rice (Callegaro et al., 1996).

Contrary to expectations, whole grains rice (all grain compounds are used) had the lowest level of total Cr (< LOQ = 10 µg kg^{-1}) measured in all rice samples, followed by arborio rice brand, as can see in Fig. 3.5.

3.3.4. Variation of total Cr levels in various foodstuffs

An overview of the average levels and the variation of total Cr in all food groups analyzed in this study is shown in Fig. 3.6.

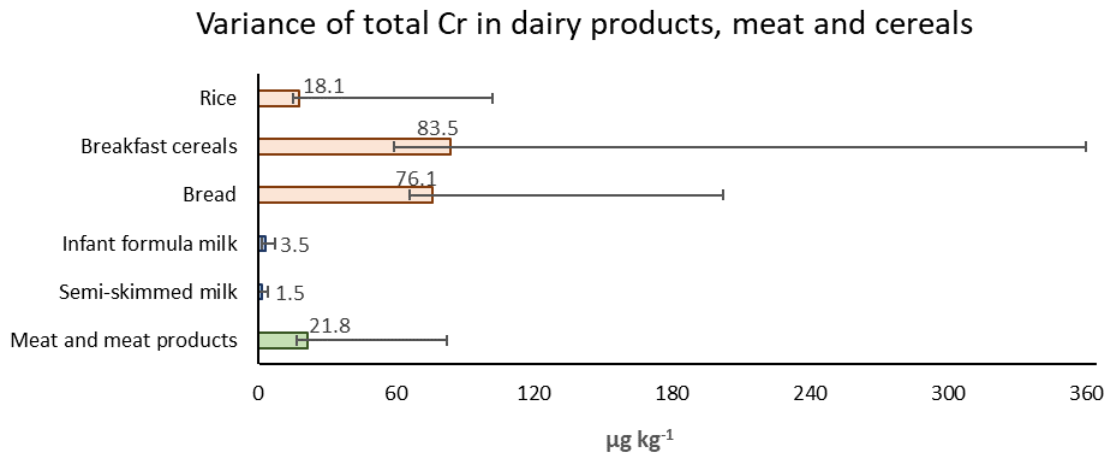

Figure 3.6. Average of total Cr concentration in different foodstuffs (µg kg^{-1}) with error bars (minimum and maximum of the value found)

As can be seen, the foodstuff with the lowest level of total Cr is the semi-skimmed milk, with levels between LOD (0.9 µg kg^{-1}) and 4.4 µg kg^{-1}, followed by infant formula milk with values between LOD (0.9 µg kg^{-1}) and 7.2 µg kg^{-1}. Amongst the food types analysed here, rice is raking the 3rd in terms of total Cr levels, ranging between the LOD (3 µg kg^{-1}) and 102.2 µg kg^{-1}, followed by meat & meat products showing levels between the LOD (3 µg kg^{-1}) and 81.9 µg kg^{-1}.

The breakfast cereals showed the highest levels of total Cr, but also the highest variation (shown in Fig. 3.6 in terms of error bars). On the opposite, the lowest levels and also lowest variation of total Cr were found in semi-skimmed and infant formula milk. For bread made with different types of flour the levels of total Cr varied between LOQ (10 µg kg^{-1}) and 202.4 µg kg^{-1}.

Total Cr levels in bread found in other studies were 8.9 to 92.2 µg kg^{-1} (Kovács et al., 2007), 47.3 to 50.9 µg kg^{-1} (Soares et al., 2010), 16 to 74 µg kg^{-1} (Vacchina et al., 2015), 57.6 to 156.1 µg kg^{-1} (Mathebula et al., 2017) and 70-182 µg kg^{-1} (Hernandez et al., 2017). Total Cr levels (mean = 76.1 µg kg^{-1}) for bread samples obtained in our study are in agreement with those found by these authors. Total Cr levels found by Mathebula et al. (2017) in breakfast cereals, varied between 162 and 774 µg kg^{-1}, higher levels compared to those obtained in this study, with mean of 83.5 µg kg^{-1}.

Total Cr levels in milk obtained by other authors was between 0.95-2.70 µg kg^{-1} (Lameiras et al., 1998), 33.2 to 57.1 µg kg^{-1} (Ambushe et al., 2009) and 36 to 43 µg kg^{-1} (Hernandez et al., 2017). The milk samples analyzed in this study have lower total Cr levels, approaching the values obtained by Lameiras et al., 1998.

For meat samples, the Cr levels found were 15-185 µg kg^{-1} (Vacchina et al., 2015) and 40.6-408 µg kg^{-1} (Son et al., 2016) being in agreement with those reported in the samples used in this study, with mean of 21.8 µg kg^{-1}.

The values of total Cr found by other authors were between 270-410 µg kg^{-1} (Rittirong et al., 2018) and between 40.6-408 µg kg^{-1} by Chen et al., 2020. The samples analyzed in this study have lower total Cr levels with a maximum of 102.2 µg kg^{-1} with mean of 18 µg kg^{-1}.

This study showed that the levels of Cr in cocoa products are remarkably high, in particular the cocoa added to breakfast cereals, suggesting that cocoa may have an important contribution to Cr exposure. A risk assessment related to Cr exposure from the consumption of chocolate powder drink was carried out in 2018 due to the total Cr levels found varying between 81 and 4790 µg kg^{-1} (n=34). This study showed, however, that the risk associated to the ingestion of Cr via the chocolate drink is not likely to cause harmful effects for adults, but the monitoring of Cr in cocoa products is recommended (Peixoto et al., 2018).

The presents could therefore be completed by the determination of Cr in cocoa products depending on the percentage and origin of the cacao and also perform a risk assessment but in terms of Cr speciation.

3.4. Conclusion

The analytical method employed in this study to determine total Cr in food samples, which was based on the microwave acidic digestion and quantification by ICP-MS allowed to obtain good quality with low limits of detection and quantification.

Total Cr levels measured in foodstuffs analyzed here are in agreement with those reported by other authors. The food group showing the lowest levels of total Cr was the semi-skimmed milk, followed by infant formula milk and the rice samples. The highest total Cr levels were measured in the breakfast cereals, particularly in the cacao cereals (two brands). This study could therefore be pursuit by the analysis of a larger panel of breakfast cereals containing cacao in order to accurately assess the human exposure to Cr via this food item.

CHAPTER 4
CHROMIUM SPECIATION ANALYSIS IN FOOD SAMPLES

Abstract

This chapter describes the development of two analytical approaches for chromium speciation analysis in various foodstuffs by using species-specific isotope dilution (SS-ID) in combination with high performance liquid chromatography (HPLC) and inductively coupled plasma-mass spectrometry (ICP-MS). The study seeks to clarify whether the presence of Cr(VI) in food as reported by some authors is real or is misleading due to the formation analytical artefacts. For this purpose, SS-ID, a primary analysis method, was employed for the accurate quantification of Cr(III) and Cr(VI) and correcting their interconversions in the same analytical run.

As mentioned above, two analytical procedures were developed and validated, one for Cr speciation in milk (infant formula and semi-skimmed) and meat and meat products and one for bread, breakfast cereals and rice. In both cases, the species extraction was achieved by sequential complexation of genuine Cr(III) with ethylenediaminetetraacetic acid (EDTA) and of Cr(III) originating from Cr(VI) with 1,5-diphenylcarbazone (DPCO) in the same analytical run. The HPLC separation of Cr(III)-EDTA and Cr(III)-DPCO complexes was carried out by anion exchange HPLC; excellent baseline separation was achieved in < 3 min. The main differences between the two approaches developed in this chapters differs in terms of extraction temperature and time.

The first approach (applied to milk and meat) was validated using the accuracy profile approach. The measurement bias corresponding to the validity domain ranged from 0.01 to 0.11%, whereas the coefficient of variation in terms of repeatability (CVr) varied from 2.9 to 11.6% (depending on the analyte level) for Cr(III) and from 6.7 to 11.8% for Cr(VI). The coefficient of variation in terms of intermediate reproducibility (CV_R) ranged from 6.8 to 13% for Cr(III) and from 6.8 to 25.9% for Cr(VI), respectively. The limit of quantification (LOQ) was 0.013 μg kg^{-1} for Cr(III) and 0.049 μg kg^{-1} for Cr(VI) (wet weight).

In case of the second approach, the measurement bias ranged from -0.31 to 0.49%, whereas CV_r varied from 1.3 to 4.4% for Cr(III) and from 0.6 to 7.9% for Cr(VI). Similarly, CV_R ranged from 1.3 to 4.4% for Cr(III) and from 2.0 to 8.9% for Cr(VI), whereas the LOQ were very similar with those of the first approach (0.014 μg kg^{-1} for Cr(III) and 0.047 μg kg^{-1} for Cr(VI), respectively).

The two analytical approaches were applied to the analysis of a selection of food samples such as milk and steak beef, bread/breakfast cereals and rice samples. Cr(VI) was not quantified in any of these samples while Cr(III) levels ranged between 0.22 (infant formula milk) up to 350 μg kg^{-1} (breakfast cereals). In all cases, Cr(III) levels are highly compatible with the levels of total Cr analysed in the same samples by using ICP-MS. This supports the EFSA statement related to the absence of Cr(VI) in foods and that Cr in this type of samples is found solely as Cr(III) species. The SS-ID employed in this chapter for development and validation of two analytical approaches applicable to Cr speciation analysis in a relatively large panel of food samples 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.

4.1. Introduction

Amongst the potentially toxic (trace) elements (PTE), chromium (Cr) has attracted special attention in the recent years because of the very different chemistry and toxicity of its main species, namely Cr(III) and Cr(VI). Actually, C(III) was for a long time considered as a beneficial element for the human health whereas Cr(VI) has been recognized for several decades as extremely toxic, genotoxic, and carcinogenic. Additionally, it is worth to highlight that in 2014, EFSA stated that Cr(VI) species is not present in foodstuffs due to the specific features (reductive potential) of this type of matrix which reduces Cr(VI) to Cr(III), and hence total Cr in foodstuffs consists of Cr(III) solely (EFSA, 2014). However, in recent years several studies reported the presence of Cr(VI) in various foodstuffs such as bread, cereals, chicken meat and tea. This leads to the need to develop accurate analytical approaches capable to quantify both Cr species in foodstuffs.

Cr speciation analysis in foods is still a complex task, particularly because of the trace and ultra-trace levels of Cr species, and also because of the complexity of food matrices, hence this process requires the use of highly sensitive and selective analytical approaches (Novotnik et al., 2013).

In the last two decades, Cr speciation analysis has been typically carried out based on the selective complexation of Cr(III) or Cr(VI) followed by the determination of each species by measuring the total Cr content in the extract without prior chromatographic separation *(off-line approaches)*, such as inductively coupled plasma mass spectrometry (ICP-MS) (Chen et al., 2014), atomic absorption spectroscopy (AAS) (Soares et al., 2010, Vieira et al., 2014), localized surface plasmon resonance (LSPR) (Fahnestock et al., 2009) and X-ray fluorescence (XRF) (Tsuyumoto and Maruyama, 2011). Several studies have shown that these approaches are prone to the formation of analytical artefacts arising mainly from the extraction step, because species interconversion may occur without any possibility to assess this side-effect when total Cr is measured in the extract fraction (Novotnik et al., 2013, Vacchina et al., 2015, Hamilton et al., 2018).

More recently, Cr speciation analysis shifted to species separation by high performance liquid chromatography (HPLC) before detection using atomic spectrometry, especially the inductively coupled plasma mass spectrometry (ICP-MS) (on-line approaches) (Pyrzynska, 2016, Marcinkowska et al., 2016, Hernandez et al., 2017, 2018; Novotnik et al., 2015; Vacchina et al., 2015; Lin et al., 2016; Hernandez et al., 2017/2018; Séby and Vacchina, 2018; Shittu et al., 2020). On-line coupling of HPLC with a highly sensitive and selective detector such as ICP-MS is currently the methodology of choice (Vacchina et al., 2015; Milačič and Ščančar, 2020; Markiewicz et al., 2015; Milačič and Ščančar, 2020). Nevertheless, the on-line approaches are not per se capable to assess species interconversion during the extraction or HPLC separation steps. Actually, species specific (SS) isotope dilution (ID) mass spectrometry (SS-ID-MS) has proven to be the only analytical approach

suitable for simultaneous speciation analysis of Cr(III) and Cr(VI) in foodstuffs due to its capability to quantify the species and to correct for their interconversion during the same analytical run (Martone et al. 2013; USEPA 2014).

The chapter presents the development and validation of two analytical approaches for simultaneous (single run) speciation analysis of Cr(III) and Cr(VI) in foodstuffs by HPLC-ICP-MS and SS-ID using selective species complexation with full capacity to correct for any species interconversion and hence accurately quantify Cr(III) and Cr(VI). These approaches were successfully applied to the analysis of a selection of real-life foodstuffs, such as meat and dairy products, bread, rice and breakfast cereals.

4.2. Analytical procedures

4.2.1. Sample preparation

The samples analysed in this study were steak beef, baby milk and semi-skimmed milk purchased in retail shops in Maisons-Alfort (France). Meat samples (~ 1 kg) were homogenized using a stainless steel free automatic mixer with titanium blades. The liquid samples (baby-milk and semi-skimmed milk) were homogenized during 4 h using a rotary shaker.

After homogenization, one third of the sample was employed for method optimization (storage at ≈ 5 °C) while another third was used for the method validation. In the latter case, the baby milk (500 mL) was spiked with 0.25 ng mL^{-1} Cr(VI), the semi-skimmed milk (500 mL) with 1 ng mL^{-1} of Cr(VI) and the steak beef (200 g) with 0.5 ng g^{-1} of Cr(VI). The remaining bulk samples were kept as a backup and stored at -18 ºC. The spike and standard solutions were stored in the dark at ≈ 5 °C.

4.2.2. Sequential extraction Cr(III) and Cr(VI) in a single run

In the Anses' laboratory, the extraction of the Cr species from food samples was carried out by using a heating block system while at DTU, a water bath system was employed for this purpose.

(a) Milk, meat and meat products

A subsample of 0.3 g was mixed with the required amount of Cr(III) and Cr(VI) spikes directly in the 50 mL polypropylene tube. Then, 16 mL of EDTA solution was added to reach a final concentration of 2.4 mM and the mixture was heated at 70 ºC during 25 minutes. The solution was cooled down (≅ 15 min) and then 200 µL of DPC solution was added to reach a final concentration of 0.02 mM; the final volume was made up to 20 mL with ultrapure water. After addition of DPC solution, the mixture was heated again at 70 ºC for 25 minutes. The extract was cooled down to room temperature (≈ 25 ºC) and then filtered using 0.45 µm polyvinylidene fluoride syringe filters prior to the analysis in the same day. To carry out SS-ID, to each standard and sample, ^{50}Cr(III) and ^{53}Cr(VI) spikes were added and the blend solution was thoroughly mixed to achieve isotopes equilibration. All samples were prepared in triplicate.

(b) Bread, breakfast cereals and rice samples

For bread, breakfast cereals and rice, the complexation of Cr(III) was carried with EDTA at the same concentration but at a higher temperature and times, namely at 90 ºC during 60 minutes. 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; the final volume was made up to 20 mL with ultrapure water. After addition of DPC solution, the mixture was heated again at 90 ºC during 25 min. The extract was cooled down to room temperature (≈ 30 ºC for 15 min) and then filtered using 0.45 µm polyvinylidene fluoride syringe filters prior to the analysis (the same day).

The optimum ICP-MS and HPLC instrumental parameters for Cr speciation analysis were reported in the Papers I and III.

4.2.3. HPLC separation

The HPLC separation of complexed species was carried out using a short (5 cm) microbore anion-exchange HPLC column and a mobile phase consisting of 0.01 mol L^{-1} HNO_3 + 2.5% (v/v) MeOH + 0.30 mol L^{-1} EDTA (pH=2) in isocratic elution mode.

4.2.4. ICP-MS detection

The main characteristics of ICP-MS are high sensitivity, multi-elemental detection, robust analysis and extremely low detection limits (Tomlinson et al., 1995; Cubadda, 2007).

This technique was used for the precise measurement of four isotopic ratios: $^{50}Cr/^{52}Cr$ and $^{53}Cr/^{52}Cr$ for Cr(III) and Cr(VI) for the application of the isotopic dilution (ID) technique in several samples under study.

4.2.5. Species-specific isotope dilution

SS-ID has already been studied by other authors in the quantification of Cr(VI) in soil (Guidotti et al., 2015; Caporale et al., 2019), water (Kingston et al., 1998; Tirez et al., 2003; Ma et al., 2008) and river sediments (Drinčić et al., 2018). The quantification of Cr species in foods with the application of SS-ID has only been applied to bread and tea samples (Novotnik et al., 2013/2015).

During sampling, storage, extraction and measurement processes in food samples, interconversions between Cr(III) and Cr(VI) have been frequently observed, leading to inaccurate speciation results. These analytical biases can be corrected mathematically and quantitatively by speciated isotope-dilution (or species specific) (SS-ID) in combination with HPLC-ICP-MS couplings (Unceta et al., 2010).

SS-ID is based on the spiking a sample with known amounts of the analyte/species with having an altered isotopic composition (spike). The most critical point in SS-ID is the equilibration of sample and spike species. Once the equilibrium is achieved the species interconversions can be quantitatively corrected (Rodríguez-González and Alonso, 2019; Gaffney, 2017).

To carry out SS-ID for chromium speciation analysis in this study to each standard and sample, ^{50}Cr(III) and ^{53}Cr(VI) spikes were added and the blend solution was thoroughly mixed to achieve isotopes equilibration. The natural isotopic abundances, standard solution Cr(III) and Cr(VI) abundances and abundance of ^{50}Cr in the Cr(III) spike and ^{53}Cr in the Cr(VI) spike are presented in Table 4.1, as provided by the manufacturer's certificate. The natural abundances of the various Cr isotopes used for SS-ID calculations are those reported by the Internal Union of Pure and Applied Chemistry (IUPAC, 2016).

Table 4.1. Abundances certified of Cr(III) and Cr(VI) and respectively enriched isotope solution

	Isotopic abundance [a] (%)		
Cr isotope	**Natural**	**^{50}Cr(III)$_{spike}$ [b]**	**^{53}Cr(VI)$_{spike}$ [c]**
50	4.35	97.4	0.1
52	83.79	2.3	6.8
53	9.50	0.3	92.4
54	2.36	0.1	0.7

[a] IUPAC, 2016
[b] ISC-SCIENCE, 20160129-1
[c] ISC-SCIENCE, 20180912-1

To carry out SS-ID, the samples were spiked with known amounts of ^{50}Cr(III) and ^{53}Cr(VI) spikes to achieve isotope ratios (^{50}Cr/^{52}Cr and ^{53}Cr/^{52}Cr) in the blend samples ≈1.

The equilibration of the samples species and the spikes is a critical step in SS-ID. In order to ensure the equilibration, the blends were stirred manually for 10 minutes and then left for 15 minutes to reach equilibrium. It is important to note that after isotopic equilibrium is achieved, subsequent sample losses or incomplete recoveries during the analytical procedure will not affect the results (Novotnik et al., 2013; Rodriguez-Gonzalez and Alonso, 2019).

The main procedural steps to carry out SS-ID are described in the next paragraphs.

(i) Preparation and characterisation of the spike solutions

Solution of ^{50}Cr(III) and ^{53}Cr(VI) spikes were prepared daily, in some cases at different concentration, depending on the optimisation to be carried out and of the concentration of Cr(III) and Cr(VI) in foodstuffs. The concentration of each spike solution was determined with reverse isotope dilution (RID) using the equations 1-2.

$$C_{spike}^{III} = C_{std} \text{ x } \frac{m_{std}}{M_{std}} \text{ x } \frac{M_{spike}}{m_{spike}} \text{ x } \frac{R_m \text{ x } A_{std,b} - A_{std,a}}{A_{spike,a} - R_m \text{ x } A_{spike,b}} \quad (1)$$

$$C_{spike}^{VI} = C_{std} \text{ x } \frac{m_{std}}{M_{std}} \text{ x } \frac{M_{spike}}{m_{spike}} \text{ x } \frac{R_m \text{ x } A_{std,b} - A_{std,c}}{A_{spike,c} - R_m \text{ x } A_{spike,b}} \quad (2)$$

where:

C_{spike}^{III} and C_{spike}^{VI}, concentration of Cr(III) and Cr(VI) in the spike solution (µg kg^{-1}) (to be determined)

C_{std}, concentration of standard solution Cr(III) or Cr(VI) (µg kg^{-1})

m_{std}, mass of the Cr(III) or Cr(VI) standard solution add (g)

M_{std}, molar mass of the Cr(III) or Cr(VI) standard solution (g mol^{-1})

M_{spike}, molar mass of the ^{50}Cr(III) or ^{53}Cr(VI) spike solution (g mol^{-1})

m_{spike} , mass of the ^{50}Cr(III) or ^{53}Cr(VI) spike solution add (g)

R_m , ratio measured after correction by K value (eq. 3)

$A_{std,a,b,c}$, natural abundance of the standard solution for each isotope (a=50, b=52 and c=53)

$A_{spike,a,b,c}$, natural abundance of the spike solution for each isotope (a=50, b=52 and c=53)

(ii) Spiking and species equilibration

The sample is spiked with a known amount of ^{50}Cr(III) and ^{53}Cr(VI) spikes to obtain ^{50}Cr(III)/^{52}Cr(III), ^{53}Cr(III)/^{52}Cr(III), ^{50}Cr(VI)/^{52}Cr(VI), and ^{53}Cr(VI)/^{52}Cr(VI) ratios ≅1 , which is prerequisite for high accuracy isotope ratio measurements.

(iii) Isotope ratio measurement by ICP-MS

The chromatographic peaks corresponding to Cr(III) and Cr(VI) species allow the measurements of the isotopic ratios mentioned above in each analysis.

The measured isotope ratios are also corrected with the mass bias factor (K) being determined during the analytical session for both ^{50}Cr/^{52}Cr on the peak Cr(III) and ^{53}Cr/^{52}Cr ratios on the peak Cr(VI) using Eq. (3).

$$K = \frac{R_t}{R_m} \quad (3)$$

where:

R_t, R_m, theoretical (or certified) and measured isotope ratio for a given couple of isotopes.

(iv) Calculation of Cr(III) and Cr(VI) concentrations and the mutual interconversion factors

The concentration of each species as well as the species interconversion factors (α and β) are determined by means of the mathematical model already reported in Paper 1 (as Supplementary data). An example of an Excel sheet used to calculate the concentrations of Cr(III) and Cr(VI) and interconversion factors (α and β) by SS-ID is provided in the appendix (Figs A2-A6)**.**

4.3. Cr speciation analysis in food by SS-ID HPLC-ICP-MS

This section presents the analytical development and validation of two novel analytical approaches for the simultaneous speciation analysis of Cr(III) and Cr(VI) in two food groups, namely (i) milk and meat (Paper I) and (ii) bread, breakfast cereals and rice samples (Paper III and IV) by means of SS-ID by HPLC-ICP-MS. For this purpose, the main parameters related to the species extraction, separation and detection were optimized.

Table 4.2 shows an overview of the literature data on Cr speciation analysis by type of food sample. The optimisation of each step of the analytical processes is reported in the next sections.

Table 4.2. Overview of the literature data on Cr speciation analysis by samples: Comparison of extraction procedure and detection method used with Cr(VI) levels reported

Samples	Extraction procedure	Detection	Cr(VI) (µg/kg)	Reference
Dairy products:				
UHT Milk	Selective extraction using chromabond NH_2 column following precipitation of proteins	ETAAS	< 0.15-1.20	Lameiras et al., 1998
Powdered Milk formula	Selective extraction using chromabond NH_2	ETAAS	10-75	Soares et al., 2000
Cow Milk	Selective extraction using chromabond NH_2 column following precipitation of proteins	DRC-ICP-MS	0.61-1.44	Ambushe et al., 2009
Cheese, yoghurts and milk	Cr(III): Microwave digestion; Cr(VI) : Alkaline extraction (NH_4OH)	HPLC-ICP-MS	No Cr(VI) detected	Hernandez et al., 2018
Meat and meat products:				
Beef meat	Incubated in water bath for 1h at 63°C followed by 10min. sonication (amplitude of 50)	HPLC-ICP-MS	6.3-14.3a	Shittu et al., 2020
Cereals:				
Bread	Microwave assisted digestion: HNO3 + H2O2	GFAAS	No Cr(VI) detected	Kovács et al., 2007
Bread	Alkaline extraction using 0.01M NaOH + 1M NH4NO3	ETAAS	5.65-6.82	Soares et al., 2010
Bread	Bread: Alkaline extraction using 0.01M NaOH + 1M NH4NO3	SIDMS	No Cr(VI) detected	Novotnik et al., 2013
Bread and cereals	Alkaline extraction using NH4NO3 (pH=11,5)	HPLC-ICP-MS	No Cr(VI) detected	Vacchina et al., 2015
Bread and breakfast cereals	Alkaline extraction using 0.1M Na2CO3	HR-CS AAS	20.4-470.4	Mathebula et al., 2017
Bread	Cr(VI) : Alkaline extraction (NH_4OH)	HPLC-ICP-MS	No Cr(VI) detected	Hernandez et al., 2017
Rice	surfactant-assisted emulsification dispersive liquid-liquid microextraction	ETAAS	No Cr(VI) detected	Dokpikul et al., 2018
Rice	Alkaline extraction with TMAH	IC-ICP-MS	5.93-142	Chen et al., 2020
Drinks and beverages:				
Tea	Alkaline extraction using 0.1M Na_2CO_3	ETAAS	0.12-0.48[a]	Panichev et al., 2005
Drinking water	Acid digestion pH=1	Spectrofluorometric	0.91-21.75	Hosseini et al., 2009
Tea	Alkaline extraction using 0.1M Na_2CO_3	GF-AAS	0.46-8.07[a]	Elci et al., 2010
Tea	Tea: Alkaline extraction using 0.1M Na_2CO_3	SIDMS	No Cr(VI) detected	Novotnik et al., 2013
Black, green and herbal teas	Alkaline extraction using 0.1M Na_2CO_3	GF-AAS	0.03-3.15[a]	Mandiwana et al., 2011
Tea leaves and infusion	Solid phase extraction adsorbent by Titanium dioxide (TDNTs)	ICP-MS	No Cr(VI) detected	Chen et al., 2014
Lager beer	Selective extraction using chromabond NH_2 column following precipitation of proteins	GFAAS	0.94-2.51	Vieira et al., 2014
Tea leaves and tea infusion	Microwave assisted digestion: HNO_3 + H_2O_2	SPE-ICP-MS	0.12[a]	Chen et al., 2014
Wine, beer	Coud point extraction of AgNPs by TritonX-114	ETAAS	0.51-0.135	López-Garcia et al., 2015
Neem power and tea infusions	Alkaline extraction 0.1M Na_2CO_3 and 0.1M $MgCl_2$	HPLC-ICP-MS	No Cr(VI) detected	Novotnik et al., 2015
Fruits and vegetables:				
Mushrooms	Alkaline extraction using 0.01M NaOH/1M NH_4NO_3	ETAAS	0.103-0.143[a]	Figueiredo et al., 2007
Red lentils	Selective extraction using Amberlite XAD-1180 resin	FAAS	No Cr(VI) detected	Narin et al., 2008
Apple, parsley, wheat, marrow, quince	Selective extraction of Cr(III) using synthesised chelating resin	FAAS	No Cr(VI) detected	Çimen et al., 2013
Fats and others				
Edible animal oils	Microwave assisted extraction using HF, TritonX-100, TBAP, EDTA	HPLC-ICP-MS	No Cr(VI) detected	Lin et al., 2016
Turmeric	Complexation with 1,5-diphenylcarbazide using Cloud point extraction (CPE)	DRS-FTIR	3190-5410	Tiwari et al., 2017

[a] µg/g; [b] µg/mL

4.3.1. Single run sequential extraction of Cr(III) and Cr(VI) from multi-food matrices

In this study, Cr(III) was selectively complexed using EDTA, while Cr(VI) (represented here as chromate anion, CrO_4^{2-}) was derivatized by reaction with DPC. Both chemical reactions are shown in Fig. 4.1 a-b. It 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 et al., 2018; Mathebula et al., 2017; Soares eta al., 2009), this approach is used here for the first time for simultaneous speciation analysis of Cr(III) and Cr(VI) in foodstuffs.

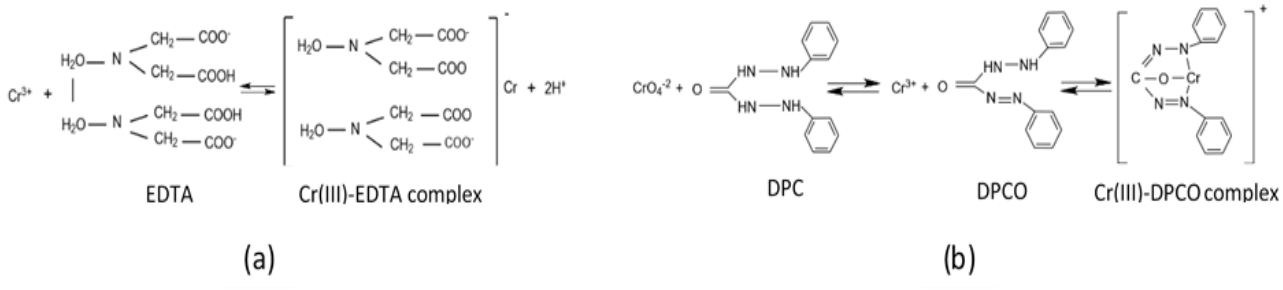

Figure 4.1. Simplified reaction schemes of Cr(III) with EDTA (a) and of Cr(VI) with DPC (b) (for simplicity, proton contribution and charge balance are neglected for the reaction b)

As can be seen in Fig. 4.1a, the Cr(III)-EDTA complex is negatively charged and consequently Cr^{3+} complexation with EDTA leads to an inversion of the charge of this species. Similarly, the reaction of Cr(VI) (in CrO_4^{2-} form) with DPC (Fig. 4.1b) involves firstly the reduction of Cr(VI) to Cr(III) and subsequently the complexation of the resulting Cr^{3+} with dyphenilcarbazone (DPCO) (the oxidized form of DPC) leading to Cr(III)-DPCO complex, which is positively charged. Therefore, the reactions of Cr(III) with EDTA and Cr(VI) with DPC both lead to charge inversion of the corresponding species, hence providing the possibility of their further HPLC separation by using ion exchange. It is also important to note that whereas Cr(III) is truly complexed with EDTA, in the case of Cr(VI), this species is firstly reduced to Cr(III) and then subsequently complexed by DPCO. Therefore, when carrying out the reactions of Cr(III) and Cr(VI) with EDTA and DPC, respectively, in the same analytical run, there is a competition between the genuine Cr(III) and Cr(III) resulted from Cr(VI) reduction by DPC. Hence, it is necessary to carry out the complexation of genuine Cr(III) by EDTA before the reaction of Cr(VI) with DPC takes place. In this way, the complexation of genuine Cr(III) with EDTA prevents on one hand, its oxidation to Cr(VI) during the analysis and on the other hand, its complexation with DPCO; actually, DPCO must react only with Cr(III) resulted from Cr(VI) reduction.

The main challenge of this step consisted of the optimisation of the sequence of ETDA and DPC addition to the Cr(III)-Cr(VI) mixture. To optimise Cr(III) complexation with EDTA and Cr(VI) derivatisation with DPC, a mix solution of Cr(III) and Cr(VI) at 5 µg L^{-1} (each species) was employed and the results were compared with those obtained by the analysis of individual solutions of Cr(III) and Cr(VI) at the same concentration as in the mixture. The various tests for this purpose were carried out in triplicates.

Initially, both EDTA and DPC were added simultaneously to the Cr(III)-Cr(VI) mixture but a quasi-total reduction of Cr(VI) was observed in this case (see Fig. 4.2, blue bar). Subsequently, EDTA and DPC were added to the species mixture at different times, first EDTA and then DPC (orange bars), and then DPC followed by EDTA (green bars). As can be seen in Fig. 4.2, the best complexation of Cr(III) with EDTA and Cr(VI) derivatisation with DPC was obtained when EDTA is added before the DPC (orange color in).

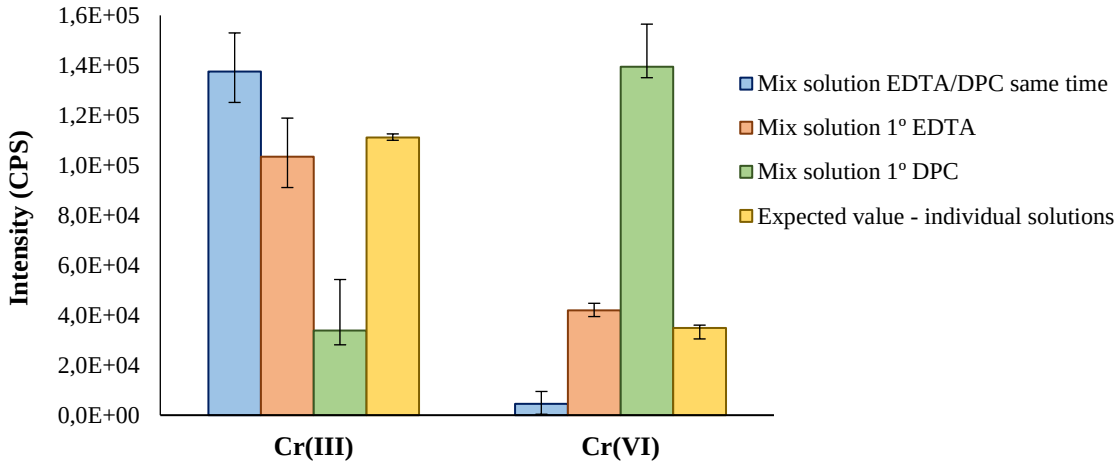

Figure 4.2. Comparison of the signals obtained for the analysis of a mix solution of Cr(III) and Cr(VI) at 5 µg kg^{-1} (each species) and those obtained by the analysis of the individual species at the same concentration (the expected signals are in yellow color)

4.3.1.1. Optimisation of the extraction of Cr(III) and Cr(VI) from milk and meat samples

(a) Optimisation of the extraction time and temperature

The extraction time and temperature were optimised to ensure complete complexation/derivatisation of Cr species from food samples. For this purpose, the temperature was varied between 30 to 90 ºC and times between 5 to 35 min (5, 15, 25 and 35 min).

The results obtained for various couples temperature/time are shown in Fig. 4.3.

At temperatures < 50 ºC, complexation of Cr(III) with EDTA and DPCO is very scarce (or absent) or the most efficient complexation of Cr(III) (genuine and from Cr(VI)) with EDTA and DPCO was obtained at 70 °C during 25 min. To validate these conditions, three certified reference materials, namely CRM041 (Cr(VI) in soil), NIST 1849a - Infant/adult nutritional formula (for total Cr) and NIST 8436 - durum wheat flour (total Cr) were analysed.

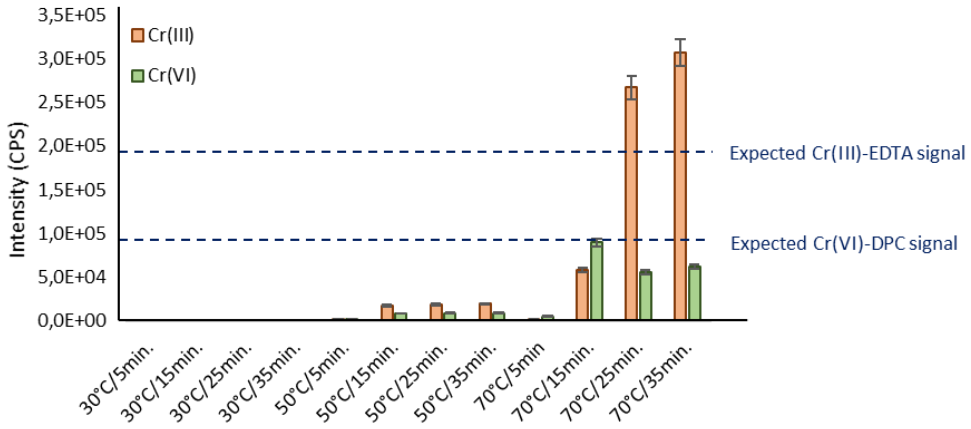

Figure 4.3. Intensity obtained for the analysis of a mix solution of Cr(III) and Cr(VI) at 5 µg L^{-1} (each species) by SS-ID HPLC-ICP-MS at different extraction temperatures and times compared with the expected signals corresponding to the analysis of individual solutions of Cr(III) and Cr(VI) (at 5 µg L^{-1})

In the latter cases, the validation was assessed by means of the species balance, comparing the sum of Cr(III) and Cr(VI) levels with the certified total Cr, as can be seen in Table 4.3. It is worth to note that so far there is no any CRM available for Cr speciation analysis in food.

Table 4.3. Values obtained for the various certified reference materials used to assess the quality of the method in the quantification of Cr(III) and Cr(VI)

Sample (n=6)	**Cr_{total} (mg kg^{-1})**	**Recovery (%)**
CRM041 [a]	127.3 ± 2.46 *	98
NIST 1849a [b]	1.132 ± 0.125	106
NIST 8436 [c]	0.022 ± 0.002	96

Reference value (mg kg^{-1}): [a] 130 ± 3.42; [b] 1.072 ± 0.03; [c] 0.023 ± 0.009
* Only a certificate reference value for Cr(VI)

(b) Optimisation of the extraction pH

The effect of the pH on the efficiency of Cr(III) complexation and Cr(VI) derivatisation was also optimized in the range between 2 and 10; for adjusting the pH, nitric acid and ammonia solutions, respectively, were used.

The results in terms of Cr(III) and Cr(VI) recovery factors obtained at different pH are presented in Fig. 4.4.

As can be seen, pH =2 and pH=4 ensured maximum efficiency of Cr(III) complexation with EDTA and Cr(VI) derivatisation with DPC. For practical reasons, optimum pH=4 was used for further experiments, which in agreement with other studies (Threeprom et al., 2005; Soares et al., 2009).

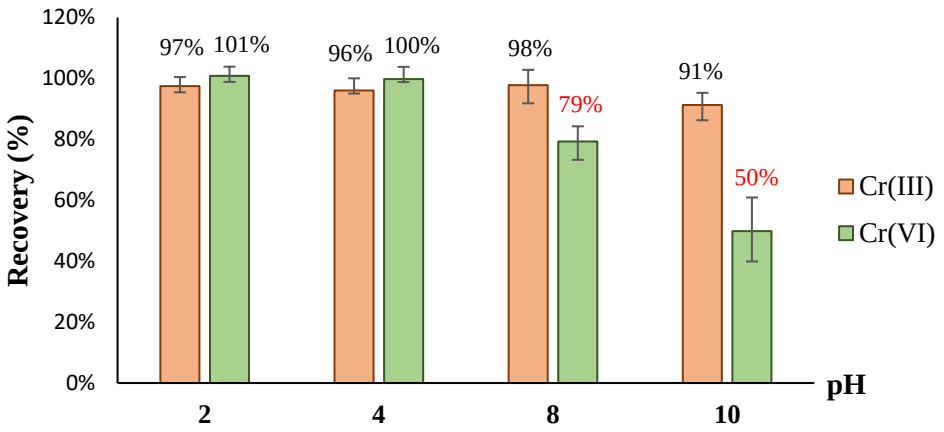

Figure 4.4. Recovery (%) obtained for the analysis of a mix solution of 5 µg L^{-1} of Cr(III) and Cr(VI) at different pHs

4.3.1.2. Optimisation of the extraction of Cr(III) and Cr(VI) from bread an breakfast cereals

The optimum parameters such as temperature and time suitable for the extraction of Cr(III)-Cr(VI) from milk and meat samples turned out to be non-efficient for the extraction, of Cr species from bread and breakfast cereals. Actually, although excellent recovery was found for the total Cr, practically all Cr measured in bread an breakfast cereals was found in Cr(VI) form (see Table 4.4).

Table 4.4. Total Cr and Cr speciation (mean ± SD) in different bread by ICP-MS and SS-ID HPLC-ICP-MS, respectively

Samples	Mass fraction ± SD (µg kg^{-1}, n=3)			
	Cr_{total} (ICP-MS)	Cr(III) (SS-ID)	Cr(VI) (SS-ID)	Recovery (%)
Baguette	36.1 ± 2.1	<LOD	34.8 ± 0.8	96
Sandwich bread	70.0 ± 3.2		71.0 ± 1.2	101
Corn bread	54.4 ± 1.9		53.9 ± 0.6	99
Toasted bread - wheat flour	39.1 ± 2.7		39.3 ± 1.7	101
Toasted bread - whole grain	31.3 ± 4.2		32.8 ± 2.3	105
French bread	26.7 ± 1.5		28.5 ± 0.5	107

This finding is most probably due to an analytical artefact, as described below.

It appears that Cr(III) in bread is more difficult to decomplex from the matrix and therefore would only be released in the second phase of heating the sample with DPC, appearing as peak Cr(VI).

To overcome this behaviour, the extraction was re-optimized by increasing the extraction temperature for the simultaneous determination of Cr(III) and Cr(VI) from a bread without any addition of Cr(VI). For this purpose, extraction temperature in the first step was increased at 90 ºC while the extraction time was optimized between 25 and 120 min. The results are shown in Fig. 4.5.

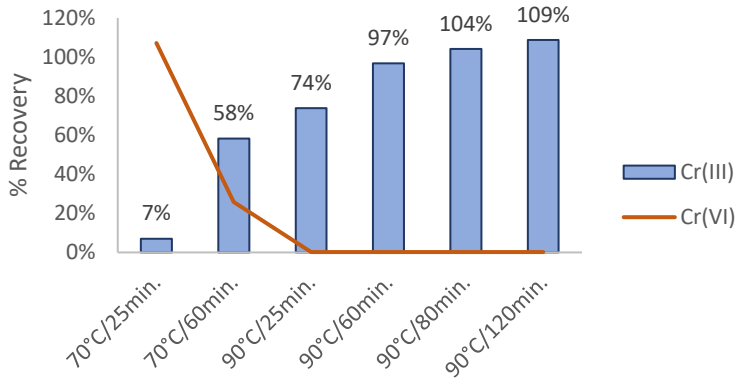

Figure 4.5. Recovery of Cr(III) (blue bars) and Cr(VI) (red line) from bread samples with the increase of extraction temperature and time in the first step while the extraction conditions of the second step remained unchanged (as reported in the section 4.3.1.1)

As can be seen, in Fig. 4.5, with the increase in extraction time and temperature of Cr(VI) artefact level decreases considerably with simultaneous increase in the Cr(III) levels. For further experiments, the extraction was carried out at 90 °C during 60 min.

In order to validate the extraction procedure for Cr(III)-Cr(VI) determination in bread (without the formation of the Cr(VI) artefact), two different types of bread (with different levels of natural Cr (III)) were spiked with different concentrations of Cr(III) and the results were compared with those obtained for the analysis of the same sample without any Cr(III) addition. The results are shown in the Table 4.5.

Table 4.5. Levels of Cr(III) and the corresponding recovery factors obtained for the analysis of two bred types spiked with various levels of Cr(III) and analyzed by SS-ID HPLC-ICP-MS

Spiking levels of Cr(III) (µg kg^{-1})	**0**	**5**	**10**	**15**
Bread 1				
Cr(III)	130 ± 1.6	137 ± 3.8	153 ± 1.3	158 ± 2.5
Recovery factor (% ± SD n=2) [a]	97 ± 1.2	99 ± 2.7	106 ± 0.9	105 ± 1.2
Cr(III) spiked	-	4.8 ± 0.7	9.7 ± 0.3	14.4 ± 1.2
Recovery factor (% ± SD n=2) [b]	-	96 ± 2.3	97 ± 1.2	96 ± 1.1
Bread 2				
Cr(III) (µg kg^{-1})	26.8 ± 1.6	31.8 ± 0.9	41.2 ± 2.1	44.7 ± 0.7
Recovery factor (% ± SD n=2) [a]	101 ± 2.2	101 ± 3.0	113 ± 5.7	107 ± 1.7
Cr(III) spiked	-	4.9 ± 0.3	10.5 ± 0.2	14.9 ± 1.1
Recovery factor (% ± SD n=2) [b]	-	98 ± 2.0	105 ± 2.4	99 ± 1.8

[a] Recovery compared with total Cr measured by ICP-MS

[b] Recovery of Cr(III) spiked in bread measured by ID-HPLC-ICP-MS

As can be seen in Table 4.5, good recovery (97 to 113%) of Cr(III) at all spiking levels was obtained for the two type of bread. Equally good recoveries were found for Cr(III) spiked in bread with values between 96 and 105%.

The results related to the optimisation of the Cr(III) extraction from bread samples demonstrate the complexity of Cr speciation and the easiness of formation of (Cr(VI)) analytical artefact, hence leading to erroneous conclusions regarding the presence of this species in foodstuffs. Additionally, it appears that Cr(III) and Cr(VI) from food is pretty much matrix dependant, despite the use of SS-ID. The extraction temperature and time of the second extraction phase (after adding the DPC) was also optimized, by using 70 °C and 90 ºC for extraction while varying the time between 25 to 60 min. and the results obtained are shown in Table 4.6.

Table 4.6. Levels of Cr(III) and Cr(VI) (mean ± SD) obtained by SS-ID HPLC-ICP-MS at 70 °C and 90 °C (heating in a water bath) while the time was varied between 25 and 60 min. in the same bread sample (n=3)

			Mass fraction ±SD (µg kg^{-1}, n=3)			
EDTA step	**DPC step**	**Cr_{total} (ICP-MS)**	**Cr(III) (SS-ID)**	**Cr(VI) (SS-ID)**	**Cr(III)+Cr(VI) (SS-ID)**	**Recovery [b] (%)**
90°C/60min.	70°C/25min.	26.6 ± 3.8	1.8 ± 0.29	28.5 ± 2.79	30.3 ± 2.4	114 ± 5
	70°C/60min.		15.5 ± 0.75	6.6 ± 0.95	22.4 ± 1.9	84 ± 2
	90°C/25min.		19.6 ± 0.34	< 0.05 (LOQ)	19.6 ± 1.8	75 ± 3
	90°C/30min.		25.8 ± 0.40	< 0.05 (LOQ)	25.8 ± 1.5	97 ± 1
	90°C/35min.		27.7 ± 0.42	< 0.05 (LOQ)	27.7 ± 2.0	104 ± 2
	90°C/40min.		28.9 ± 0.10	< 0.05 (LOQ)	28.9 ± 2.1	109 ± 6

[a] Cr(III)+Cr(VI) (SSID);
[b] The recovery is calculated by comparison of the sum of Cr(III) + Cr(VI (SS-ID) with total Cr by ICP-MS

As can be seen in Table 4.6, the increase in temperature to 90 ºC in the second extraction phase showed a reduction in Cr(VI) levels with a consequent increase in Cr(III) levels, with good recoveries between 75 and 114%.

For simplicity, the same temperature (90 °C) was adopted for the second extraction step while optimizing the time was optimized more deeply by its variation between 5 and 30 min. For this purpose, a bread sample was spiked with Cr(VI) at 1 µg kg^{-1} and the recovery for this species extracted at different times was calculated. As can be seen in the Table 4.7., the best recovery (102 ± 3%) was obtained while carrying out the second step extraction at 90°C for 25 min., hence these conditions were adopted for the further experiments.

Table 4.7. Cr(VI) level and the corresponding spike recovery obtained for Cr(VI) from a bread sample spiked Cr(VI) (at 1 µg kg^{-1}) by SS-ID-HPLC-ICP-MS at 90 °C while carrying the extraction time between 5 and 30 min (the first extraction was carried out at 90 °C for 30 min)

Extraction time (min) (DPC step)	$Cr(VI)_{spiked}$ (µg kg^{-1})	Recovery (%)
5	2.14 ± 0.01	214 ± 9
10	1.94 ± 0.08	194 ± 6
15	1.48 ± 0.14	148 ± 8
20	1.23 ± 0.04	123 ± 4
25	1.02 ± 0.03	102 ± 3
30	1.06 ± 0.04	106 ± 4

In conclusion, the optimal extraction conditions for the single run speciation analysis of Cr(III)-Cr(VI) from bread consisted of a first step (EDTA Cr(III) complexation) heating at 90 °C for 60 minutes and at 90 °C for 25 minutes in the second step (Cr(VI) derivatisation by DPC).

It is also worth to note that no Cr(VI) was found in any of the bread samples analyzed during the method development and validation, which is in agreement with EFSA and other studies (EFSA 2014; Kovács et al., 2007; Novotnik et al., 2013; Vacchina et al., 2015; Hernandez et al., 2017; Milačič and Ščančar, 2020).

4.3.2 Species separation by HPLC

(a) The choice of the separation mechanism

In this study, several types of columns such as IC Dionex™ IonPac™ CS5A (4 x250 mm x 9 µm), Dionex Ion PAC AS7 column (4x250mm), Dionex Ion Pac AG-7 (50 × 4 mm) were initially tested by the analysis of Cr(III) and Cr(VI) individually and mixed. Due to the ionic nature of the Cr(III)-EDTA and Cr(DPCO) species, all the columns mentioned above have anion exchange mechanisms. Each of these columns were tested by using different mobile phases, as described below.

(b) Optimisation of the mobile phase

The mobile phase consisted of a solution of HNO_3 whose concentration was optimized in the range 0.6 mol L^{-1} up to 0.01 mmol L^{-1} which correspond to a variation in pH between 0.2 and 4.5. For this purpose, a standard mixture of Cr(III)-EDTA and Cr(VI)-DPC at 5 µg kg^{-1} was analyzed in triplicate by SS-ID HPLC-ICP-MS.

As can be seen in Fig. 4.6, when the Dionex Ion PAC AG7 column was employed with the mobile phase at pH=0.15 and pH=1, a single peak corresponding to Cr(III)-EDTA is obtained, hence indicating a complete reduction of Cr(VI) during the separation in these conditions.

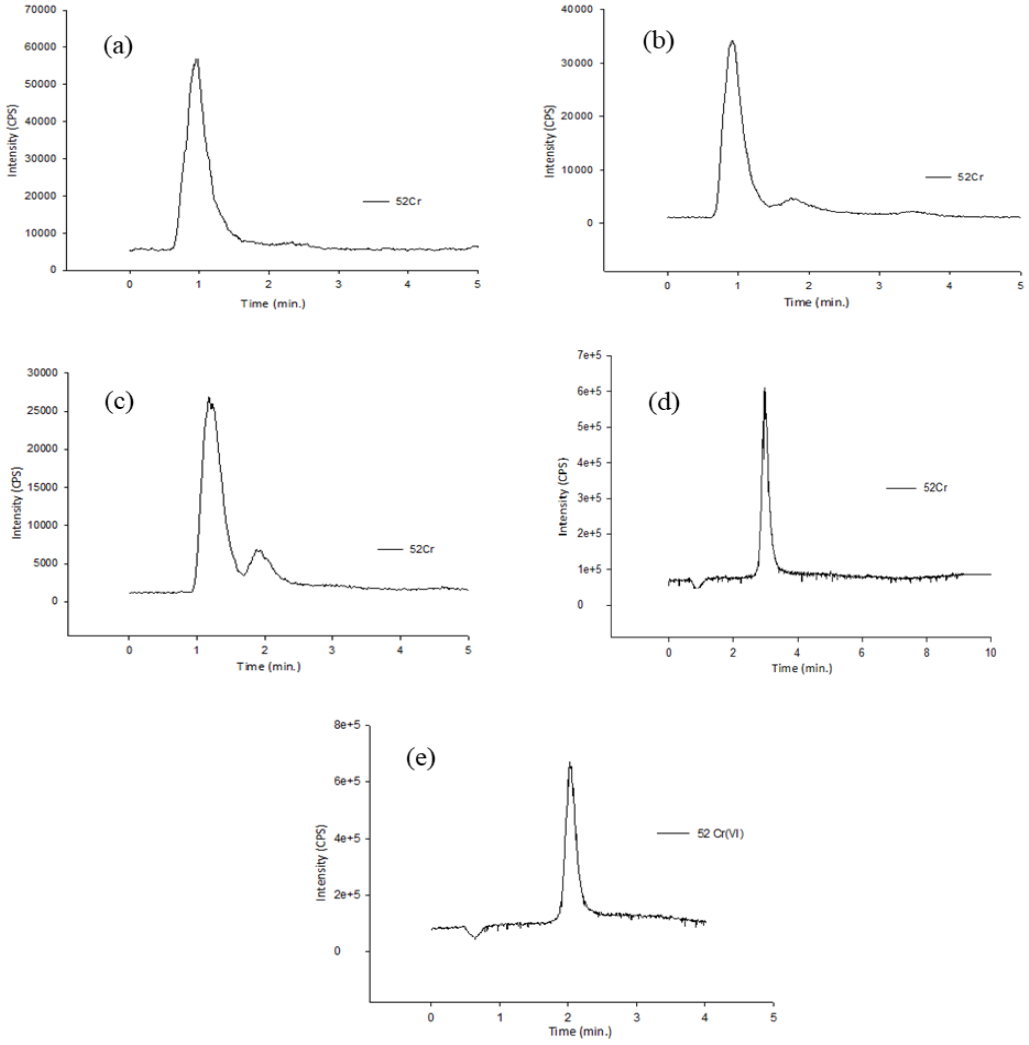

Figure 4.6. Chromatograms obtained by SS-ID HPLC-ICP-MS for the analysis of a mix solution of Cr(III) and Cr(VI) at 5 µg kg^{-1} with the mobile phase at pH=0.15 (a), pH=1.04 (b), pH=2.10 (c), pH=3.75 (d) and pH=4.55 (e). *Note:* Both peaks (at a retention time of 1 min) in figs. (a)-(b) correspond to the Cr(III)-EDTA peak, whereas the peaks in the figs. (d)-(e) correspond to Cr(III)-DPCO peak

The optimum separation was achieved at pH≈2 (10 mM HNO_3). It is worth to note that at pH<2, a single peak corresponding to the Cr(III)-EDTA complex was obtained, whereas at pH>2, a single peak corresponding to Cr(III)-DPCO complex. This confirms the importance of the pH to ensure optimum separation of Cr(III) complexes (with EDTA and DPCO) by anion exchange HPLC.

Further optimisation of the HPLC separation efficiency was carried out by the assessment of the effect of methanol in the mobile phase at 1.0, 2.5 and 5% (v/v), respectively. The concentration of MeOH at 2.5 % assured the best separation, with a resolution factor of ≥ 1.5.

Further, in order to prevent the de-complexation of Cr(III)-EDTA species during the HPLC separation due to the difference between the optimum complexation pH (4) and that of the HPLC separation (2), EDTA was also added to the mobile phase, as recommended in other studies (Uenderson et al., 2017; Lin et al., 2016; Danuta et al., 2013; Andrew et al., 2009; Kuo et al., 2007; Wolf et al., 2007; Chang and Jiang, 2001). For this purpose, EDTA concentration in the mobile phase was optimized in the range from 0 to 0.60 mM and the most efficient separation ensuring quantitative complexation of Cr(III) with EDTA was obtained at 0.30 mmol L^{-1} (recovery of 105% for Cr(III)-EDTA and 97% for Cr(III)-DPCO).

Overall, the optimum HPLC mobile phase consisted of a mixture of HNO_3 (10 mmol L^{-1}), EDTA (0.30 mmol L^{-1}) and MeOH (2.5%, v/v) at pH=2.

(c) Optimisation of the column temperature

Finally, the separation column temperature was optimized in the range between 20-50°C (20, 25, 30, 35, 40, 45 and 50°C). The optimum resolution factor (≥1.5) was obtained at 30°C, which was chosen for the rest of the experimental work.

4.3.3 Species detection by ICP-MS

Two ICP-MS systems were used, iCAP Q initially used during my stay in France and iCAP TQ in Denmark. Only the iCAP Q ICP-MS system was optimized, at the beginning of this study, for the determination of Cr(III) and Cr(VI) and the optimized parameters were later used in the iCAP TQ ICP-MS system, working perfectly without the need for a second optimization of these parameters. The data presented below correspond to the iCAP Q ICP-MS system.

(a) Dwell time optimisation for the iCAP Q ICP-MS

The dwell time was optimized in the range between 10 and 1000 ms (10, 20, 50, 75, 100, 250, 500 and 1000 ms). The best condition was obtained for 250 ms. For simplicity, these data are not shown.

(b) Optimisation of the plasma power

Plasma power was also optimized in the range from 1400 to 1600 W to ensure maximum efficiency of species ionization. The results obtained for the analysis of a mix solution of 5 µg L^{-1} of Cr(III) and Cr(VI) are presented in Fig. 4.7. As can be seen, the highest ionisation efficiency of Cr was obtained at 1550W for both species, which was used for further experiments.

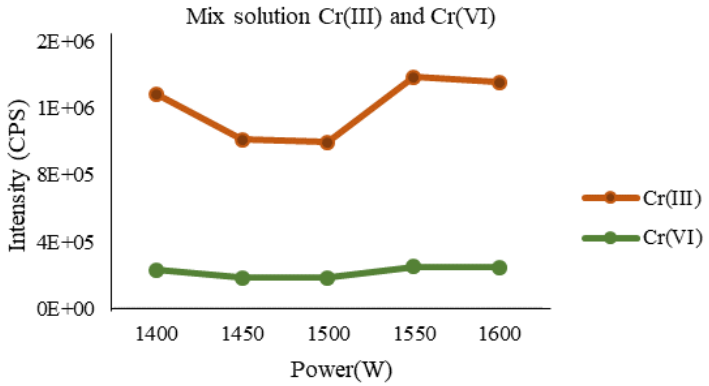

Figure 4.7. Intensity obtained for the analysis of a mix solution of Cr(III) and Cr(VI) at 5 µg kg^{-1} at different plasma powers.

(c) Optimisation of the detection mode: standard versus collision cell approach

In order to minimize the spectral interferences, all ICP-MS are nowadays equipped with collision/reaction cells based on kinetic energy discrimination (KED) using H_2 (reaction mode) or He (collision mode) (Tanner et al., 2002; Koppenaal et al., 2004; Quarles et al., 2014). Despite the capability of the KED mode to minimize the spectral interferences (e.g. $^{40}Ar^{12}C$ on ^{52}Cr isotope), a side effect of this technology is a reduction in sensitivity (Hagendorfer and Goessler, 2008). Therefore, the flow of the collision gas need to be optimized to ensure the maximum signal/noise ration while reducing the spectral interferences.

In this work, the flow of He used here as collision gas was optimized in the range between 3.0-5.5 mL min^{-1}. For this purpose, the CRM041-30G (Chromium VI in soil) material was analysed by SS-ID HPLC-ICP-MS (in triplicate) and the results in terms of Cr(VI) recovery are shown in Table 4.8.

Table 4.8. Results obtained for different gas flow (He) in the analysis of Cr(VI) in CRM041 (mean ± SD)

Gas flow (mL min.)	Cr(VI) mass fraction (mg kg^{-1})	Recovery (%)[a]
3.0	175 ± 9	134 ± 3
3.5	154 ± 6	118 ± 4
4.0	143 ± 8	110 ± 2
4.5	138 ± 5	106 ± 4
5.0	**131 ± 4**	**101 ± 2**
5.5	115 ± 6	89 ± 2

As can be seen in Table 4.8, the best recovery of Cr(VI) (101 ± 2%) from this CRM was obtained when using a gas flow of 5.0 mL min^{-1}, which was chosen for further analysis.

A chromatogram obtained for the analysis of a standard mixture of Cr(III) and Cr(VI) at 5 µg kg^{-1} by SS-ID HPLC-ICP-MS conditions optimized in the sections above is shown in Fig. 4.8.

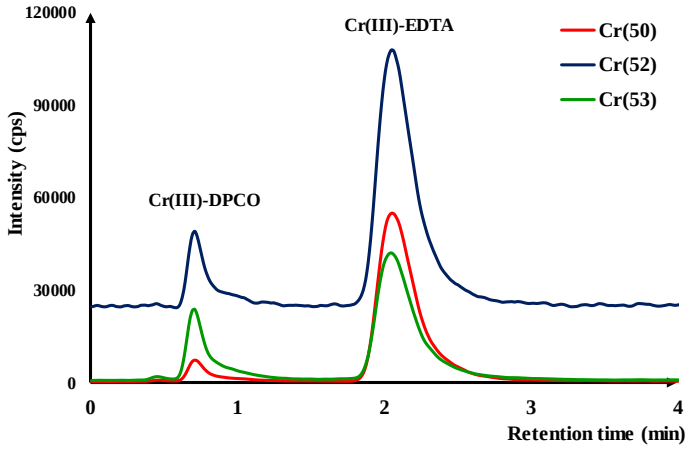

Figure 4.8. Chromatogram obtained for the analysis a standard mixture of Cr(III) and Cr(VI) at 5 µg kg^{-1} by SS-ID HPLC-ICP-MS in optimum conditions

4.4. Method validation by means of the accuracy profile approach

The method was validated based on the accuracy profile approach according to guidelines described in detail elsewhere (IUPAC, 2002; AFNOR, 2010; Ghosn et al., 2020) for milk and meat samples (Paper I) and for bread samples (Paper III). Briefly, this approach takes into account the simultaneous assessment of the method accuracy and precision. 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 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 (≥5) corresponding to natural (CRM, if available) or spiked matrices. Further, a tolerance interval is calculated for each level; β_{TI} is the interval in which a prediction can be made for a known proportion of the results, which is represented on the accuracy profile.

It is worth to note that CV_R (and hence the expanded uncertainty) is dependent on the analyte measurement level. In this study, for simplicity, CV_R is reported for two concentration domains, namely < 2×LOQ and ≥ 2×LOQ.

In the absence of CRMs for speciation of chromium in foods, extraction controls were performed using spiked samples. The certificate reference material - BCR® 544 for quantification of Cr(III), Cr(VI) and total Cr in lyophilised solution is no longer available.

4.4.1. Method validation for milk and meat analysis

The validation was performed using three food matrices: infant formula milk, semi-skimmed milk and bovine meat (Paper I). The spiked samples at different levels were carried out on homogenized samples before extraction. The method was validated by means of the accuracy profile approach by carrying out six measurement series in duplicate on (six) different days during two months.

For Cr(III), six different levels were taken into account: A= ½ LQ (0.013 µg kg^{-1}), B= LQ (0.024 µg kg^{-1}), C= 2xLQ (0.048 µg kg^{-1}), D= Cr(III) naturally present in infant formula milk (2.70 µg kg^{-1}), E= Cr(III) naturally present in semi-skimmed milk (4.31 µg kg^{-1}) and F= Cr(III) naturally present in bovine meat (4.67 µg kg^{-1}).

The spike levels of Cr(VI) taken into account were: A= ½ LQ (0.032 µg kg^{-1}), B= LQ (0.051 µg kg^{-1}), C= 2xLQ (0.099 µg kg^{-1}), D= Cr(VI) spiked in infant formula milk (0.259 µg kg^{-1}), E= Cr(VI) spiked in bovine meat (0.535 µg kg^{-1}) and F= Cr(VI) spiked in semi-skimmed milk (0.817 µg kg^{-1}).

The analytical performances for Cr(III) and Cr(VI) speciation analysis by SS-ID-HPLC-ICP-MS following the method validation by means of the accuracy profile are presented in Table 4.9.

Table 4.9. Analytical performance characteristics for Cr(III) and Cr(VI) speciation analysis by SS-ID-HPLC-ICP-MS in milk and meat samples

Sample	Level[a] (µg kg^{-1})	Bias (%)	CV_r		CV_R		LOD [d] (ng kg^{-1})	LOQ [d] (ng kg^{-1})
			Level 1[b]	Level 2[c]	Level 1[b]	Level 2[c]		
Cr(III)								
Infant formula milk	2.70	-0.01	11.6	10.8	11.6	11.4	4.2	13
Semi-skimmed milk	4.31	-0.11	8.2	6.8	8.2	6.8		
Bovine meat	4.67	-0.08	11.2	2.9	13.0	8.1		
Cr(VI)								
Infant formula milk	0.26	-0.02	8.2	11.2	13.7	11.4	14.7	49
Semi-skimmed milk	0.82	-0.09	8.8	11.8	25.9	11.8		
Bovine meet	0.54	-0.05	6.7	10.0	6.8	10.0		

[a] genuine level for Cr(III) and spiking level for Cr(VI); [b] Level < 2×LOQ; [c] Level ≥2×LOQ; [d] sample weight of 0.3 g

The LOD and LOQ were 0.004 and 0.013 µg kg^{-1} for Cr(III), respectively, and 0.015 and 0.049 µg kg^{-1} for Cr(VI), respectively (Table 4.9). The measurement bias corresponding to the validity domain ranged from 0.01 to 0.11%, whereas the coefficient of variation in terms of repeatability (CV_r) varied from 2.9 to 11.6 % (depending on the analyte level) for Cr(III) and from 6.7 to 11.8% for Cr(VI). Similarly, the coefficient of variation in terms of intermediate reproducibility (CV_R) ranged from 6.8 to 13% for Cr(III) and from 6.8 to 25.9% for Cr(VI), respectively, as can be seen in Table 4.9.
Accuracy profile for Cr(III) and Cr(VI) speciation analysis by SSID-HPLC-ICP-MS are present in the appendix (Fig. A7 a,b).

4.4.2. Method validation for bread and breakfast cereals analysis

The validation was performed using three bread samples (Paper III). The spiked samples at different levels of Cr(VI) (5, 10 and 15 µg kg^{-1}) were carried out on homogenized samples before extraction. The method was validated by means of the accuracy profile approach by carrying out in duplicate on five different days to assess accuracy, repeatability and reproducibility intermediate.
The validity domain was defined between the limit of the quantification method (LOQ) and the upper concentration tested (15 µg kg^{-1}).
The main analytical performance characteristics are summarised in Table 4.10.

Table 4.10. Analytical performance characteristics for Cr(III) and Cr(VI) speciation analysis in bread samples by SS-ID HPLC-ICP-MS

Sample	Mass fraction [a] (µg kg^{-1})	Bias (%)	CV_r (%)	CV_R (%)	LOD[b] (ng kg^{-1})	LOQ[b] (ng kg^{-1})
Cr(III)						
"Campagne" bread with wheat flour	26.6	0.30	4.4	4.4		
Bread with spelt flour	37.8	-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%)	219.3	0.01	1.3	1.3		
Cr(VI)						
Bread + 5 µg kg^{-1}	5.1	0.03	0.6	3.3		
Bread + 10 µg kg^{-1}	9.9	0.14	7.9	8.9	14	47
Bread + 15 µg 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

As can be seen, the trueness, expressed 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 obtained 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) and 2.0-8.9% for Cr(VI) determination.The LOQ obtained were 14 ng kg^{-1} and 47 ng kg^{-1} for Cr(III) and Cr(VI), respectively.

4.5. Application of the SSID HPLC-ICP-MS approaches for the speciation analysis of Cr(III) and Cr(VI) in foodstuffs

In this study, Cr(III) and Cr(VI) were determined in different brands of infant formula milk, semi-skimmed milk, meat and meat products (Paper I), bread, breakfast cereals (Paper III) and rice (Paper IV) by SS-ID HPLC-ICP-MS.

All samples were first analyzed in terms of total Cr by ICP-MS, these values have been presented previously (section 3.1.4). The total Cr levels were used to calculate the recovery of Cr(III) in the food samples analysed here.

(a) Dairy products

For this group of foods, two types of milk were tested: ten samples of semi-skimmed milk and another ten of infant formula milk. For semi-skimmed milk, an almond vegetable drink was included because its fat percentage is similar to other samples of cow's milk (≈1-2%). A sample of raw milk was also analyzed before the pasteurization process.

Table 4.11 shows Cr(III) and Cr(VI) levels measured in a selection of infant formula and semi-skimmed milk by SS-ID-HPLC-ICP-MS and recovery (%) compared with total Cr levels for same samples by ICP-MS.

Table 4.11. Cr(III) and Cr(VI) levels measured in a selection of infant formula and semi-skimmed milk by SS-ID-HPLC-ICP-MS and recovery (%) compared with total Cr levels for same samples by ICP-MS

	Mass fraction ± SD (µg kg^{-1}, n=2)		
Sample [a]	**Cr(III)**	**Cr(VI)**	**Recovery (%)** [e]
Semi-skimmed milk			
Brand 1	0.68 ± 0.07		[b]
Brand 2	0.74 ± 0.12		[b]
Brand 3	0.57 ± 0.03		[b]
Brand 4	0.48 ± 0.01		[b]
Brand 5	0.66 ± 0.04	< LOD	[b]
Brand 6	0.67 ± 0.09	(0.015 µg kg^{-1})	[b]
Brand 7	1.05 ± 0.04		[b]
Brand 8 (almond drink)	4.38 ± 0.09		99
Brand 9	0.69 ± 0.04		[c]
Brand 10 (raw)	1.93 ± 0.33		[b]
Infant formula milk			
Brand 1	7.61 ± 0.84		105
Brand 2	2.25 ± 0.45		[c]
Brand 3	2.06 ± 0.04		[c]
Brand 4	2.18 ± 0.09		[d]
Brand 5	5.45 ± 0.40	< LOD	103
Brand 6	5.42 ± 0.01	(0.015 µg kg^{-1})	100
Brand 7	2.42 ± 0.10		[c]
Brand 8	2.90 ± 0.08		[c]
Brand 9	1.29 ± 0.03		[b]
Brand 10	0.22 ± 0.02		[b]

[a] information on the brands is not reported.
[b] Cr_{total} <LOD; [c] Cr_{total} ≥ LOD < LOQ ; [d] < LOQ
[e] Cr(III)/Cr_{total} (%)

Cr(III) levels varied between 0.48 and 4.38 µg kg^{-1} for semi-skimmed milk and 0.22 to 7.61 µg kg^{-1} for infant formula milk, with a recovery between 99 and 105%. The raw milk (Brand 10) had the highest concentration of Cr(III) (1.93 µg kg^{-1}) amongst the milk samples (except for the almond drink). It is worth to note that infant formula milk contained globally the highest levels of Cr(III) compared to semi-skimmed milk and the almond drink, especially the Brands 1 (7.61 µg kg^{-1}), 5 (5.45 µg kg^{-1}) and 6 (5.42 µg kg^{-1}).

Finally, Cr(VI) was not detected in these samples of milk, which is in agreement with other authors (Hernandez et al., 2017).

(b) Meat and meat products

Six meat samples (chicken, turkey, pork, cow, duck and veal) and four meat products (ham slice, chorizo, bacon and sausage) were analysed for Cr(III) and Cr(VI) speciation and the results are presented in Table 4.12.

Table 4.12. Cr(III) and Cr(VI) levels measured in meat and meat products by SS-ID HPLC-ICP-MS and recovery (%) compared with total Cr levels measured ion the same samples by ICP-MS

	Mass fraction ± SD (µg kg^{-1}, n=2)		
Sample [a]	**Cr(III)**	**Cr(VI)**	**Recovery (%)**[e]
Chicken cutlet	23.41 ± 2.84		93
Turkey cutlet	6.12 ± 0.05		[c]
Pork rib to grill	4.13 ± 0.05		[b]
Beef Grilled Pavé	18.11 ± 0.13		95
Duck liver	3.35 ± 0.02	< LOD	[b]
Ham slice	7.27 ± 0.22	(0.015 µg kg^{-1})	[c]
Spanish chorizo sausage	79.72 ± 2.29		97
Bacon strips	9.91 ± 0.17		98
Pork sausage (100%)	53.33 ± 0.49		102
Cutlet veal to grill	1.40 ± 0.04		[b]

[a] information on the brands is not reported; [b] Cr_{total} <LOD;
[c] $Cr_{total} \geq$ LOD < LOQ ; [d] < LOQ
[e] Cr(III)/Cr_{total} (%)

The Cr(III) levels found for meat and meat products were between 1.40 and 79.72 µg kg^{-1}, with a recovery between 93 and 102%. The lowest levels of Cr(III) were found in fresh meat (e.g. duck liver, cutlet veal to grill, etc.) while the highest levels were measured in processed meat (pork sausage at 53.3 µg kg^{-1} and Spanish chorizo at 79.7 µg kg^{-1}).

Contrary to the study reported by Shittu et al., 2020 (11.5 to 14.3 µg kg^{-1} in meat), no Cr(VI) was detected in these samples, which is in agreement with the results reported by Vacchina et al., 2015 and Hamilton et al., 2018.

(c) Bread, breakfast cereals and rice

Eleven bread samples with different types of flour (rye, wheat, corn, malt and spelt or gluten free, toasted bread), eleven breakfast cereals (with and without chocolate, toasted or no-toasted, presence or absence of nuts and fruits, gluten-free cereals, muesli, granola, and cereals composed of 100% whole grain) and ten rice samples (with different refinement processes) were analyzed in this study. The results are reported in Table 4.13. For the bread samples, Cr(III) concentration ranged between 5.2 ± 0.1 µg kg^{-1} and 176.3 ± 6.1µg kg^{-1}, and the recoveries were between 87 and 110%. For breakfast cereals, the Cr(III) concentration were between 23.8 ± 1.6 µg kg^{-1} and 350.3 ± 5.5 µg kg^{-1} considerable higher levels were found in the cereals than in bread, with a recovery between 90 to 109%.

Table 4.13 Cr speciation levels (mean ± SD) measured in cereals by SS-ID-HPLC-ICP-MS and recovery (%) compared with total Cr levels for same samples by ICP-MS

Samples [a]	Mass fraction +SD (µg kg^{-1}, n=2) Cr(III)	Cr(VI)	Recovery (%) [b]
Bread			
Bread with rye flour	87.7 ± 4.5	< LOD (0.015 µg kg^{-1})	102
Bread with malt flour	67.2 ± 3.3		101
"Burger" with wheat flour + seeds	170.1 ± 5.6		100
Bread with spelt flour	176.3 ± 6.2		87
“Pita” with wheat flour	76.9 ± 1.5		101
"Baguette" with wheat flour	130.6 ± 8.0		97
"Sandwich" with wheat flour	43.8 ± 3.3		98
"Ball" with wheat flour	5.6 ± 0.4		110
Toasted bread with wheat flour	5.2 ± 0.1		99
Toasted bread with complete wheat flour	9.6 ± 0.4		94
"Campagne" bread with wheat flour	26.8 ± 0.6		101
Breakfast cereals			
Cacao (20.1%)	350.3 ± 5.5	< LOD (0.015 µg kg^{-1})	97
All bran (100% whole grains)	63.3 ± 5.2		94
Bran flakes bio	24.8 ± 0.6		96
Corn flakes	25.8 ± 0.1		106
Weetabix 100% whole grains	25.1 ± 1.5		100
Weetabix Fruit + Nuts	62.8 ± 2.1		102
Cacao (16.5%)	238.0 ± 1.4		109
Granola bio	39.8± 3.1		96
Granola gluten free	27.4 ± 0.7		105
Fruity muesli	23.8 ± 1.6		90
Toasted muesli	39.7 ± 0.3		97
Rice			
Risotto	5.9 ± 0.1	< LOD (0.015 µg kg^{-1})	92
Sushi	9.7 ± 0.1		110
Basmati	16.9 ± 0.1		98
Black	10.9 ± 0.1		103
Red	14.3 ± 1.4		102
Brown	103.9 ± 3.7		102
Jasmin	8.2 ± 0.2		101
Arborio	4.2 ± 0.1		108
Parboiled	6.6 ± 0.1		102
Whole grains	0.6 ± 0.1		110

[a] information on the brands is not reported.
[b] Cr(III)/Cr$_{total}$ (%)

For rice samples the concentration of Cr(III) found were between 0.60 ± 0.09 and 103.9 ± 3.72 µg kg^{-1}, with recovery varying between 92 to 110%.

For bread, breakfast cereals and rice, the results obtained agree with those reported by other authors that reported also the absence of Cr(VI) (Kovács et al., 2007; Vacchina et al., 2015; Hernandez et al., 2017, Vacchina et al., 2015, Dokpikul et al., 2018). However, some authors reported the presence of Cr(VI) in cereals, Soares et al., 2010 with Cr(VI) levels between 5.68 and 6.82 µg kg^{-1} and Mathebula et al., 2017 between 20.4 and 470.4 µg kg^{-1}. For rice samples, the Cr(VI) levels found were between 6-142 µg kg^{-1} by Chen et al., 2020.

Species specific isotope dilution mass spectrometry (SSID-MS) has proven to be the only analytical approach suitable for simultaneous speciation analysis of Cr(III) and Cr(VI) in foodstuffs due to its capability to quantify the species and to correct for their interconversion during the same analytical run (USEPA, 2014; Martone et al., 2013; Milačič and Ščančar, 2020).

4.6. Conclusion

Speciation analysis of Cr(III) and Cr(VI) in various foods, such as milk, meat and meat products, bread, breakfast cereals and rice, were successfully applied with the development of two primary analysis methods based on SS-ID, the only analytical approach that allows the complete correction of the (analytical) artefacts of Cr(VI).

Cr(VI) was not found in any of the samples analysed here while Cr(III) concentration ranged between 0.22 and 350.31 µg kg^{-1}. Additionally, the species mass balance (sum of levels of Cr(III) and Cr(VI)) compared to total Cr determined by ICP-MS in the same samples was excellent (87-110 %). This supports the EFSA statement related to the absence of Cr(VI) in foods and that Cr in this type of samples is found solely as Cr(III) form.

An online approach such as HPLC-ICP-MS and species-specific isotopic dilution mass spectrometry (SS-ID-MS) was established and proved to be the only suitable analytical approach for simultaneous analysis of Cr(III) and Cr(VI) in food due to its ability to quantify species and correct their interconversion during the same analytical run.

CHAPTER 5

ASSESSMENT OF THE FATE OF CHROMIUM SPECIES DURING COOKING OF FOOD AND RELATED HEALTH RISK

Abstract

The interconversion of Cr species in foods can potentially occur during their or during the analytical process cooking due to changes in the redox status, pH and temperature during. The accurate assessment of these Cr species interconversion of species is important because they can lead to analytical artefacts, but can also lead to the formation of more toxic species (e.g. Cr(VI) *via* the oxidation of Cr(III)) with a harmful contribution to human health.

This chapter aims to investigate the behaviour of Cr and its species (Cr(III) and Cr(VI)) in infant formula milk, semi-skimmed milk and bovine meat samples during their various heating based cooking. Each type of sample was subjected to two different heating treatments in terms of time and temperature. The temperatures used for this purpose simulated those used in the daily cooking of the same samples mentioned above.

Due to the lack of Cr(VI) in most of the foodstuffs, this species was added at different levels in the samples investigated in order to simultaneously asses Cr(III) and Cr(VI) behaviour function of temperature.

The samples were analyzed in terms of total Cr, Cr(III) and Cr(VI) before and after heat treatment. ANOVA data treatment showed that there were no significant differences between the raw and cooked samples (milk and bovine meat), regardless the culinary treatments employed. This confirms that an oxidation of Cr(III) to Cr(VI) does not occur in these food samples during heat treatment.
A risk assessment was also carried out comparing the total Cr levels in raw and cooked foods; the assessed risk is very low due to the low total Cr level in the milk and meat samples.

5.1. Introduction

Speciation analysis is defined as the analytical activity that allows the identification and/or measurement of the levels of one or more individual chemical species in a given sample (IUPAC, 2000). This analytical approach generally involves the selective extraction of the species of interest, this step being considered the most critical in the analytical procedure, since the species can be poorly extracted or inter-converted. The interconversions of Cr species in foodstuffs can potentially occur during matrix modifications (changes in the redox status, pH, exposure to light, etc.) but also during the analytical procedure (formation of analytical artefacts), especially during the extraction step (Pyrzynska, 2012, Zhao et al., 2012, Mathebula et al., 2017).

As previously mentioned, conventional analytical approaches in which we only have the phase of separation (when it exists) followed by quantification, do not allow us to assess whether or not there was interconversion/transformation of species leading to errors or scientific artefacts in the results of the studies.

The application of SS-ID allows to clarify if interconversion between Cr species occurs and correct also the results of the final levels of Cr(III) and Cr(VI) in each analyzed sample. It is possible to check the interconversions of Cr species due to the calculation of two factors, α, represents the percentage of Cr(III) that was oxidized to Cr(VI) during the analytical procedure and β, the percentage of Cr(VI) reduced to Cr(III) during the analytical procedure.

Most studies in the past dealt with the impact of cooking on various food matrices such as leguminous vegetables, vegetables, fish and seafood, meat, pasta and dairy products focused almost exclusively on total Cr levels (Table 5.1). The mostly used techniques in these studies for total Cr determination were ICP-MS and ICP-AES, but AAS and GF-AAS were also frequently used. In general, the total Cr levels decreased after the culinary treatment of vegetables (Fidelis et al., 2018, Kmiecik et al., 2007), fish and other seafood (Mehdipour et al., 2018, Rasmussen et al., 2017, Perugini et al., 2014) and dairy products (Güler, 2007) while, an increase in total Cr concentration was noted for rice samples (Rittirong et al, 2018), pasta and meat (Alberti-Fidanza et al., 2002, Wang et al., 2015) after cooking.

The increase or decrease in the total Cr levels after the culinary procedure does not depend exclusively on the sample matrix, but also on the addition of salt, water, oils or others ingredients, which may increase or decrease (by a dilution effect) the Cr levels in the food. A reduction of the water content in food can also lead to an increase of total Cr in the sample.

Very few studies reported speciation analysis of Cr in raw and cooked foods such as bread, cereals and edible animal oils (Table 5.2) (Mathebula et al., 2017, Kovács et al., 2007, Lin et al., 2016) most

probably because of the analytical challenges related to the accurate determination of Cr(III) and Cr(VI) at trace and ultra-trace levels.

Mathebula et al. (2017) quantified both Cr(III) and Cr(VI) in bread and breakfast cereals and reported that in toasted bread, 33-73% of total Cr (58-156 μg kg^{-1}) exists as Cr(VI) compared to the same untoasted samples. Therefore, there is actually a controversy in terms of the conversion of Cr(III) to Cr(VI) during cooking/thermal treatment of various foods.

It is also important to note that the final output of food analyses is the health risk assessment of the contaminant under study. Currently, chemicals are ubiquitous, leading humans, flora and fauna in the environment to be exposed to an increasing number of chemical compounds, with anthropogenic and/or natural origin (Ingenbleek et al. 2021). The general principles for safety and nutritional evaluation of foods and the potential health risks associated with hazardous compounds are described as developed by the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and European Food Safety Authority (EFSA).

The main objective of CRA is to quantify chemical exposures from relevant sources (inhalation, oral, skin and water/food) in a given population or species (exposure assessment), determine the safety levels for such chemicals (hazard identification and characterization) and quantify the risk associated with such exposures (risk characterization) (Ingenbleek et al. 2021). Accurate chemical risk assessment (CRA) related to foods consumption requires a multidisciplinary approach involving chemistry, biology, toxicology, epidemiology, biostatistics and modelling (EFSA, 2010).

Information on total Cr levels solely is not adequate in terms of toxicity related to food consumption, since each species (Cr(III) and Cr(VI)) has its own degree of toxicity. Cr(VI) is genotoxic and carcinogenic and therefore ingestion or contact whenever possible should be avoided. Cr(VI) is considered 4 to 10 times more toxic than Cr(III) (Aharchaou et al., 2017), being therefore the main species of interest when carrying out a risk assessment of Cr in food. There are no convincing evidence of beneficial effects associated with Cr(III) intake in healthy people, so an tolerable daily intake (TDI) of 300 μg Cr(III)/kg b.w. per day was established by EFSA panel, 2014.

The aim of the present study was to assess the potential interconversion of Cr(III) and Cr(VI) in infant formula milk, semi-skimmed milk and bovine meat during different type of (thermal) cooking. This is the first study that compares the interconversions of Cr species in relation with the heat treatment on samples of milk and meat (Article II).

Risk assessment related to raw and cooked milk and bovine meat was also performed to assess the impact of Cr exposure to human health, since the cooked food provides more realistic information than the corresponding raw foodstuffs.

5.2. Analytical procedures

5.2.1. Samples and sample preparation

The samples used for this study are described in chapter 3 and their preparation was described in detail in chapter 4 dealing with Cr speciation analysis. Cr(VI) was not present in any of the samples investigated here and hence each sample was spiked with different levels of Cr(VI) to assess the eventual interconversion of Cr(III) and Cr(VI) during the different types of cooking. The infant formula milk (500 mL) was spiked with 0.25 μg mL^{-1} Cr(VI), the semi-skimmed milk (500 mL) with 1.0 μg mL^{-1} of Cr(VI) and bovine meat (200 g) with 0.5 μg g^{-1} of Cr(VI) (Paper II).

(a) Cooking of milk and meat

Infant formula milk, semi-skimmed milk and bovine meat were subjected to two different cooking procedures based on heating. The temperatures were recorded using a food probe/thermometer (ThermoPro TP01 Digital Kitchen).

The milk samples were heated in a microwave oven at 70 ºC during 2 minutes as well as on a hot plate at 100 ºC for 5 minutes. The temperatures used in this study were comparable with those reported elsewhere (Mehdipour et al., 2018, Diaconescu et al., 2013).

The bovine meat samples were fried during 5 minutes with and without sunflower oil (≈ 30 mL) at 120 ºC (oil frying) and 95 ºC (dry frying), respectively. These temperatures were similar to those normally used in daily life for preparation of this type of food.

The milk samples were heated at temperatures considerably higher compared to those typically used in daily life; this option was chosen to assess whether species interconversion occur in these extreme conditions; if no species interconversion occur in this case, the same would be in case of use of conventional temperatures in daily life.

Milk and bovine meat were prepared in triplicate and each cooked sample was analyzed in duplicate (the same days of the cooking). The results in terms of total Cr and Cr speciation were compared to those obtained for the same raw sample (Paper II).

5.2.2. Analytical procedure

Speciation analysis of Cr(III) and Cr(VI) in raw and cooked samples was carried out using HPLC-ICP-MS and double spike species specific ID (SS-ID HPLC-ICP-MS) based on the procedure described in chapter 4 in the section 4.1.1. Briefly, the extraction of Cr(III) and Cr(VI) from raw and cooked sub-samples (0.3 g) spiked with Cr(VI) was performed by using EDTA and DPC using heating blocks as described in detail in Chapter 4.

The chromatograms obtained for the analysis of infant formula, semi-skimmed milk and bovine meat samples are shown in Fig. 5.1.

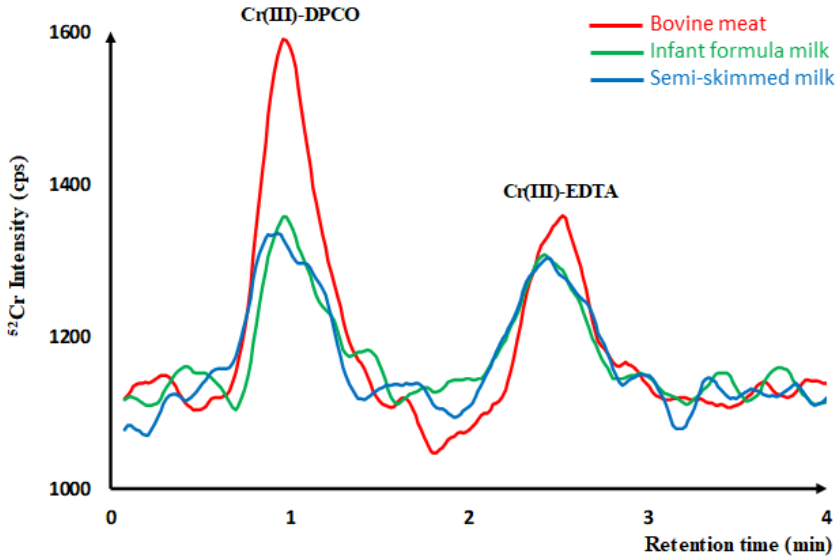

Figure 5.1. Chromatograms obtained for the analysis of infant formula (green line) and semi-skimmed (blue line) milk and bovine meat (red line) samples (with genuine Cr(III) and Cr(VI) spiked)

The same samples were also analyzed for their total Cr levels by ICP-MS based on the procedure described in the section 3.1.1.

5.3. Results and discussion

As it was mentioned previously, most studies in the past dealt with the impact of cooking on various food matrices focused almost exclusively on total Cr levels (Cr_{total}). An overview of the literature data on the effect of cooking on the total Cr content in various types of food is provided in Table 5.1. Similarly, an overview of the literature data on the effect of increased temperature on the Cr speciation in bread and breakfast cereals and animal oils in provided in Table 5.2.

Table 5.1. Overview of the literature data on the effect of cooking on the total chromium (Cr_{total}) content in various types of food

Sample	Cooking procedure	Detection technique	Cr_{total} (µg kg^{-1})	Reference
Dairy products:				
Milk and yogurt	(A) Raw; (B) Fermentation process, draining of yoghurt, cooking and salting	ICP–AES	(A) 770; (B) Strained yoghurt: 1230; Salted yoghurt: 430	Güler, 2007
Meat:				
Beef	(A) Raw; (B) Conventional cooking (80°C for 3min.); (C) Plate/Ohmic cooking (50Hz for 3 min.); (D) Plate/Ohmic cooking (10Hz for 3 min.)	ICP-MS	(A) 0.004; (B) 0.077; (C) 1.540; (D) 0.091[a]	Wang et al., 2015
Meat	(A) Raw; (B) Hotplate, grilled and roasted (without temperature information)	GF-AAS	(A) 12.2-63.0; (B) 11.8-66.7[b]	Alberti-Fidanza et al., 2002
Fish and seafood:				
Rutilus frisii kutum (White fish)	(A) Raw; (B) Microwave oven (2450 MHz, 10 min); (C) Grilled in the oven (180 °C, 20 min); (D) Fried in sunflower oil (180 °C, 4 min); (E) boiled in a stainless steel container (5min); (F) steamed in a rice cooker (10 min); (G) brine salted for 1h	AAS	(A) 2.8; (B) 2.06; (C) 2.18; (D) 2.54; (E) 2.00; (F) 2.36; (G) 2.56	Mehdipour et al., 2018
Prawns and halibut	(A) Raw; (B) Smoked; (C) Peeled prawns	ICP-MS	(A) 150; (B) 120; (C) 30	Rasmussen et al., 2017
Norway lobster	(A) Raw: 1) White meat and 2) Brown meat; (B) White meat (boiled 105°C); (C) Brown meat (boiled 105°C)	ICP-MS	(A1) 150; (A2) 620; (B) 120; (C) 380	Perugini et al., 2014
Crayfish	(A) Raw abdominal meat; (B) Raw hepatopancreas meat; (C) Abdominal meat cooked*; (D) Hepatopancreas meat cooked* *10 minutes in aluminum or stainless steel saucepan containing 4 L of tap water, 0.3 L of salt, and herbs (dill).	GF-AAS	(A) 0.019-0.025; (B) 0.042-0.050; (C) 0.018-0.088; (D) 0.13-0.18[c]	Jorhem et al., 1994
Finfish and shellfish (Mediterranean)	(A) Raw; (B) Boiled 2–3 min. in water/panfried (170°C); (C) Grilled domestic electric oven (180°C)	GF-AAS	(A) 0.05-0.26; (B) 0.06-0.52; (C) 0.05-0.13[d]	Kalogeropoulos et al., 2012
Fish, cephalopods, crustaceans and bivalves	(A) Raw; (B) Steamed (90°C for 60min.)	ICP-MS	(A) 150; (B) 250	Barbosa et al., 2018
Fish	(A) Raw; (B) Hotplate, grilled and roasted (without temperature information)	GF-AAS	(A) 44.0-88.5; (B) 44.7-87.9[b]	Alberti-Fidanza et al., 2002
Cereals:				
Rice	(A) Raw; (B) regular tap water*; (C) de-ionized water*; (D) acidic water*; (E) basic water* (*Heated using a hot plate (95°C for 2 hr))	ICP-MS	(A) 270; (B) 300; (C) 410; (D) 320; (E) 280	Rittirong et al, 2018
Pasta	(A) Raw; (B) Hotplate (without temperature information)	GF-AAS	(A) 54.9-103.5; (B) 63.1-107.7[b]	Alberti-Fidanza et al., 2002

Table 5.1. Overview of the literature data on the effect of cooking on the total chromium (Cr_{total}) content in various types of food *(Continued)*

Sample	Cooking procedure	Detection technique	Cr_{total} (µg kg^{-1})	Reference
Vegetables:				
Pea and bean	(A) Fresh vegetables; (B) After blanching; (C) After cooking in 2% brine to consumption consistency; (D) Frozen products from samples B and C, after 12 months of storage at -30°C and then prepared for consumption; (E) Frozen products from sample C were defrosted and heated in a microwave oven	ICP-AES	Broad bean (A) 0.007; (B) 0.007; (C) 0.006; (D) 0.007; (E) 0.006; Broad pea (A) 0.016; (B) 0.011; (C) 0.011; (D) 0.011; (E) 0.012 French-bean (A) 0.017; (B) 0.016; (C) 0.015; (D) 0.014; (E) 0.015[e]	Lisiewska et al., 2008
Pumpkin	(A) Raw; (B) Heated to 5 min at 100°C using gas stove	AAS	(A) 0.9- 0.1; (B) 0.6- 0.1[c]	Fidelis et al., 2018
Vegetables	(A) Raw, (B) blanched material before freezing, (C) cooked material before freezing, (D)cooked in 2% brine after 12 months frozen storage from material blanched before freezing, (E) product defrosted and heated in a microwave oven after 12 months frozen storage from material cooked before freezing	ICP-AES	(A) 0.014, (B) 0.007, (C) 0.008, (D) 0.006, (E) 0.008[e]	Kmiecik et al., 2007
Vegetables and Pulses	(A) Raw; (B) Hotplate (without temperature information)	GF-AAS	Vegetables: (A) 41.9-62.6; (B) 53.4-54.8[b] Pulses: (A) 89.4; (B) 81.8[b]	Alberti-Fidanza et al., 2002

[a] (mg/120g); [b] (mg/100 g dry weight); [c] (mg/kg fresh weight); [d] (µg/g); [e] (mg/100 g fresh matter)

Table 5.2. Overview of the literature data on the effect of increased temperature on the Cr speciation in bread and breakfast cereals and animal oils

Samples matrix	Cooking procedure	Detection technique	Cr_{total} (µg kg^{-1})	Cr(VI) (µg kg^{-1})	Reference
Cereals:					
Bread and Breakfast cereals	(A) Untoasted; (B) Toasted (for 3 min. on an Essential 900 W)	HR-CS-AAS	(A) 57.6-156.1	(A) 19.3 - 63.9; (B) 33.65 - 76.3	Mathebula et al., 2017
Bread	The temperature was increased gradually in melting furnace to 900°C using 50°C/30 min. heating steps.	GF-AAS	-	Cr(III) does not change to Cr(VI) during the heating process	Kovács et al., 2007
Fats:					
Edible animal oils	Microwave digestion with 0,4% HF; (A) Oil 1-buffalo/cow/cattle; (B) Oil 2- mixed animal oil; (C) Oil 3- fish oil	HPLC-ICP-MS	(A) 838; (B) 2500; (C) 131	Cr(VI) <LD (0.052)	Lin et al., 2016

5.3.1. Total Cr and Cr species determination in milk and bovine meat

One sample of infant formula milk (1 L, protected brand), a sample of semi-skimmed milk (1 L protected brand) and a sample of bovine meat (1 kg) were analyzed in duplicate for the quantification of total Cr in the samples and in six times for the quantification of Cr species, with the analytical methods described above. The levels of total Cr, Cr(III) and Cr(VI) in raw and after cooking processes are reported in Table 5.3.

Table 5.3. Levels (mean± SD) of total Cr (by ICP-MS) and Cr(III)-Cr(VI) (SS-ID HPLC-ICP-MS) in raw and spiked samples undergoing different cooking processes (the recovery of both Cr(VI) and Cr_{total} is also reported)

Sample	Mass fraction ±SD (µg kg^{-1}, n=6)				Recovery ± SD (%, n=6)[d]		
	Cr_{total} [a] (ICP-MS)	Cr(III) (SS-ID)	Cr(VI)[b] (SS-ID)	Cr_{total} [c] (SS-ID)	Cr(VI)[b] (SS-ID)	Cr_{total} (SS-ID)	β±SD (%)[e]
Infant formula milk							
Raw	2.97±0.15	2.63±0.05	0.25±0.02	2.88±0.06	100±2	97±1	117±10
Microwave	2.95±0.13	2.62±0.10	0.23±0.02	2.84±0.13	92±8	96±5	109±6
Hot Plate	2.92±0.12	2.79±0.07	0.27±0.01	2.96±0.12	108±4	101±6	115±11
Semi-skimmed milk							
Raw	5.22±0.11	4.55±0.04	0.92±0.01	5.47±0.04	92±3	105±0	106±5
Microwave	5.20±0.13	4.47±0.15	0.85±0.04	5.23±0.06	85±6	101±1	118±7
Hot Plate	5.21±0.10	4.38±0.15	0.88±0.12	5.26±0.11	88±4	101±3	112±3
Bovine meat							
Raw	5.21±0.12	4.49±0.17	0.49±0.03	4.98±0.09	98±10	96±3	100±2
Fried without oil	5.20±0.09	4.62±0.05	0.48±0.05	5.10±0.10	96±11	98±5	113±5
Fried with oil	5.25±0.11	4.78±0.05	0.46±0.04	5.24±0.14	92±10	100±1	110±4

[a] Cr natural + Cr(VI) spiked (n=3)
[b] spiked
[c] Cr(III)+Cr(VI) (SS-ID)
[d] The recovery for Cr(VI) determination (SS-ID) is calculated by comparison with the spiking level whereas for Cr_{total} (SS-ID) the recovery factor is assessed by comparison with total chromium level of the blend sample analyzed by ICP-MS.
[e] Yield of Cr(VI) conversion to Cr(III) during the analytical process

In general, total Cr levels for all samples do not differ between different heating treatments. Total Cr levels in raw, microwave and hot plate heated milk were of 2.97±0.15, 2.95±0.13 and 2.92±0.12 µg kg^{-1}, respectively. Similarly, for semi-skimmed milk, the total Cr levels measured in raw, microwave and heated by hot plate were of 5.22±0.11, 5.20±0.13 and 5.21±0.10 µg kg^{-1}, respectively.

For bovine meat, the values in raw, fried without oil and fried with oil were 5.21±0.12, 5.20±0.09 and 5.25±0.11 µg kg^{-1}, respectively. A recent study in terms of total Cr determination by ICP-MS in infant formula (milk, soy and corn based) showed very high levels compared to our samples, namely between 41 to 348 µg kg^{-1} with the mean value 168±0.143 µg kg^{-1} (Elaridi et al., 2020).

The concentrations of total Cr in cooked foods were slightly lower compared to raw foods, as seen in Table 5.3., in agreement with other similar studies for total Cr concentration (Perugini et al., 2014; Mehdipour et al., 2018; Rasmussen et al., 2017).

Excellent recovery was obtained for the analysis of both from raw and cooked samples spiked with Cr(VI) at various levels, as can be seen in Table 5.3. It is worth to note that all samples were spiked

with Cr(VI) one week before their analysis hence these results show the stability of Cr(VI) spiked in these matrices. Nevertheless, this does not necessarily infirm the EFSA hypothesis related to the absence of Cr(VI) in foodstuffs (EFSA, 2014a) because the behavior of Cr(VI) in natural foodstuffs may be different than that of Cr(VI) artificially spiked to the same samples types.

The species interconversions that may occur during the analytical procedure can be characterized by two factors, alpha (α) (CrIII→CrVI) and beta (β) (CrVI→CrIII). It is worth to note that SS-ID is capable to correct these interconversions, even if an extensive (100%) conversion occurs. A high conversion rate of a species indicates that it is highly unstable in the sample matrix (USEPA, 2014) or during the analytical process; the latter case was encountered in our study where a complete reduction of Cr(VI) (β≅100%) occurred during the sample analysis of the samples spiked with Cr(VI), as can be seen also in Table 5.3. Therefore, without the capability of SS-ID, Cr(VI) eventually incorporated into foodstuffs would not be accurately assessed because of its reduction to Cr(III) for the reasons mentioned above.

5.3.2. Statistical treatment of the results of Cr(III) and Cr(VI) in milk and bovine meat

Analysis of variance (one-way ANOVA) was performed to assess the significant differences between total Cr and Cr species (III and VI) concentrations in raw and cooked milk and bovine meat samples. All analyses were carried out with six replicates for Cr(III) and Cr(VI) and in triplicate for total Cr. F-test and the Student t-test were performed at a significance level of 0.05, using the Excel software. The results obtained for total Cr (by ICP-MS) in raw and heated milk and bovine meat and Cr(III) and Cr(VI) concentration by SS-ID HPLC-ICP-MS, also, in raw and cooked milk and bovine meat are reported in Table 5.4. An ANOVA test was used to compare the mean total Cr levels between and within groups, as seen in Table 5.4 (Paper II).

Table 5.4. One-way ANOVA data treatment in terms of total Cr determination in raw and cooked food (Infant formula milk, semi-skimmed milk and bovine meat) by ICP-MS (n=3)

	Cr_{total}				
Food group	**SS between groups**	**SS within groups**	**F**	**p-value**	**F_{crit}**
Infant formula milk	0.0027	0.0020	4.0893	0.0758	5.1433
Semi-skimmed milk	0.0005	0.0005	2.8893	0.1322	5.1433
Bovine meat	0.0022	0.0003	4.1342	0.0541	5.1433

For all sample types, no significant differences in terms of total Cr for the different sample treatments were observed. These findings are comparable with those reported in similar studies for fish, vegetables, pasta and pulses (Mehdipour et al., 2018; Rittirong et al., 2018; Rasmussen et al., 2017; Perugini et al., 2014; Alberti-Fidanza et al., 2002), in which total Cr levels were comparable between

raw and cooked samples. F average for infant formula milk, semi-skimmed milk and bovine meat were 4.01, 2.89 and 4.13, respectively, which are in all cases lower than F_{crit} (5.14) (Paper II).

The samples were analyzed in the first instance without any addition of Cr(III) or Cr(VI) to study the fate of genuine Cr(III) solely. Cr(III) mass fraction measured in the raw and cooked samples are reported in Table 5.5.

Table 5.5. Levels of Cr(III) (mean ± SD, n=2) in raw and cooked food as well as the Cr(III) fraction of Cr(III) in the samples after cooking obtained by SS-ID HPLC-ICP-MS

Samples	**Cr(III) (µg kg^{-1})**	**$Cr(III)_{cooked}$/ $Cr(III)_{raw}$ (%)**
Infant formula milk		
Raw	2.72±0.06	-
Microwaved	2.63±0.17	97±5
Hot plate	2.51±0.12	93±4
Half-fat Milk		
Raw	4.50±0.04	-
Microwaved	4.43±0.06	98±1
Hot plate	4.34±0.17	97±4
Bovine meat		
Raw	4.48±0.09	-
Fried without oil	4.46±0.13	98±4
Fried with oil	4.62±0.18	103±5

For both samples and their respective culinary treatments, excellent recoveries (93-103%) were obtained for Cr(III) compared to the raw samples.

ANOVA showed no significant differences for raw and cooked samples (milk and bovine meat) for Cr(III) species (see Table 5.6) since for all averages the F value was lower compared to the Fcrit value (F= 2.70, 3.44 and 1.74 < Fcrit.= 3.68). This confirms the stability of Cr(III) in this type of samples, even when subjected to heating at high temperatures, since the ratio $Cr(III)_{cooked}/Cr(III)_{raw}$ found for the three food samples studied were between 93 and 103% (Table 5.5).

ANOVA was also used to assess the differences between the Cr(VI) levels in raw and cooked samples (see Table 5.6). For all samples and regardless the cooking procedure studied here, there were no significant differences between the groups, since for all averages the F value was lower compared to the Fcrit value (F= 1.41, 1.59, 1.29 < Fcrit.= 3.68).

Table 5.6. One way ANOVA data treatment in terms of Cr(III) and Cr(VI) determination in raw and cooked food (semi-skimmed milk, infant formula milk and meat) by SSID HPLC-ICP-MS (n=6)

Cr(III)					
Group	SS between groups	SS within groups	F	p-value	F crit
Infant formula milk	0.1261	0.3503	2.7008	0.0996	3.6823
Semi-skimmed milk	0.0732	0.1597	3.4385	0.0590	3.6823
Steak	0.0909	0.3931	1.7349	0.2100	3.6823
Cr(VI)					
Infant formula milk	0.0495	0.1889	1.4070	0.2247	3.6823
Half-fat milk	0.0178	0.0840	1.5930	0.2359	3.6823
Steak	0.0041	1.2918	1.2918	0.3036	3.6823

5.4. Risk assessment related to Cr(III) species in raw and cooked milk and meat samples

The main objective of chemical risk assessment (CRA) is to quantify exposures to chemical contaminants and relevant sources (inhalation, oral, skin and water/food) in a given population or species (exposure assessment), determine the safety levels for such chemicals (hazard identification and characterization) and quantify the risk associated with such exposures (risk characterization) (as shown in Fig. 5.2).

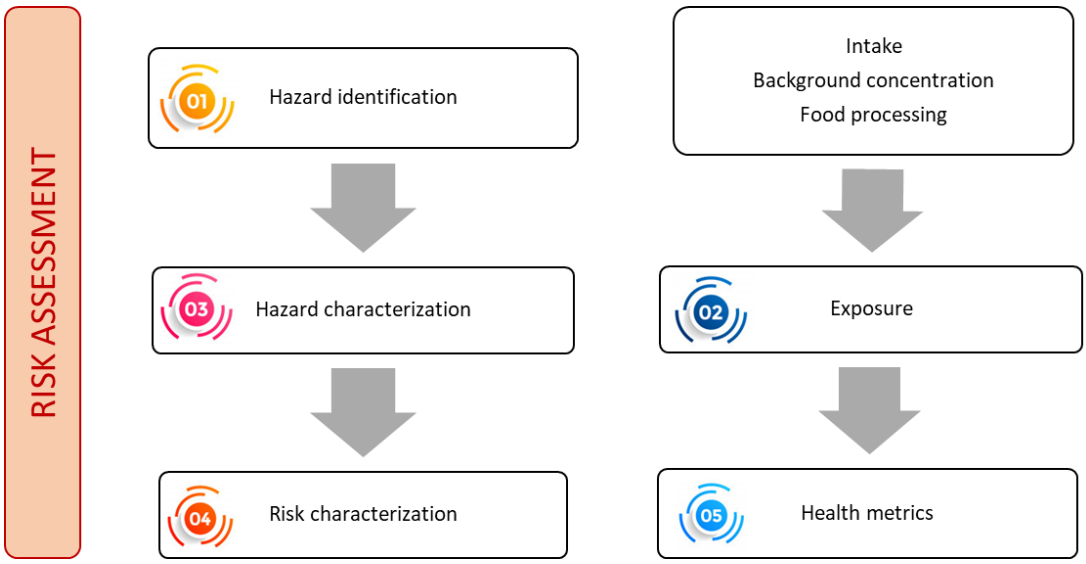

Figure 5.2. The main steps of the risk assessment as recommended by EFSA Scientific Committee (Source: Scientific Opinion on Risk Assessment Terminology. EFSA Journal 10(5) (2012), 2664. DOI: 10.2903/j.efsa.2012.2664)

To assess the exposure, it is necessary to investigate how much the organism is exposed to a chemical contaminant, through the detection and quantification of chemical residues in foods (occurrence data) and the level of consumption of the food. For this, it is of great importance to consider the relevant analytical techniques available and the food consumption databases, available by FAOSTAT website (http://www.fao.org/faostat/en/#data/FBS).

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

Three main aspects regarding chemical toxicity are considered: (*i*) the rate of the elimination of the contaminant from the human body (toxicokinetics - elimination and/or absorption), (*ii*) the mechanism of elimination of the contaminant and (*iii*) determination of the safe levels of the chemical hazard in humans.

Therefore, in brief, risk identification includes identifying the hazard (Cr and Cr species) and the sources of exposure (food) that cause or may cause adverse health effects while the risk characterisation brings the exposure and hazard dimensions together and provides means to quantify

health risks for a given population after single or multiple chemical exposures (chemical mixtures or "cocktail effects").

The diversity of chemicals that risk assessors deal with in the food and feed sectors is rather large and includes pesticides, biocides, food and feed additives, pharmaceuticals, air pollutants as well contaminants of anthropogenic (e.g. persistent organic pollutants, heavy metals, perfluoroalkyl substances, brominated flame retardants, dioxins etc.) or natural (marine biotoxins, mycotoxins etc.) origin. Cr(III) levels were used here to assess exposure and then compare exposure to reference values, because it provides more relevant information than total Cr, since toxicokinetics and toxicodynamics depend on the species rather than the total element level. As Cr(VI) was not detected in any of the samples analyzed, the exposure assessment was not performed for this species.

5.4.1 Exposure assessment

In the evaluation of the exposure related to Cr, it is important to accurately know how much Cr species an organism exposed to, taking into account the basic principle of toxicology: *''Sola dosis facit venenum''* which means that only the dose causes a substance to become a poison. Dietary exposure is the result of the sum of the concentration of the chemical substance in food multiplied with the amount of food eaten daily and is usually normalized to body weight (Eq.1).

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

where:

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

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

L_k concentration of the food chemical (total Cr and/or Cr species - μg kg^{-1}).

Wi body weight of the individual (kg).

It is important to note that proper assessments of dietary exposure depends on the availability of food consumption data, data of concentrations found in these same foods and also the anthropometric data of the populations under study.

5.4.2 Exposure to Cr from milk and meat in France and Denmark

There are usually two ways to assess the consumption of a given food: *(i)* by estimating the availability of the food in a country, with an estimate of the national production plus the importation of a given food, minus the quantities exported and *(ii)* from a global database, such as the FAOSTAT, which provides open access to food and agriculture data for more than 245 countries and territories

and covers all FAO regional groupings from 1961 to the most recent year available (2017 for milk and bovine meat in Denmark and France).

In this study, the consumption data provided by FAOSTAT and the food consumption reports for Denmark ([b] meat and meat products and [c] milk) and France ([d] bovine meat and milk) were used (see Table 5.7).

Table 5.7. Annual production, food supply and consumption of bovine meat and milk in Denmark and France in 2017 (latest data available)

Bovine meat	**Unit**	**Denmark (5 732 274 persons)**	**France (64 842 509 persons)**
Annual production	tonnes	124 000	1 433 000
Food supply quantity	(kg/capita/yr)	25.38	23.15
Consumption [a]	(kg/capita/day)	0.0695	0.0634
Consumption	(kg/capita/day)	0.0605 [b]	0.0692 [d]
Milk - excluding butter			
Annual production	(tonnes)	5 557 000	26 509 000
Food supply quantity	(kg/capita/yr)	307.03	260.11
Consumption [a]	(kg/capita/day)	0.8412	0.7225
Consumption	(kg/capita/day)	0.3051 [c]	0.1723 [d]

[a] Source: FAOSTAT website - Food and Agriculture Organization - value calculated from food supply quantity for each food group

[b] Source: Nordic Council of Ministers (NCM). Tetens I. Nutritional evaluation of lowering consumption of meat and meat products in the Nordic context (2013) No. 2013:506. DOI: https://doi.org/10.6027/TN2013-506 **(n= 2025)**

[c] Source: Agnes N. Pedersen, Dietary habits in Denmark 2011-2013 **(n= 6032)**

[d] Source: ANSES REPORT – INCA3 study on the Update of the food consumption database and the estimation of nutritional intakes of individuals living in France through the implementation of the 3rd national individual study of food consumption (2017) **(n=2 121)** DOI: INCA3 study.pdf

The consumption of bovine meat was different depending on whether the data was provided by FAOSTAT or by the Nordic council of Ministers (Denmark), in the latter with the lowest consumption of bovine meat per day by the Danes.

In the case of France, the opposite was verified, that is, the consumption per day of bovine meet was higher in the study carried out in France than in the general study carried out by FAOSTAT.

In case of milk consumption, the variations were even higher; actually, the consumption was much lower in the studies carried out in the Danish and French population (0.305 and 0.172 kg/day, respectively) in this case only the milk entered in the calculation, while that FAOSTAT study included milk and milk products present values of 0.841 and 0.723 kg/day, respectively.

These differences may be due to the FAOSTAT estimate being based in production data while French and Danish estimates being based on what people actually eat (by conducting surveys).

The data are related to the total Cr, since only these data are available via FAOSTAT and in the internal reports (Danish and French), but these data were extrapolated to Cr(III) because with this

study it was demonstrated that in the analyzed samples the levels of total Cr are similar to Cr(III). The chemical exposure related to Cr (Eq.1) was assessed using the consumption data of bovine meat and milk, Cr(III) levels found raw and cooked foods and average body weight of 70 kg, as presented in Table 5.8. The values used for the calculations are reported in the appendix (Table A3).

Table 5.8. Estimated exposures related to Cr(III) due to the consumption of raw and cooked milk and bovine meat (different cooking types) in France and Denmark

	Denmark					
Exposure µg kg bw per day	**Bovine meat**			**Milk**		
	Raw	Fried without oil	Fried with oil	Raw	Microwave	Hot plate
FAOSTAT	0.0045	0.0046	0.0047	0.0547	0.0537	0.0526
Danish report	0.0039	0.0040	0.0041	0.0198	0.0195	0.0191

	France					
Exposure µg kg bw per day	**Bovine meat**			**Milk**		
	Raw	Fried without oil	Fried with oil	Raw	Microwave	Hot plate
FAOSTAT	0.0041	0.0042	0.0043	0.0470	0.0461	0.0452
French report	0.0044	0.0046	0.0047	0.0112	0.0110	0.0108

(a) Global exposure to Cr(III) due to the consumption of milk and bovine meat

The estimated contribution arising from both bovine meat and milk in Denmark and France, using the two different approaches (FAOSTAT and Internal study) were calculated and the results are shown in Table 5.9.

Table 5.9. Total contribution to Cr(III) exposure of bovine meat + milk samples in Denmark and France calculated with two different approaches to average consumption

	Denmark			France		
	Exposure (µg kg^{-1} bw day^{-1}) (contribution of bovine meat + milk)					
	Raw	Fried without oil	Fried with oil	Raw	Microwave	Hot plate
FAOSTAT	0.0592	0.0583	0.0574	0.0511	0.0503	0.0495
Internal country study	0.0237	0.0235	0.0232	0.0156	0.0156	0.0155

Comparing the values obtained in the risk assessment with dietary reference intakes (DRI) for Cr (µg/day) of 30 to 35 for adult males and 20-25 for adults females (IOM, 2001), the values are very low, between the various treatments and in the two countries represented, with values between 0.0155 and 0.0592 µg kg^{-1} body weight per day. When compared to the tolerable daily intake (TDI) of Cr at 300 µg kg^{-1} body weight per day (EFSA CONTAM Panel), the risk is further reduced since estimated exposures are well below TWI, exposures are little or no concern.

A graphical representation of the values of the exposure estimate with the consumption of Cr(III) in raw and cooked milk and bovine meat are present in Fig. 5.3. As can be seen, the exposure estimate is higher for the Danish population perhaps because the estimated consumption of milk and bovine meat is slightly higher when compared to the French population.

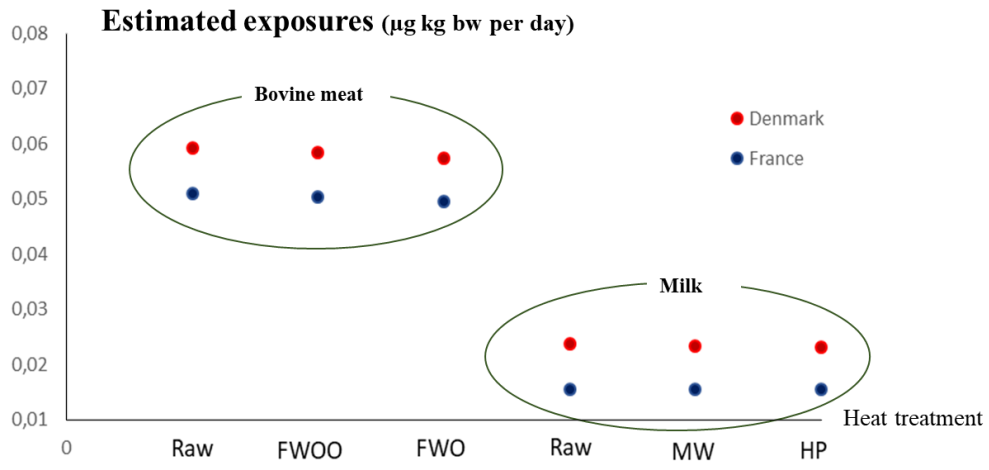

Figure 5.3. Representation of the risk values with the consumption of Cr(III) in raw and cooked milk and bovine meat; Acronym legend: FWOO – fried without oil; FWO – fried with oil; MW – Microwaves; HP – Hot plate

5.5. Conclusion

This study aimed to assess the behaviour of Cr(III) and Cr(VI) species during different cooking types of milk and beef samples using a powerful analytical approach (SS-ID) allowing the quantification of Cr(III) and Cr(VI) and simultaneously correction of their interconversion during the analytical process. Cr(VI) added to the milk and meat samples was entirely reduced ($\beta \approx 100\%$) to Cr(III) during the analytical process but SS-ID allowed its accurate quantification due to its ability to correct for the species interconversion.

ANOVA tests showed no significant differences in terms of Cr(III) and Cr(VI) between uncooked (raw) and cooked samples (milk and bovine meat) ones, regardless of the culinary treatments employed. This confirms that an oxidation of Cr(III) to Cr(VI) does not occur in these food samples during their conventional cooking. In all cases, the Cr(III) levels were comparable with those of total Cr, which supports the EFSA statement in terms of the presence of Cr in foodstuffs exclusively as Cr(III) species.

An exposure assessment was also carried out for cooked milk and meat for the first time, hence showing a more realistic approach (samples as consumed) compared to the raw samples. The assessment showed extremely low exposure to Cr related to the consumption of bovine meat and milk, both in Denmark and France.

CHAPTER 6
GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The main scientific goal of this Ph.D. project was to develop and apply novel analytical approaches for Cr speciation analysis in foodstuffs. For this purpose, a primary method based on species-specific isotope dilution and HPLC-ICP-MS was employed throughout. The novel analytical methods were applied to the speciation analysis of Cr(III) and Cr(VI) in infant formula and semi-skimmed milk, meat and meat products, bread, breakfast cereals and rice.

The conventional methods for Cr(III) and Cr(VI) speciation analysis rely on the selective and sequential extraction of Cr(III) and Cr(VI) followed by the determination of total Cr in the extracted fraction (*off-line* approaches). The more advanced approaches rely on the *on-line* separation of Cr(III) and Cr(VI) by high performance liquid chromatography (*on-line* approaches). Nevertheless, in both cases (on and off line approaches), the mutual interconversion of Cr(III) and Cr(VI) during the analytical process cannot be accurately pictured. Actually, the only analytical approach capable to address the quantification and the correction of the mutual species inter-conversion is the species-specific isotope dilution whose principle was presented in detail in Chapter 4. This approach was employed thoroughly for simultaneous (single run) speciation analysis of Cr(III) and Cr(VI) in foodstuffs (Obj. 1). This method provides accurate, precise and reproducible measurements at very low limits of quantification.

The application of the new analytical method based on SS-ID to assess the stability of Cr(III) and Cr(VI) species during various types of cooking in milk and meat was also carried out (Obj. 2). The results showed no significant differences between the raw and cooked (milk and bovine meat) samples in terms of Cr(III) and Cr(VI).

An exposure assessment related to Cr(III) in raw and cooked milk and bovine meat in Denmark and France was also carried out; the contribution of these foods to exposure of Cr(III) were extremely low in France and Denmark.

Several tests, in an attempt to clarify the difficult possibility of the presence of Cr(VI) in bread, breakfast cereals and rice (Obj.3), were successfully carried out

All the occurrence data obtained in this study showed that Cr(VI) is not present in any of the samples analyzed here hence confirming EFSA's statement in terms of the absence of Cr(VI) in foodstuffs (Obj. 3). It is worth to note that in all samples analyzed in this project, the levels of Cr(III) were compatible in most cases with total Cr levels in the same samples, hence confirming indirectly the absence of Cr(VI). However, the study must be pursuit by enlarging the panel of samples types, including the assessment of Cr species status in such samples after their cooking.

The Ph.D. project was highly innovative, due its very complex nature, but also because it addressed the most powerful analytical approach nowadays-available (SS-ID) for trace and ultra-trace speciation analysis of Cr in various aspects, including the assessment of Cr species behavior during various types of cooking. All the objectives proposed for this Ph.D. were successfully met.

The complex professional experience that I acquired during this doctoral project will certainly allow me to address new scientific challenges in the general field of analytical chemistry, with easy adaptability in other areas, such as environmental, biomedical (food supplements) and others.

The finding of this study related to the absence of Cr (VI) in foods leads to a simplification of the risk assessment for Cr species in foods, since the attention falls only on Cr(III).

As stated above, the Ph.D. project was indeed at the scientific forefront in terms of analytical chemistry and its applications. Nevertheless, it is worth to highlight that the analytical approaches currently employed for food analysis provide only a static picture of the actual contaminants status. Actually, even though extremely accurate measurements are ensured, they provide information about the "free" species in a sample. With other words, little (if any) information is provided regarding the actual status of the species within the sample matrix: is the species present in a free state or it is bound to matrix components, such proteins and lipids, found abundantly in most of the foodstuffs? Can a cooking process release a species from the matrix (without actual loss), hence making it more available and ultimately more toxic? This information, complementary to the knowledge regarding the levels of the species in the sample, is important because it is highly probable that the actual toxicity and mobility of a chemical species is determined by its interactions with the molecular environment of the sample.

The project may therefore be enlarged to the assessment of the interactions of Cr species with the samples matrices. For this purpose, techniques providing information on the proteins fractions such as size-exclusion chromatography or asymmetric field flow fractionation can be employed. This would provide a truly status of toxic species in a food matrix, including its eventual gain in mobility during the food cooking.

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APPENDICE

Table A1: Estimates of atmospheric Cr emission from anthropogenic sources in U.S (U.S. Geological Survey, 2019)

Source category	Number of sources	Cr emission (metric tons/year)	Cr(VI) fraction (%)
Combustion of coal and oil	Many	1723	0.2
Chromium chemical manufacturing	2	18	67
Chemical manufacturing cooling tower	2039	43	100
Petroleum refining cooling towers	475	32	100
Specialty/steel production	18	103	2.2
Primary metal cooling towers	224	8	100
Chrome plating	4000	700	100
Comfort cooling towers	38000	7.2-206	100
Textile manufacturing cooling towers	51	0.1	100
Refractory production	10	24	1.3
Ferrochromium production	2	16	5.4
Sewage sludge incineration	133	13	<0.1
Tobacco cooling towers	16	0.2	100
Utility industry cooling towers	6	1.0	100
Chrome ore refining	6	4.8	<0.1
Tire and rubber cooling towers	40	0.2	100
Glass manufacturing cooling towers	3	0.01	100
Cement production	145	3	0.2
Municipal refuse incineration	95	2.5	0.3
National total		**2700-2900**	

Table A2. Cr species and their physical characteristics depending on the oxidation state (EFSA, 2014a; Chemistry.LiberTexts, 2019; PubChem., 2019)

Cr species	Chemical Formula	Oxidation state	Characteristic	CAS no.
Chromium	Cr	0		7440-47-3
Chromium Oxides and Hydroxides				
Chromium oxide	CrO	(II)	Insoluble black powder	12018-00-7
Chromium oxide	Cr_2O_3	(III)	Amphoteric and while it is insoluble in water Producing a dark green colour	1308-38-9
Chromium chloride	$CrCl_2$	(II)	White crystalline solid The primary hazard is the threat to the environment. Used to make other chemicals and as an oxygen absorbent	10049-05-5
Chromium dioxide	CrO_2	(IV)	Ferromagnetic properties long, glass rod like crystals	
Chromium trioxide	CrO_3	(VI)	React with water to form chromic acid Dark red-orange granular complex Extremely toxic	1333-82-0
Chromate	CrO_4^{2-}		Salt Yellow colour in basic conditions Poor oxidizing agent	11104-59-9
Dichromate	$Cr_2O_7^{2-}$		Strong orange colour in acidic conditions Strong oxidizing agent but bad precipitating agent	13907-47-6
Sodium chromate	Na_2CrO_4	(VI)	Yellow crystalline solid Pigments for paints and inks, wood preservative.	7775-11-3
Potassium chromate	K_2CrO_4	(VI)	Yellowish, crystalline, inorganic compound that emits toxic chromium fumes upon heating Highly corrosive and strong oxidizing agent Used in the manufacture of dyes and in textile dyeing processes	7789-00-6
Strontium chromate	$SrCrO_4$	(VI)	Yellowish, crystalline, inorganic compound that emits toxic chromium fumes upon heating Used as a colorant Highly corrosive and is a strong oxidizing agent.	7758-97-6
Zinc chromate	ZnCrO4	(VI)	Yellowish, crystalline, inorganic compound that emits toxic chromium fumes upon heating. Highly corrosive and strong oxidizing agent. Used as a corrosion inhibitor, metal conditioner and paints, varnishes and oil colours	13530-65-9
Lead chromate	PbCrO4	(VI)	Yellow, orange or red coloured, crystalline, inorganic compound that emits toxic chromium fumes upon heating. Highly corrosive and strong oxidizing agent. used in printing inks, paints and to colour vinyl, rubber and paper	7758-97-6

Sodium dichromate	$Na_2Cr_2O_7$	(VI)	Orange to red coloured, crystalline, inorganic compound that emits toxic chromium fumes upon heating. Highly corrosive and strong oxidizing agent. Mainly used to produce other chromium compounds, but used in drilling muds, in metal treatments, in wood preservatives, in the production of dyes and organic chemicals and as a corrosion inhibitor.	10588-01-9
Ammonium dichromate	$(NH_4)_2Cr_2O_7$	(VI)	Bright orange red crystalline solid. Readily ignited and burns producing a voluminous green residue. Strong oxidizing agent if mixed with or contaminated with combustible material. Soluble in water.	7789-09-5
Potassium dichromate	$K_2Cr_2O_7$	(VI)	Bright orange-red crystals Used in dyeing, staining, tanning leather, as bleach, oxidizer, depolarizer for dry cells, etc. Medically, used externally as an astringent, antiseptic, and caustic. Corrosive poison.	7778-50-9
Chromic acid	H_2CrO_4	(VI)	Oxidising agent Conjugate acid of a hydrogenchromate	7738-94-5
Lead Sulfochromate Yellow	CrO_5Pb_2	(VI)	Pigment: yellow 34 Pigment is the colouring material of choice in a number of applications. Extremely low solubility, significantly less bio-available than other lead containing substances including basic lead oxides.	1344-37-2
Lead chromate molybdate sulfate red C.I. Pigment Red 104	$PbCrO_4$, $PbMoO_4$, $PbSO_4$	(VI)	Solid solution of lead chromate, lead molybdate and lead sulfate. Fine, dark-orange or light-red powder. Pigment in printing inks, paints, plastics.	12656-85-8

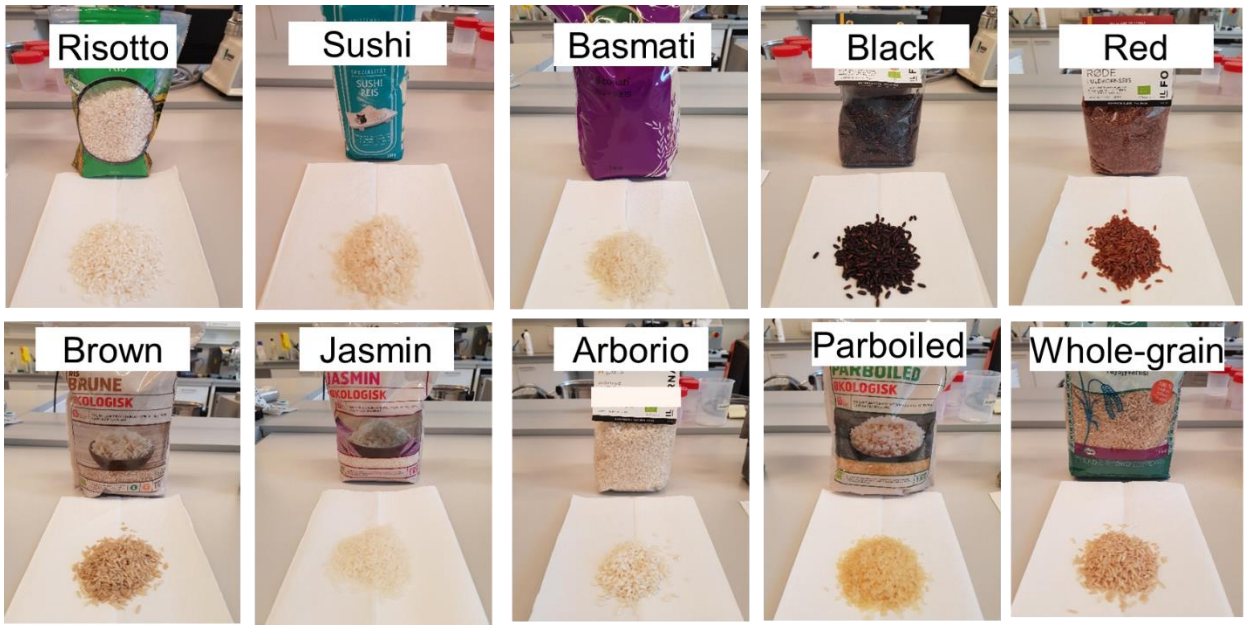

Figure A1: Rice samples types tested with the proper color depending on the different levels of processing

Date	05.02.2020							
	5ppb Cr(III)-EDTA	50Cr(III)	52Cr(III)	$R_{Experimental}$ III (50/52)		5ppb Cr(VI)-DPC	52Cr(VI)	53Cr(VI)	$R_{Experimental}$ VI (53/52)
	inj.1	38064	787690	**0,048**		inj.1	224546	26776	**0,119**
	inj.2	36989	749391	**0,049**		inj.2	230307	27066	**0,118**
	Moyenne	**37526**	**768540**	0,049		Moyenne	**227427**	**26921**	0,118
	Ecart-type	**760**	**27081**	***0,07%***		Ecart-type	**4073**	**205**	***0,12%***
	RSD (%)	**2,0**	**3,5**	**< 1%***		RSD (%)	**1,8**	**0,8**	**< 1%***

$A(^{50}Cr)_{nat}$	$A(^{52}Cr)_{nat}$	$R_{theorique}(^{50}/_{52})$
0,0435	0,8379	0,05

$A(^{52}Cr)_{nat}$	$A(^{53}Cr)_{nat}$	$R_{theorique}(^{53}/_{52})$
0,8379	0,0950	0,11

K Cr(III)	K Cr(VI)
1,06	**0,96**
-6%	***4%***
< 20%	

Figure A2: Calculations for the K (mass bial factor) value for Cr(III) and Cr(VI) - these values are measured daily. The values result from the injection of 5 µg kg^{-1} of standard solution of Cr(III) and Cr(VI) separately.

	Abundances Cr							
Isotope	Natural	Spike ^{50}Cr(III)	Molar Mass Cr (g/mol)	Molar Mass ^{50}Cr spike (g/mol)	Facteur :		[ ] ^{50}Cr(III) spike (µg/kg)	[ ] ^{53}Cr(VI) spike (µg/kg)
50	4,35	97,4	49,946				1015	1007
52	83,79	2,3	51,941	50,0019	1,16		103,99	100,24
53	9,50	0,2	52,941				20,28	20,07
54	2,37	0,1	53,939				10,05	9,97
			51,996				0,99	0,98
							0,05	0,05
	Abondances Cr						0,00	0,00
Isotope	Natural	Spike ^{53}Cr(III)	Molar Mass Cr (g/mol)	Molar Mass ^{53}Cr spike (g/mol)	Facteur :			
50	4,35	0,1	49,946					
52	83,79	2,9	51,941	51,9961	1,10			
53	9,50	92,4	52,941					
54	2,37	0,0	53,939					
			51,996					
Date:	05.02.2020							
Samples Type:	Danish bread							

Dilution isotopique (DI)	Rug I	Rug II	Malt I	Malt II	Burguer I	Burguer II	Spelt I	Spelt II
Conc Cr(III) (µg/kg) sample	25,6	25,6	24,2	24,2	34,9	34,9	37,8	37,8
Conc Cr(VI) (µg/kg) sample	0	0	0	0	0	0	0	0
Sample mass (g)	**0,3**	**0,3**	**0,3**	**0,3**	**0,3**	**0,3**	**0,3**	**0,3**
conc ^{50}Cr(III) (µg/kg)	20,28	20,28	20,28	20,28	20,28	20,28	20,28	20,28
vol ^{50}Cr(III) to add (mL=g)	**0,326**	**0,326**	**0,308**	**0,308**	**0,444**	**0,444**	**0,481**	**0,481**
conc ^{53}Cr(VI) (µg/kg)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
vol ^{53}Cr(VI) to add (mL=g)	**#DIV/0!**	**#DIV/0!**	**#DIV/0!**	**#DIV/0!**	**#DIV/0!**	**#DIV/0!**	**#DIV/0!**	**#DIV/0!**

Figure A3: Calculations to know how much to add from each spike depending on the concentration of Cr(III) and Cr(VI) found in the food samples, in this case, various types of bread.

Date	05.02.2020

Mass Bial Facteur	
1,06	0,96

Samples	$^{50}Cr^{III}$	$^{52}Cr^{III}$	$^{53}Cr^{III}$	$^{50}Cr^{VI}$	$^{52}Cr^{VI}$	$^{53}Cr^{VI}$	Rm 50/52 Cr(III)	Rm 53/52 Cr(III)	Rm 50/52 Cr(VI)	Rm 53/52 Cr(VI)
Rug I	43174	25910	239	547	3230	830	1,77	0,01	0,16	0,25
	44557	21403	299	538	4326	827	2,21	0,01	0,12	0,18
Malt I	53623	23293	278	525	3687	633	2,44	0,01	0,14	0,16
	53334	23297	336	531	3483	622	2,43	0,02	0,15	0,17
Burguer I	65181	24885	258	502	3423	603	2,78	0,01	0,14	0,17
	63288	24516	273	575	3659	607	2,74	0,01	0,15	0,16
Spelt I	71291	34555	270	434	3448	700	2,19	0,01	0,12	0,19
	74926	33244	232	418	3663	725	2,39	0,01	0,11	0,19

Figure A4: Calculation of the four ratios found after injection of the samples for the three differents chromium isotopes.

M_{spike}^{III} (µg/µmol)	50,002	$^{50}A_{nat}$	4,3%
M_{spike}^{VI} (µg/µmol)	52,909	$^{52}A_{nat}$	83,8%
$^{50}A_{spike}^{III}$	97%	$^{53}A_{nat}$	9,5%
$^{52}A_{spike}^{III}$	2,3%	W_{ant}	0,006
$^{53}A_{spike}^{III}$	0,2%	M_{nat} (µg/µmol)	51,996
$^{50}A_{spike}^{VI}$	0,1%		
$^{52}A_{spike}^{VI}$	2,9%		
$^{53}A_{spike}^{VI}$	92,4%		

Date	05.02.2020

Identification de l'échantillon	**Rug I**	**Rug II**	**Malt I**	**Malt II**	**Burguer I**	**Burguer II**	**Spelt I**	**Spelt II**
C_{spike}^{III} (µMole/g)	0,0004	0,0004	0,0004	0,0004	0,0004	0,0004	0,0004	0,0004
C_{spike}^{VI} (µMole/g)	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
m_{spike}^{III} (g)	0,3318	0,3295	0,3114	0,3133	0,4483	0,4481	0,4850	0,4833
m_{spike}^{VI} (g)	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
m_x (g)	0,3073	0,3113	0,3087	0,3013	0,3040	0,3018	0,3020	0,3064
R $^{50/52}$ Cr(III)	1,77	2,21	2,44	2,43	2,78	2,74	2,19	2,39
R $^{53/52}$ Cr(III)	0,01	0,01	0,01	0,02	0,01	0,01	0,01	0,01
R $^{50/52}$ Cr(VI)	0,16	0,12	0,14	0,15	0,14	0,15	0,12	0,11
R $^{53/52}$ Cr(VI)	0,25	0,18	0,16	0,17	0,17	0,16	0,19	0,19
$^{53}A_{sp.}III * m_{sp.} * C_{sp.}III$	0,0000003	0,0000003	0,0000003	0,0000003	0,0000004	0,0000004	0,0000004	0,0000004
$^{52}A_{sp.}III * m_{sp.} * C_{sp.}III$	0,0000031	0,0000031	0,0000029	0,0000029	0,0000042	0,0000042	0,0000045	0,0000045
$^{50}A_{sp.}III * m_{sp.} * C_{sp.}III$	0,0001311	0,0001302	0,0001230	0,0001238	0,0001771	0,0001770	0,0001916	0,0001909
$^{52}A_x * m_x$	0,2574836	0,2608352	0,2586566	0,2524563	0,2547186	0,2528752	0,2530428	0,2567295
$^{53}A_x * m_x$	0,0291935	0,0295735	0,0293265	0,0286235	0,0288800	0,0286710	0,0286900	0,0291080
$^{50}A_x * m_x$	0,0133522	0,0135260	0,0134130	0,0130915	0,0132088	0,0131132	0,0131219	0,0133131
$^{53}A_{sp.}VI * m_{sp.} * C_{sp.}VI$	0,0000000	0,0000000	0,0000000	0,0000000	0,0000000	0,0000000	0,0000000	0,0000000
$^{52}A_{sp.}VI * m_{sp.} * C_{sp.}VI$	0,0000000	0,0000000	0,0000000	0,0000000	0,0000000	0,0000000	0,0000000	0,0000000
$^{50}A_{sp.}VI * m_{sp.} * C_{sp.}VI$	0,00000000	0,00000000	0,00000000	0,00000000	0,00000000	0,00000000	0,00000000	0,00000000
α	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*
β	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*	*#DIV/0!*
C_x (III) (µg/kg)	**24,39**	**25,89**	**26,77**	**25,20**	**33,06**	**37,59**	**37,81**	**35,50**
C_x (VI) (µg/kg)	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**
C_x (III) (µg/kg) average	**25,14**	**Expecte Value**	**25,98**	**Expecte Value**	**35,33**	**Expecte Value**	**36,65**	**Expecte Value**
C_x (III) (µg/kg) stdev.	**1,06**	25,6	**1,11**	24,2	**3,21**	34,9	**1,63**	37,8
C_x (III) (%) RSD	**4%**	**98%**	**4%**	**107%**	**9%**	**101%**	**4%**	**97%**
C_x (VI) (µg/kg) average	**0,00**	**Expecte Value**	**0,00**	**Expecte Value**	**0,00**	**Expecte Value**	**0,00**	**Expecte Value**
C_x (VI) (µg/kg) stdev.	**0,00**		**0,00**		**0,00**		**0,00**	
C_x (VI) (%) RSD	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Figure A5: Concentration found for Cr(III) and Cr(VI) in bread samples

M_{spike}^{III} (µg/µmol)	50,002	$^{50}A_{nat}$	4,3%
M_{spike}^{VI} (µg/µmol)	52,909	$^{52}A_{nat}$	83,8%
$^{50}A_{spike}^{III}$	97%	$^{53}A_{nat}$	9,5%
$^{52}A_{spike}^{III}$	2,3%	W_{ant}	0,006
$^{53}A_{spike}^{III}$	0,2%	M_{nat} (µg/µmol)	51,996
$^{50}A_{spike}^{VI}$	0,1%		
$^{52}A_{spike}^{VI}$	2,9%		
$^{53}A_{spike}^{VI}$	92%		

Samples	Microwave 2min. T=70°C 1	Microwave 2min. T=70°C 2	DigiPrep 100°C/5min. 1
C_{spike}^{III} (µMole/g)	0,00020	0,00020	0,00020
C_{spike}^{VI} (µMole/g)	0,00001	0,00001	0,00001
m_{spike}^{III} (g)	0,0701	0,0717	0,0750
m_{spike}^{VI} (g)	0,1477	0,1471	0,1467
m_x (g)	0,3029	0,3010	0,3042
$R^{50/52}$ Cr(III)	0,90	0,92	0,92
$R^{53/52}$ Cr(III)	0,21	0,19	0,19
$R^{50/52}$ Cr(VI)	0,61	0,53	0,49
$R^{53/52}$ Cr(VI)	0,65	0,67	0,59
$^{53}A_{sp.}III * m_{sp.} * C_{sp.}III$	0,0000000	0,0000000	0,0000000
$^{52}A_{sp.}III * m_{sp.} * C_{sp.}III$	0,0000003	0,0000003	0,0000003
$^{50}A_{sp.}III * m_{sp.} * C_{sp.}III$	0,0000137	0,0000140	0,0000146
$^{52}A_x * m_x$	0,2537969	0,2522049	0,2548861
$^{53}A_x * m_x$	0,0287755	0,0285950	0,0288990
$^{50}A_x * m_x$	0,0131610	0,0130785	0,0132175
$^{53}A_{sp.}VI * m_{sp.} * C_{sp.}VI$	0,0000013	0,0000013	0,0000013
$^{52}A_{sp.}VI * m_{sp.} * C_{sp.}VI$	0,0000000	0,0000000	0,0000000
$^{50}A_{sp.}VI * m_{sp.} * C_{sp.}VI$	0,00000000	0,00000000	0,00000000
K11	-0,000062	-0,000062	-0,000064
K12	0,000000	0,000000	0,000000
K21	0,000002	0,000002	0,000002
K22	-0,000053	-0,000064	-0,000063
K31	-0,000095	-0,000114	-0,000128
K32	0,000000	0,000000	0,000000
K41	0,000001	0,000001	0,000001
K42	-0,000009	-0,000009	-0,000010
d1	0,00	0,00	0,00
K	0,00	0,00	0,00
α	-2%	0%	0%
β	122%	100%	104%

Figure A6: Interconversion factors (α and β) in infant formula milk cooked

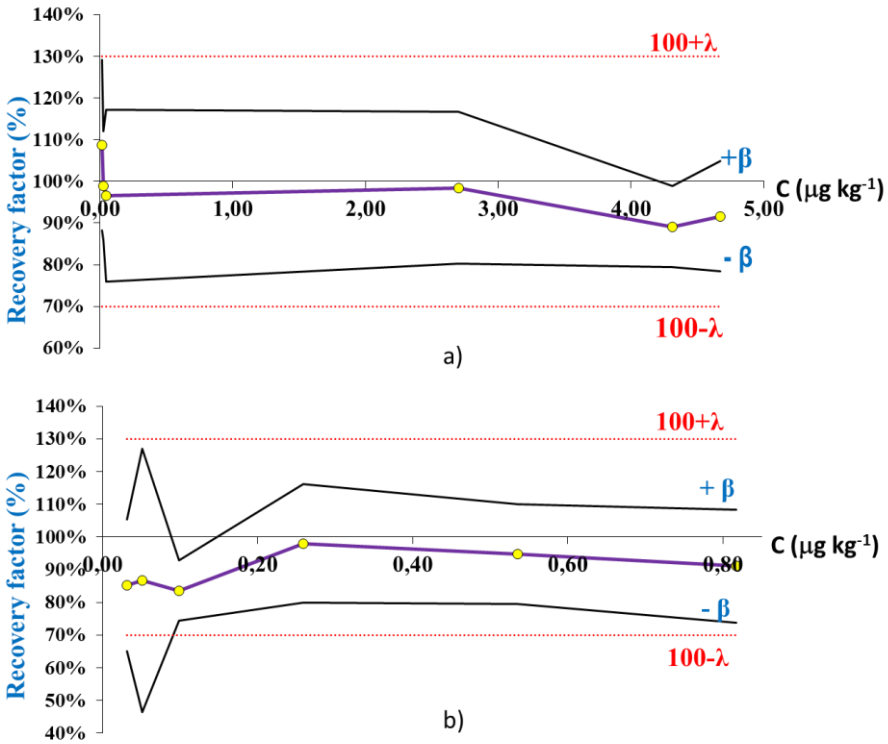

Figure A7: Accuracy profile for Cr(III) (a) and Cr(VI) (b) speciation analysis by SS-ID HPLC-ICP-MS.

Table A3: Risk assessment with two different approaches (FAO and Internal country study) to meat and milk consumption by the Danish and French population

	Bovine meat									***Milk***								
	FAOSTAT [a]	Internal study [b]	(L_k) Cr(III) Concentration g kg^{-1}			(E_j) Exposure assessment µg kg^{-1} Cr in bovine meat/person/day				FAOSTAT [a]	Internal study [b]	(L_k) Cr(III) Concentration µg kg^{-1}			(E_j) Exposure assessment µg kg^{-1} Cr in milk/person/day			
	$C_{i,k}$ Consumption (kg/day)		Raw	Fried without oil	Fried with oil	Raw		Fried without oil		Fried with oil		$C_{i,k}$ Consumption (L/day)		Raw	Microwave	Hot plate	Raw		Processed by microwave		Processed by hot plate	
Denmark	**0.0695**	**0.061**	4.49	4.62	4.78	0.0045	0.0039	0.0046	0.0040	0.0048	0.0041	**0.8412**	**0.305**	4.55	4.47	4.38	0.0547	0.0198	0.0537	0.0195	0.0526	0.0191
France	**0.0634**	**0.069**				0.0041	0.0044	0.0042	0.0046	0.0043	0.0047	**0.7225**	**0.172**				0.0469	0.0112	0.0461	0.0110	0.0452	0.0107

Body weight (Wi) = 70 kg, considered the average value

[a] source: FAOSTAT website - Food and Agriculture Organization - value calculated from food supply quantity for each food group

[b] (Denmark_Meat) Source: Nordic Council of Ministers (NCM). Tetens I. Nutritional evaluation of lowering consumption of meat and meat products in the Nordic context (2013) No. 2013:506. DOI: https://doi.org/10.6027/TN2013-506 **(n= 2025)**; (Denmark_Milk) Source: Agnes N. Pedersen, Dietary habits in Denmark 2011-2013 **(n= 6032);** (France) Source: ANSES REPORT – INCA3 study on the Update of the food consumption database and the estimation of nutritional intakes of individuals living in France through the implementation of the 3rd national individual study of food consumption (2017) **(n=2 121)** DOI: INCA3 study.pdf

Article I

Marina Saraiva, Rachida Chekri, Axelle Leufroy, Thierry Guérin, Jens J. Sloth and Petru Jitaru

Development and validation of a single run method based on species specific isotope dilution and HPLC-ICP-MS for simultaneous species interconversion correction and speciation analysis of Cr(III)/Cr(VI) in meat and dairy products

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Development and validation of a single run method based on species specific isotope dilution and HPLC-ICP-MS for simultaneous species interconversion correction and speciation analysis of Cr(III)/Cr(VI) in meat and dairy products

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ABSTRACT

This study presents the development, validation and application of a new analytical approach for the simultaneous speciation analysis of Cr(III) and Cr(VI) in meat and dairy products by high-performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) and double spike species specific-isotope dilution (SS-ID). The species extraction was achieved by sequential complexation of Cr(III) with ethylenediaminetetraacetic acid (EDTA) and of Cr(VI) with 1,5-diphenylcarbazide (DPC) in the same analytical run. The HPLC separation of complexed species was carried out using a short (5 cm) microbore anion-exchange HPLC column and a mobile phase consisting of 0.01 mol L^{-1} HNO_3 + 2.5% (v/v) MeOH + 0.30 mol L^{-1} EDTA (pH = 2) in isocratic elution mode with excellent baseline separation achieved in less than 3 min. The method was validated by means of the accuracy profile approach by carrying out 6 measurement series in duplicate on (six) different days over a timespan of two months. The quantification limit was 0.013 μg kg^{-1} for Cr(III) and 0.049 μg kg^{-1} for Cr(VI), respectively. The measurement bias corresponding to the validity domain ranged from 0.01 to 0.11%, whereas the coefficient of variation in terms of repeatability (CV_r) varied from 2.9 to 11.6% (depending on the analyte level) for Cr(III) and from 6.7 to 11.8% for Cr(VI). Similarly, the coefficient of variation in terms of intermediate reproducibility (CV_R) ranged from 6.8 to 13% for Cr(III) and from 6.8 to 25.9% for Cr(VI), respectively. The method was successfully applied to the analysis of a selection of food samples such as baby and semi-skimmed milk and steak beef samples. Cr(VI) was not quantified in any of these samples while Cr(III) levels ranged between 2.7 and 4.7 μg kg^{-1}, which were comparable with the levels of total chromium analysed in the same samples by ICP-MS (accredited method). The method presented here with combined use of species specific isotope dilution 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 a chemical element with different physico-chemical and toxicological characteristics, depending on its species. It is still used extensively in various industrial processes such as electroplating, metal finishing, leather tanning, and production of pigments and it is still discharged into the surrounding air, water, and terrestrial environments and hence exerting adverse effects on the ecosystem and ultimately on the human health via the food chain [1–3].

The most stable oxidation states of Cr are Cr(III) and Cr(VI), having very different toxicity and mobility in the environment and also in the human body. Cr(III) is poorly soluble and relatively immobile in aqueous environments compared to Cr(VI), which is highly mobile, soluble and consequently, more bioavailable [4,5].

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0039-9140/

Cr(VI) has been recognized for several decades as being carcinogenic, whereas Cr(III) was for a long time considered as a beneficial element for the human health [6]. Nevertheless, the European Food Safety Authority (EFSA) stated relatively recently that there is no convincing evidence of beneficial effects of Cr(III) for healthy people [7]. Hence the interest in Cr speciation, which was focused almost entirely on Cr(VI), shifted to the determination of both Cr species (Cr(III) and Cr(VI)), especially in foodstuffs. Nevertheless, simultaneous speciation analysis of Cr(III) and Cr(VI) is a challenging task mainly because of their high chemical instability. In addition, both Cr species are generally present at ultra-trace levels in food samples, which requires the use of very sensitive and highly selective analytical tools for their determination.

The speciation analysis of Cr(III) and Cr(VI) has been mostly carried out based on a selective complexation of Cr(III) or Cr(VI) followed by the determination of each species by measuring the total Cr content in the extract without prior chromatographic separation (*off-line* approaches) [8–10]. Several studies have shown that these approaches are prone to the formation of analytical artefacts arising mainly from the extraction step, because the co-extraction of both species may occur without any possibility to assess these side-effects when total Cr is measured in the extract fraction [11–13].

Cr speciation analysis shifted relatively recently towards carrying out species separation by using high performance liquid chromatography (HPLC) before the on-line detection by atomic spectrometry, especially inductively coupled plasma-mass spectrometry (ICP-MS) (*on-line* approaches) [6,14–17]. Nevertheless, the on-line approaches are not *per se* capable to assess species interconversion during the extraction or HPLC separation steps. Actually, species specific (SS) isotope dilution (ID) mass spectrometry (SS-ID-MS) has proven to be the only analytical approach suitable for simultaneous speciation analysis of Cr(III) and Cr (VI) in foodstuffs due to its capability to quantify the species and to correct for their interconversion during the same analytical run [18,19]. Briefly, ID-MS is based on the measurement of isotope ratios in a sample whose analyte isotopic composition is altered by the addition of a known amount of an isotopically modified analyte (*spike*) [20]. Apart from the quantification and species interconversion correction in the same analytical run, which was already demonstrated in previous studies [21–25], another important advantage of SS-ID-MS is that once the isotopic equilibrium between the sample and the spike(s) is achieved, the matrix (physical) effects, the species loss (e.g., incomplete extraction) or transformation (e.g. oxidation and/or reduction) during the analytical process does not influence the method accuracy, due to the measurement of isotopic ratios rather than the absolute or relative signals as in the case of conventional analytical methods [20]. It is also worth to highlight that SS-ID-MS is a primary analytical method, hence its application counteracts the lack of certified reference materials (CRM) in terms of Cr speciation analysis [26–29].

In 2014, EFSA stated that Cr(VI) species is not present in foodstuffs due to the specific features (reductive potential) of this type of matrix which reduces Cr(VI) to Cr(III), and hence total Cr in foodstuffs consists of Cr(III) solely [7]. However, in recent years several studies reported the presence of Cr(VI) in various foodstuffs such as bread, cereals, chicken meat and tea [30–35]. However, the quantification of Cr(VI) in these samples has been disputed as being most likely due to an analytical artefact because of its co-extraction or the conversion of Cr(III) to Cr(VI) during the extraction and/or the HPLC separation, given that this processes cannot be assessed by conventional speciation analysis methods (other than SS-ID) [11,30–35].

The aim of the present study was to develop and validate a new analytical approach for simultaneous (single run) and accurate speciation analysis of Cr(III) and Cr(VI) in foodstuffs by HPLC-ICP-MS and SS-ID using selective species complexation with full capacity to correct for any species interconversion and hence accurately quantify Cr(III) and Cr (VI). The method was successfully applied to the analysis of a selection of real-life foodstuffs (meat and dairy products) hence proving its full capacity to correct for species interconversion and accurately quantify Cr(III) and Cr(VI) in a single analytical run.

2. Material and methods

2.1. Reagents and chemicals

Ultrapure water (18.2 MΩ cm^{-1}, Millipore Milli-Q™, Merck Millipore, Saint Quentin in Yvelines, France) was used throughout the study. Ethylene diamine tetraacetic acid (EDTA, 99.995%, Sigma-Aldrich, France) was used as complexing agent for Cr(III) while 1.5-Diphenylcarbazide (DPC, ≥ 99%, Sigma-Aldrich) was used as a derivatisation agent for Cr(VI) determination. HNO_3 (67% v/v, Suprapur) used as an eluent was purchased from VWR (Fontenay sous-Bois, France).

Standard stock solutions (1000 mg L^{-1}) of Cr(III) and Cr(VI) (Inorganic Ventures, Christiansburg, USA) were used to prepare the calibration standards for the speciation analysis while isotopically enriched solutions (*spikes*) of ^{50}Cr(III) and ^{53}Cr(VI) (100 μg mL^{-1}) (ISC Science, Oviedo, Spain) were used for SS-ID. The intermediate solutions were prepared daily by diluting the stock standard solutions with ultrapure water. The standard solutions used for the external calibration (speciation analysis method) were prepared by mixing the required amount of intermediate solution with 16 mL EDTA (0.9 g L^{-1}) solution, 200 μL of DPC (0.5 g L^{-1}) solution and the necessary amount of ultrapure water to reach a final volume of 20 mL.

For the determination of total Cr by ICP-MS (external calibration), a standard solution of Cr and scandium (Sc, internal standard) of 1000 mg L^{-1} (SCP Science, Quebec, Canada) were used.

All stock and intermediate standard solutions were stored in the refrigerator at 4–5 °C during maximum one month.

EDTA solution at 0.9 g L^{-1} was prepared by dissolving 0.45 g of reagent into 500 mL of ultrapure water and the mixture was sonicated for 15 min at 25 °C (sonication bath Model 86483, Fischer BioBlock, Rungis, France) at maximum power. DPC solution at 0.5 g L^{-1} was prepared by dissolving 50 mg of the reagent in 10 mL of methanol (≥99.8%, AnalaR NORMAPUR® ACS, VWR, France); then, 50 mL of ultrapure water containing 2.8 mL of H_2SO_4 (95–97% m/m, Merck KGaA, France) was added by continuously stirring. The obtained DPC solution was made up to 100 mL with ultra-pure water in a volumetric flask and it was stored during maximum one month at room temperature in the dark conditions [36].

2.2. Instrumentation

Sample preparation (species complexation and extraction) was carried out by heating using a heating block system type DigiPREP MS (SCP Science, Courtaboeuf, France). For total Cr determination, the samples were digested in a closed microwave system (Multiwave 3000, Anton Paar, Courtaboeuf, France).

The online separation of Cr(III) and Cr(VI) was carried out by HPLC using a Dionex ICS-5000$^+$ system (ThermoFisher Scientific, France) equipped with a Dionex IonPac™ AG7 column (2 mm internal diameter, id × 50 mm length × 10 μm particles diameter) (ThermoFisher Scientific, France).

An ICP-MS iCAP Q (ThermoFisher Scientific, Courtaboeuf, France) equipped with a PFA standard nebulizer and a cyclonic quartz spray chamber was used for on-line Cr species detection after their HPLC separation. Kinetic energy discrimination in high sensitivity mode (KED) using helium (He) as a collision gas was employed throughout the study. The ICP-MS was directly connected with the outlet of the HPLC column via a 0.18 mm i.d. PEEK tubing (80 cm length).

ICP-MS measurement conditions were optimized daily by running short term stability tests in standard mode and also using the cell collision mode (He). Data were processed using ThermoFisher Scientific Qtegra software.

For total Cr determination, an ICP-MS 7700 from Agilent

Technologies (Courtaboeuf, France) equipped with a third-generation Octopole Reaction System and a CETAC ASX-500 auto-sampler (CETAC Technologies, Omaha, NE, USA) was employed. The optimum operating conditions of the HPLC-ICP-MS (iCAP-Q ICP-MS) coupling and the Agilent 7700 ICP-MS (for Cr_{total} determination) are summarized in Table 1.

2.3. Analytical procedures

2.3.1. Sample preparation and storage

The samples analysed in this study were steak beef, baby milk and semi-skimmed milk purchased in retail shops in Maisons-Alfort (France). Meat samples (≅ 1 kg) were homogenized using a stainless steel free automatic mixer (Retsch Grindomix GM 200, Haan- Germany) with titanium blades. The liquid samples (baby-milk and semi-skimmed milk) were homogenized during 4 h using a rotary shaker (Heidolph reax 2, Merck KGaA, Darmstadt, Germany). After homogenization, one third of the sample was employed for method optimization (storage at ≈ 5 °C) while another third was used for the method validation. In the latter case, the baby milk (500 mL) was spiked with 0.25 ng mL^{-1} Cr(VI), the semi-skimmed milk (500 mL) with 1 ng mL^{-1} of Cr(VI) and the steak beef (200 g) with 0.5 ng g^{-1} of Cr(VI). The remaining bulk samples were kept as a backup and stored at −18 °C. The spike and standard solutions were stored in the dark at ≈ 5 °C.

2.3.2. Determination of total chromium by ICP-MS

Cr_{total} levels were determined by ICP-MS using an accredited method by employing samples digestion using concentrated HNO_3 (67% v/v) in a closed microwave digestion system on the basis of the procedure described in a previous study [37]. In brief, approximately 0.5 g of meat or 2.0 g of milk were accurately weighed into 80 mL quartz vessels directly and then thoroughly mixed overnight with 3 mL of concentrated HNO_3; 3 mL of ultra-pure water was then added before the microwave treatment. The digests were quantitatively transferred to polypropylene tubes (50 mL) and then filled up with ultrapure water after the addition of the internal standard. The analysis was carried out by ICP-MS using the external calibration approach (and Sc as internal standard). For this purpose, five standards with concentrations ranging from 1.0 to 10 μg L^{-1} (1, 2.5, 5, 7.5 and 10) were analysed to obtain the calibration curve.

Table 1
Optimum ICP-MS and HPLC instrumental parameters for Cr speciation analysis and Cr_{total} determination.

Parameter	ICP-MS	
	Thermo iCAP Q (Cr speciation)	Agilent 7700 (Cr_{total})
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	1.0 mm
Skimmer cone orifice (Ni)	0.5 mm	0.77 mm
KED gas flow (He)	≅ 5.0 mL min^{-1}	≅ 4.3 mL min^{-1}
Plasma power	1550 W	1500 W
Dwell time	250 ms	100 ms
Monitored isotopes	^{50}Cr, ^{52}Cr, ^{53}Cr, ^{13}C	^{52}Cr
HPLC separation		
Column	Dionex IonPac™ AG7: 2 mm × 50 mm, 10 μm	
Mobile phase	10^{-2} mol L^{-1} HNO_3 + 2.5% (v/v) MeOH + 0.30 mol L^{-1} EDTA (pH = 2.1)	
Injection volume	20 μL	
Flow rate	0.20 mL min^{-1}	
Elution mode	Isocratic	
Injector temperature	5 ± 3°C	
Column temperature	30 ± 1°C	
Total acquisition duration	5 min	

2.3.3. Single run speciation analysis of Cr(III) and Cr(VI) by HPLC-ICP-MS and SS-ID following selective Cr(III) and Cr(VI) complexation

A subsample of 0.3 g was mixed with the required amount of Cr(III) and Cr(VI) spikes directly in the 50 mL polypropylene tube and then placed in the heating block system. Then, 16 mL of EDTA solution was added to reach a final concentration of 2.4×10^{-3} mol L^{-1} (or 2.4 mM) and the mixture was heated at 70 °C during 25 min. The solution was cooled down (≅ 15 min) and then 200 μL of DPC solution was added to reach a final concentration of 0.02 mM; the final volume was made up to 20 mL with ultrapure water. The mixture was heated again at 70 °C for 25 min. The extract was cooled down to room temperature and then filtered using 0.45 μm polyvinylidene fluoride syringe filters (Merck Millipore, Cork, Ireland) prior to the analysis (the same day).

To carry out SS-ID, to each standard and sample, ^{50}Cr(III) and ^{53}Cr(VI) spikes were added and the blend solution was thoroughly mixed to achieve isotopes equilibration. The abundance of ^{50}Cr in the Cr(III) spike was 97.4% while that of ^{53}Cr in the Cr(VI) spike was 92.4% (as provided by the manufacturer's certificate). The natural abundances of the various Cr isotopes used for SS-ID calculations are those reported by the Internal Union of Pure ad applied chemistry (IUPAC) [38].

Finally, it must be emphasized that a standard mixture of Cr(III) (5.0 ng L^{-1}) and Cr(VI) (2.5 ng L^{-1}) was analysed daily in order to assess the overall instrumental accuracy and the stability of ^{50}Cr(III) and ^{53}Cr(VI) spikes used for SS-ID.

2.3.4. Method validation approach

The method was validated based on the accuracy profile approach according to guidelines described in detail elsewhere [39–41]. 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 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 (≥5) corresponding to natural (CRM, if available) or spiked matrices. Further, a tolerance interval (β_{TI}, Eq. (1)) is calculated for each level; β_{TI} is the interval in which a prediction can be made for a known proportion of the results, which is represented on the accuracy profile.

$$\beta_{TI} = k_{TI} \times s_{TI} \quad (1)$$

where:

β_{TI}, expected probability of the tolerance interval
k_{TI}, student statistical test depending on the number of degrees of freedom
S_{TI}, standard deviation of the tolerance interval

For multi-series analysis with the same number of replicates carried out for each series, S_{TI} is calculated by means of Eq. (2).

$$S_{TI} = S_R \sqrt{\left(1 + \frac{1}{IJB^2}\right)} \quad (2)$$

where:

S_R, standard deviation in terms of intermediate precision
I, number of series of measurements (days)
J, number of measurement replicates per series
B, parameter depending on intra- and inter-series standard deviations (Eq. (3)):

$$B = \sqrt{\frac{A+1}{JA+1}} \quad (3)$$

where A is calculated by Eq. (4):

$$A = \frac{s_B^2}{s_r^2} \quad (4)$$

where S_r, S_B, are intra- and inter-series standard deviation, respectively.

The β-expectation tolerance is generally set to 80–90%, which means that the risk of expected results falling outside these limits is below 10–20%. The method acceptance limit (λ) is set according to the criteria expected of repeatability and intermediate precision. Based on the accuracy profile approach, a method is validated when the β-expectation tolerance interval for a given result is comprised within the acceptability limit (λ).

The accuracy profile approach allows the determination of the intermediate reproducibility (CV_R), which in this study is assumed as the principal component of the combined uncertainty (u_c). Using CV_R values, the expanded uncertainty (U) is calculated as follows (Eq. (5)):

$$U = k \times u_C = k \times \frac{CV_R \times \overline{X}}{100 \text{ x } \sqrt{n}} \quad (5)$$

where:

k, coverage factor (k = 2 for p = 95%); $\overline{X}$ = average of *n* independent replicate measurements.

It is worth to note that CV_R (and hence the expanded uncertainty) is dependent on the analyte measurement level. In this study, for simplicity, CV_R is reported for two concentration domains, namely $<2 \times$ LOQ and $\geq 2 \times$ LOQ (see Table 2).

3. Results and discussion

3.1. Optimization of the complexation of chromium species

It is known that Cr(III) is stable in acidic medium while Cr(VI) is stable at basic pH [42], hence simultaneous extraction of both species in their genuine ionic form is not feasible. A way to overcome this intrinsic disadvantage relies on a sequential complexation at optimum pH for each species rather than simultaneous complexation of both species. 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 here as chromate anion, CrO_4^{2-}) was derivatized by reaction with DPC. Both chemical reactions are shown in Fig. 1a and b. It is important to note that although both EDTA and DPC are well known for their selective affinity towards Cr(III) and Cr(VI) [36,43,44], this approach was not previously used for simultaneous speciation analysis of Cr in foodstuffs.

As can be seen in Fig. 1a, the Cr(III)-EDTA complex is negatively charged and consequently Cr^{3+} complexation with EDTA leads to an inversion of the charge of this species. Similarly, the (simplified) reaction of Cr(VI) (in CrO_4^{2-} form) with DPC (Fig. 1b) involves firstly the reduction of Cr(VI) to Cr(III) and subsequently the complexation of the resulting Cr^{3+} with dyphenilcarbazone (DPCO) (the oxidized form of DPC) leading to a Cr(III)-DPCO complex, which is positively charged. Therefore, the reactions of Cr(III) with EDTA and Cr(VI) with DPC both lead to charge inversion of the corresponding species, hence providing the possibility of their further HPLC separation by using an ion exchange mechanism. It is also important to note that whereas Cr(III) is truly complexed with EDTA, in case of Cr(VI), as it was stated above, this species is firstly reduced to Cr(III) which is subsequently complexed by DPCO. Therefore, when carrying out the reactions of Cr(III) and Cr(VI) with EDTA and DPC, respectively, in the same analytical run, there is a competition between the genuine Cr(III) and Cr(III) resulted from Cr(VI) reduction by DPC. Hence, it is necessary to carry out the complexation of genuine Cr(III) by EDTA before the reaction of Cr(VI) with DPC takes place. In this way, the complexation of genuine Cr(III) with EDTA prevents on one hand, its oxidation to Cr(VI) during the analysis and on the other hand, its complexation with DPCO; actually, DPCO must react only with Cr(III) resulted from Cr(VI) reduction.

3.1.1. Optimization of the complexation pH

The pH controlling the complexation/extraction step was optimized by the analysis (in duplicate) of a standard solution consisting of 5 μg kg^{-1} of Cr(III) and Cr(VI) at different pH ranging from 2 to 10 (nitric acid or ammonia were used for the pH adjustment). As can be seen in Fig. 2, the recovery factors for both Cr(III) and Cr(VI) range between 85-100% and 50–111%, respectively. The optimum pH for reaction of Cr (III) by EDTA and of Cr(VI) by DPC in the same analytical run was obtained at 4, which is in agreement with other studies [45].

3.1.2. Optimization of the reaction temperature and time

For the temperature optimization, the complexation of Cr(III) and derivatisation of Cr(VI) was carried out at 30, 50, 70 and 90 °C, respectively (duplicate analysis) using a reaction time of 25 min. It turned out that the complexation/derivatisation of Cr(III) and Cr(VI) is incomplete at 30 °C while at 90 °C, consistent oxidation of Cr(III) to Cr (VI) occurs (not shown here), which is in agreement with other studies [6,43]. Quantitative derivatisation reactions of Cr(III)-EDTA (101%) and Cr(VI)-DPC (98%) was obtained at 70 °C.

The reaction time was optimized by the analysis of a mixture of Cr (III) and Cr(VI) at 5 μg kg^{-1} at the optimum temperature (70 °C) in the range between 5 and 30 min. As can be seen in Fig. 3, the optimum reaction time (recovery ≅100%) was obtained at 25 min for each species.

Table 2
Analytical performance characteristics for Cr(III) and Cr(VI) speciation analysis by SS-ID-HPLC-ICP-MS.

Sample	Level[a] (μg kg^{-1})	Bias (%)	CV_r		CV_R		LOD[d] (μg kg^{-1})	LOQ[d] (μg kg^{-1})
			Level 1[b]	Level 2[c]	Level 1[b]	Level 2[c]		
Cr(III)								
Baby milk	2.70	−0.01	11.6	10.8	11.6	11.4	0.0042	0.014
Semi-skimmed milk	4.31	−0.11	8.2	6.8	8.2	6.8		
Steak beef	4.67	−0.08	11.2	2.9	13.0	8.1		
Cr(VI)								
Baby milk	0.26	−0.02	8.2	11.2	13.7	11.4	0.015	0.049
Semi-skimmed milk	0.82	−0.09	8.8	11.8	25.9	11.8		
Steak beef	0.54	−0.05	6.7	10.0	6.8	10.0		

[a] Genuine level for Cr(III) and spiking level for Cr(VI).
[b] Level $< 2 \times$ LOQ.
[c] Level $\geq 2 \times$ LOQ.
[d] Sample weight of 0.3 g.

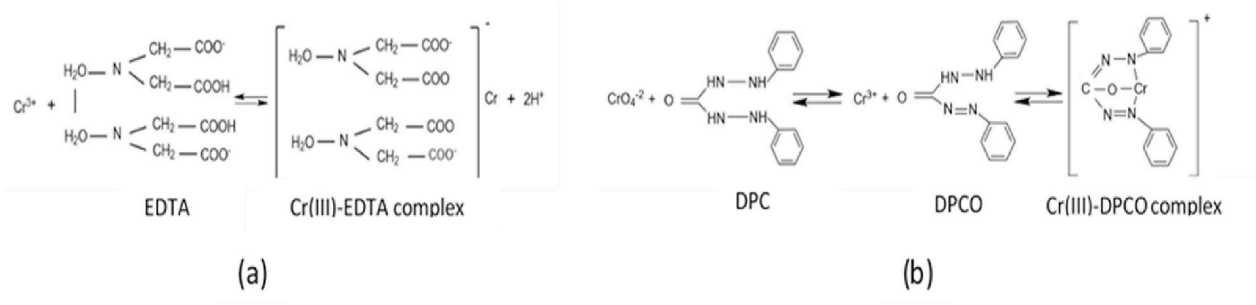

Fig. 1. Simplified reaction schemes of Cr(III) with EDTA (a) and of Cr(VI) with DPC (b) (for simplicity, proton contribution and charge balance is neglected for the reaction b).

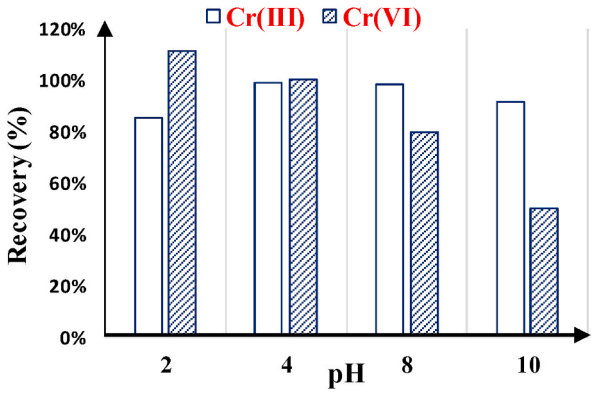

Fig. 2. Optimization of the pH for the complexation/derivatisation of Cr(III) with EDTA and Cr(VI) with DPC by the analysis of a standard mixture at 5 μg kg^{-1} (each species) by SS-ID HPLC-ICP-MS.

3.1.3. Optimization of EDTA and DPC concentration

The concentrations of EDTA and DPC were optimized by the analysis of a mixture solution of Cr(III) and Cr(VI) at 50 μg kg^{-1} (each species) by SS-ID HPLC-ICP-MS. For a sample amount of 0.2 g and a final extract solution of 20 g, these levels would correspond to 5 mg kg^{-1} in a real sample, which may reflect the worst case scenario case of foodstuffs contaminated with Cr species.

The optimization of EDTA concentration used for Cr(III) complexation was carried out in the range from 0.60 to 2.40 mM, which corresponds to the addition of volumes of EDTA solution between 4 and 16 mL for a final reaction volume of 20 mL. The concentration of DPC used for Cr(VI) derivatisation was optimized by carrying out the reaction at five different (DPC) levels (0.01, 0.02, 0.03, 0.04 and 0.05 mM). The optimum concentrations of EDTA and DPC for the complexation of Cr (III) and Cr(VI) at 50 μg kg^{-1} were 2.40 and 0.02 mM, respectively and they were used throughout the study.

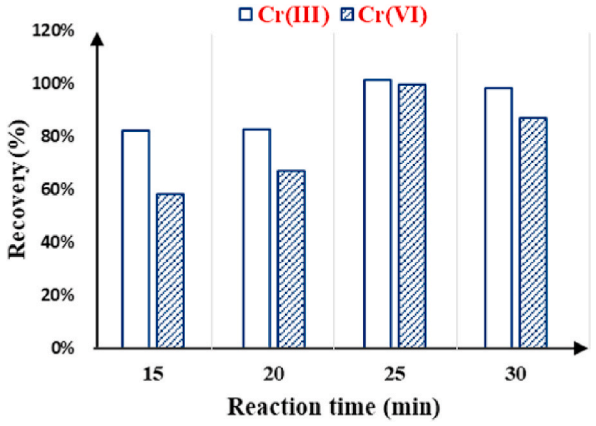

Fig. 3. Optimization of the complexation/derivatisation time of Cr(III) with EDTA and Cr(VI) with DPC by the analysis of a standard mixture at 5 μg kg^{-1} (each species) by SS-ID HPLC-ICP-MS.

3.2. Optimization of the HPLC separation

The separation of Cr(III)-EDTA and Cr(III)-DPCO complexes was carried out by using anion-exchange HPLC. It is known that pH affects the surface charge of the stationary phase as well the speciation of a trace metal hence controlling the HPLC separation [46,47]; therefore, the main parameters affecting the separation, such as the pH and the composition of the mobile phase and the temperature were optimized.

The pH of the mobile phase consisting of a solution of HNO_3 was optimized in the range from 0.2 (0.6 mol L^{-1} HNO_3) up to 4.5 (0.01 mmol L^{-1} HNO_3) by the analysis of a standard mixture of Cr(III) and Cr (VI) at 5 μg kg^{-1}. As can be seen in Fig. 4, the optimum baseline separation was achieved at pH = 2. It is worth to note that at pH<2, a single peak corresponding to the Cr(III)-EDTA complex was obtained, whereas at pH>2, a single peak corresponding to Cr(III)-DPCO complex was also observed (not shown here), which confirms the importance of the pH in optimum separation of Cr(III) complexes (with EDTA and DPCO) by anion exchange HPLC.

To prevent the decomplexation of Cr(III)-EDTA species during the HPLC separation due to the difference between the optimum complexation pH (4) and that of the HPLC separation (2), EDTA was also added to the mobile phase, as it was recommended elsewhere [48–54]. EDTA concentration in the mobile phase was optimized in the range from 0 to 0.60 mmol L^{-1} and the most efficient separation ensuring quantitative complexation of Cr(III) with EDTA was obtained at 0.30 mmol L^{-1} (recovery of 105% and 97% for Cr(III)-EDTA and Cr(III)-DPCO,

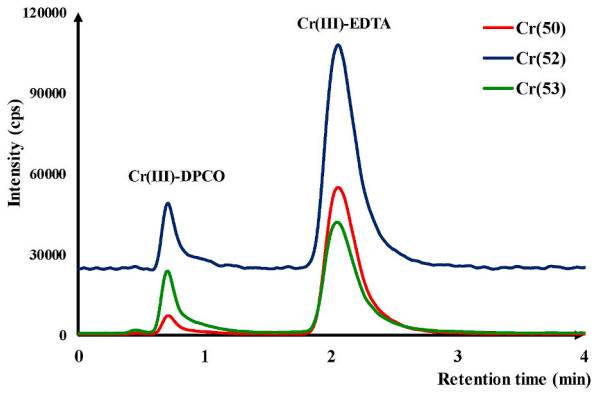

Fig. 4. Chromatogram obtained by the analysis of a standard mixture of Cr(III) (Cr(III)-EDTA complex) and Cr(VI) (Cr(III)-DPCO complex) at 5 μg kg^{-1} (each species) by SS-ID HPLC-ICP-MS.

respectively).

Further optimization of the HPLC separation efficiency was carried out by addition of methanol in the mobile phase at 1.0, 2.5 and 5% (v/v), respectively. The concentration of MeOH at 2.5% assured the best separation, with a resolution factor of ≥1.5.

Overall, the optimum HPLC mobile phase consisted of a mixture of HNO_3 (10 mmol L^{-1}), EDTA (0.30 mmol L^{-1}) and methanol (2.5%, v/v) at pH = 2.

Finally, the separation temperature was optimized in the range between 20 and 50 °C (20, 25, 30, 35, 40, 45 and 50 °C). The optimum resolution factor (≥1.5) was obtained at 30 °C, which was chosen for the rest of the experiments.

Fig. 4 shows a chromatogram obtained for the analysis of a standard mixture of Cr(III) and Cr(VI) at 5 μg kg^{-1} by SS-ID HPLC-ICP-MS under optimized conditions.

3.3. Optimization of species specific isotope dilution

To carry out SS-ID, the samples were spiked with known amounts of ^{50}Cr(III) and ^{53}Cr(VI) spikes to achieve isotope ratios ($^{50}Cr/^{52}Cr$ and $^{53}Cr/^{52}Cr$) in the blend samples ~1.0. For this purpose, the mass of each spike solution added to each sample was calculated based on Eq. (6) (example provided here for Cr(III) spike solely).

$$M_S^{50} = \frac{\left(C^{III} \times m_x\right)/F^{50/52}}{C_S^{50}} \quad (6)$$

where:

M_S^{50}, mass of ^{50}Cr(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 ^{50}Cr(III) spike and the natural abundance of ^{52}Cr(III)
C_S^{50}, concentration of the ^{50}Cr(III) spike solution (μg kg^{-1})

The equilibration of analytes and the corresponding spikes in the sample matrix is a critical step in ID-MS. In this work, the equilibration was achieved by manually shaking the blend for 10 min and then leaving it for 15 min to reach equilibrium. It is worth to note that after the isotopic equilibration is achieved, subsequent losses of sample or incomplete recoveries during the analytical procedure will not affect the final results [20].

SS-ID requires the determination of two isotope ratios for each species, namely ^{50}Cr(III)/^{52}Cr(III), ^{53}Cr(III)/^{52}Cr(III), ^{50}Cr(VI)/^{52}Cr(VI), and ^{53}Cr(VI)/^{52}Cr(VI), respectively. The concentration of each species as well as the species interconversion factors (α and β) are determined by means of the mathematical model provided in the Supplementary data [18]. The measured isotope ratios are also corrected with the mass bias factor (k) being determined during the analytical session for both $^{50}Cr/^{52}Cr$ and $^{53}Cr/^{52}Cr$ ratios using Eq. (7) [18].

$$k = R_t/R_m \quad (7)$$

where R_t, R_m, theoretical (or certified) and measured isotope ratio for a given couple of isotopes.

3.4. Method validation by means of the accuracy profile approach

In this study, the first three levels of the validity domain consisted of standard solutions of Cr(III) and Cr(VI) at levels corresponding to $^1/_2$ × LOQ, LOQ and 2 × LOQ, respectively; the other 3 levels corresponded to spiked samples (no CRM is currently available).

LOQs was initially estimated based on the IUPAC criterion (10 × σ_0), where σ_0 is the standard deviation determined from the measurements of 21 blank samples under repeatability conditions. The LOQs were 0.030 μg L^{-1} and 0.049 μg L^{-1} for Cr(III) and Cr(VI), respectively.

For constructing the accuracy profile, six measurement series were carried out in duplicate on (six) different days over a timespan of two months. The probability β was set to 85%, which means that the risk of future expected results falling outside these limits is below 15%. The acceptance limits (λ) were set to ± 30%, which corresponds to intermediate precision coefficient of variation estimated at ≅ 10% for k = 3 (p = 99%). The accuracy profiles obtained for Cr(III) and Cr(VI) determination by SS-ID HPLC-ICP-MS are provided in Fig. 5a and b.

As can be seen in Fig. 5a, β-expectation tolerance interval for Cr(III) was comprised within the acceptability limits for the whole validity domain assessed here, which corresponds to levels between 0.013 and 4.670 μg kg^{-1}.

For Cr(VI) determination, β-expectation tolerance interval were comprised within the acceptability limits except for the first level (Fig. 5b); this corresponds to a validity domain between 0.086 and 0.817 μg L^{-1}.

The intermediate precision (S_R) is calculated based on the accuracy profile by taking into account the intra- and inter-series variability (Eq. (8)) [55].

$$S_R = \sqrt{S_r^2 + S_B^2} \quad (8)$$

where S_r, intra-series standard deviation; S_B, inter-series standard deviation.

The main analytical parameters characteristics are summarized in Table 2. As can be seen, the trueness, expressed here in terms of bias (%) was in all cases < 0.1%, hence showing the excellent capability of the method for simultaneous quantification of Cr(III) and Cr(VI).

The repeatability (expressed in terms of relative standard deviation (CV_r) for both domains (<2 × LOQ and ≥2 × LOQ) for Cr(III) was comprised between 7 and 12% for both species and all matrices, apart from the steak beef for which considerably better repeatability (CV_r = 2.9%) was obtained. The intermediate precision (CV_R) was slightly higher, being generally between 7 and 14% for both species except for semi-skimmed milk in the domain <2 × LOQ, for which CV_R was ≅ 26%.

Whereas for CV_r no distinct trend is observed depending on analyte and/or matrix, as can also be seen from Table 2, CV_R was slightly higher for the Cr(VI) determination compared with Cr(III); actually, the mean

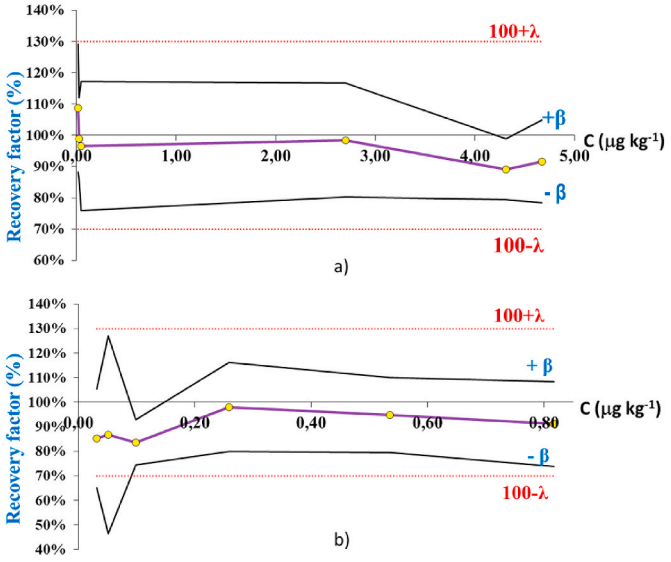

Fig. 5. Accuracy profile for Cr(III) (a) and Cr(VI) (b) speciation analysis by SS-ID HPLC-ICP-MS.

CV_r for Cr(III) determination taking into account all matrices was ≅ 11% for levels <2 × LOQ while it was ≅ 9% for levels ≥ 2 × LOQ. In turn, the mean CV_R for Cr(VI) determination was 15% (levels <2 × LOQ) and 11%, respectively (levels ≥ 2 × LOQ).

The accuracy profile approach allows also to obtain an operationally defined LOQ, which corresponds to the intersection of the acceptability (λ) and the tolerance limits (β). LOD is calculated as 3/10 × LOQ based on the LOQ assessed based on the accuracy profile [40]. As can be seen in Table 2, excellent LOQ were obtained for both species determination, being less than 50 ng kg^{-1}. It is worth to note that the LOQ for Cr(VI) determination is ≅ 3 times higher compared to that of Cr(III) determination, most probably due to lower sensitivity and lower signal/noise ratio for Cr(VI) determination by SS-ID HPLC-ICP-MS.

As it was mentioned above, SS-ID is the only analytical approach capable to assess and correct mathematically for the species interconversion by means of the model represented by Eqs. (S1-S4). Table 3 shows the interconversion factors (α and β) obtained during the analysis of a standard solution at LOQ level as well as for baby/semi-skimmed milk and beef samples. Standard solutions at 1/2 × LOQ and 2 × LOQ were also analysed for the construction of the accuracy profile but for simplicity, α and β for these samples are not reported here (they are highly comparable with those obtained for the analysis of the standard solution at LOQ level). As can be seen in Table 3, mutual interconversion of Cr(III) and Cr(VI) took place when analysing the standard solution, with higher rate of conversion (reduction) of Cr(VI) to Cr(III) (55%) compared to the oxidation of Cr(III) to Cr(VI) (25%). On the other hand, when analysing food samples, complete reduction of Cr(VI) to Cr(III) occurred in all cases. This confirms the reducing features of food samples analysed here and hence the low probability to quantify Cr(VI) in such samples. It is also worth to underline that despite the reduction of Cr(VI) to Cr(III) during the analytical process, SS-ID approach developed in this study is capable to correct mathematically for it hence providing excellent accuracy (see Table 2).

3.5. Speciation analysis of Cr(III) and Cr(VI) in food samples by SS-ID HPLC-ICP-MS

The method developed in this study was applied to the analysis of a selection of food samples including steak beef, baby and semi-skimmed milk. Cr_{tot} was also determined in these samples and both the speciation and Cr_{tot} data are presented in Table 4. A comparison of chromatograms obtained for these three types of samples is shown in Fig. 6.

As can be seen from Table 4, Cr(VI) levels were <LOQ in all samples analysed here, whereas Cr(III) levels ranged between 2.3 and 4.7 μg kg^{-1}. Additionally, Cr(III) levels are compatibles with Cr_{tot}, which strongly supports the general assumption that Cr in food is found exclusively as Cr(III), as stated by EFSA and also found in previous studies [7,8,11,16,56].

In order to assess the method applicability for Cr(VI) speciation analysis in these samples, spiking experiments with various levels of Cr (VI) (0.25 (baby milk), 0.5 (steak beef) and 1.0 μg kg^{-1} (semi-skimmed milk) were also carried out (Table 5).

Table 3
Average values ± standard deviation (n = 12[a]) obtained for the inter-conversion factors (α and β) for Cr(III) and Cr(VI) speciation analysis in synthetic (standard solutions) samples, baby milk, semi-skimmed milk and steak beef by SS-ID-HPLC-ICP-MS during the method validation by means of the accuracy profile.

Sample	Level[b] (μg kg^{-1})	α (%)	β (%)
Standard solution at LOQ level[c]	[b]	25 ± 18	55 ± 17
Baby milk	2.70	0	111 ± 10
Semi-skimmed milk	4.31	0	112 ± 9
Steak beef	4.67	0	110 ± 6

[a] Analyses carried out in duplicate in 6 different days during 2 months.
[b] Genuine level for Cr(III) and spiking level for Cr(VI).
[c] 0.030 μg L^{-1} and 0.049 μg L^{-1} for Cr(III) and Cr(VI), respectively.

Table 4
Levels of Cr_{tot} (by ICP-MS) and chromium speciation analysis (SS-ID HPLC-ICP-MS) in baby/semi-skimmed milk and steak beef food samples.

Sample	mass fraction ± U[a] (μg kg^{-1})		
	Cr_{tot}[b]	Cr(III)[c]	Cr(VI)[c]
Baby milk	2.30 ± 0.33	2.64 ± 0.26	< LOQ (0.049)
Semi-skimmed milk	4.40 ± 0.62	4.45 ± 0.27	< LOQ (0.049)
Steak beef	4.65 ± 0.66	4.73 ± 0.34	< LOQ (0.049)

[a] Expanded uncertainty (k = 2).
[b] n = 2.
[c] n = 5.

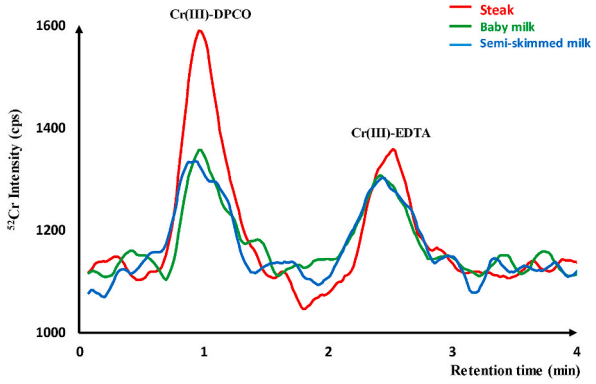

Fig. 6. Chromatograms obtained for the analysis of baby milk, semi-skimmed milk and steak beef samples by SS-ID HPLC-ICP-MS.

Good recoveries were obtained for the determination of Cr(VI) in the spiked samples, whereas Cr(III) levels are highly comparable with those obtained for the analysis of un-spiked samples as it was found also for the analysis of the same genuine samples (see Table 4). Additionally, excellent mass balance was obtained for the sum of Cr(III) and Cr(VI) levels measured by SS-ID with the levels of Cr_{tot} determined by ICP-MS, which confirms the trueness of the SS-ID HPLC-ICP-MS method for the speciation analysis of Cr(III) and Cr(VI) in these food samples.

Table 5
Levels of Cr_{tot} (by ICP-MS) and chromium speciation analysis (SS-ID-HPLC-ICP-MS) in baby/semi-skimmed milk and steak beef samples spiked with various levels of Cr(VI).[a]

Sample	Level ± U[b] (μg kg^{-1}, n = 5)					
	Cr_{tot}[c]	Cr(III)	Cr(VI)	R_Cr (VI)[d] (%)	Cr (III)+ Cr(VI)[e]	$Cr_{tot\_speciation}$/ Cr_{tot_ICP-MS} (%)
Baby milk	2.73 ± 0.38	2.70 ± 0.27	0.26 ± 0.03	104 ± 10	2.96	108
Semi-skimmed milk	5.20 ± 0.73	4.41 ± 0.27	0.82 ± 0.09	82 ± 8	5.23	101
Steak beef	5.02 ± 0.71	4.67 ± 0.34	0.54 ± 0.05	107 ± 15	5.21	104

[a] Cr(VI) spiking levels: baby milk 0.25 μg kg^{-1}; steak beef 0.5 μg kg^{-1}; semi-skimmed milk: 1.0 μg kg-1.
[b] Expanded uncertainty (k = 2).
[c] n = 2.
[d] Recovery factor for Cr(VI) determination after spiking.
[e] Sum of Cr(III) and Cr(VI) levels obtained by speciation analysis.

4. Conclusion

The present study focused on the development and validation of a novel analytical methodology for single run chromium speciation analysis in foodstuffs by sequential complexation of Cr(III) and Cr(VI) and double spike SS-ID in combination with HPLC-ICP-MS. This analytical approach allows the speciation analysis of Cr(III) and Cr(VI) at ultra-trace levels in a single run, and simultaneously addresses and corrects for any species interconversion during the analytical process, particularly the extraction step. Additionally, it provides accurate, precise and reproducible measurements at very low limits of quantification (part per trillion level).

The method was successfully applied to the speciation analysis of Cr (III) and Cr(VI) in various foodstuffs, such as baby milk, semi-skimmed milk and beef. Cr(VI) was not found in any of the samples analysed here while Cr(III) concentration ranged between 3 and 5 μg kg^{-1}. Additionally, the species mass balance (sum of levels of Cr(III) and Cr(VI)) compared to total chromium determined by ICP-MS in the same samples was excellent (101–108%). This supports the EFSA statement related to the absence of Cr(VI) in foods and that Cr in this type of samples is found solely as Cr(III) species. This method forms the basis for future studies on other types of food and can also be used to study potential species interconversion during food preparation/processing.

Credit author statement

Marina Saraiva: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Roles/Writing – original draft, Writing - review & editing. Rachida Chekri: Data curation, Methodology, Validation, Roles/Writing – original draft. Axelle Leufroy: Data curation, Methodology, Supervision, Validation. Thierry Guérin: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Roles/Writing – original draft, Writing - review & editing. Jens J. Sloth: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Roles/Writing – original draft, Writing - review & editing. Petru Jitaru: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Roles/Writing – original draft, Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.121538.

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SUPPLEMENTARY DATA

Equations of the double spike species specific isotope dilution approach

$$R_{50/52}^{III} = \frac{(A_X^{50}\, C_X^{III}\, m_X + 50A_S^{III}\, C_S^{III}\, m_S^{III})(1-\alpha)+(A_X^{50}\, C_X^{VI}\, m_X + 50A_S^{VI}\, C_S^{VI}\, m_S^{VI})\beta}{(A_X^{52}\, C_X^{III}\, m_X + 52A_S^{III}\, C_S^{III}\, m_S^{III})(1-\alpha)+(A_X^{52}\, C_X^{VI}\, m_X + 52A_S^{VI}\, C_S^{VI}\, m_S^{VI})\beta} \quad \text{(S1)}$$

$$R_{53/52}^{III} = \frac{(A_X^{53}\, C_X^{III}\, m_X + 53A_S^{III}\, C_S^{III}\, m_S^{III})(1-\alpha)+(A_X^{53}\, C_X^{VI}\, m_X + 53A_S^{VI}\, C_S^{VI}\, m_S^{VI})\beta}{(A_X^{52}\, C_X^{III}\, m_X + 52A_S^{III}\, C_S^{III}\, m_S^{III})(1-\alpha)+(A_X^{52}\, C_X^{VI}\, m_X + 52A_S^{VI}\, C_S^{VI}\, m_S^{VI})\beta} \quad \text{(S2)}$$

$$R_{50/52}^{VI} = \frac{(A_X^{50}\, C_X^{III}\, m_X + 50A_S^{III}\, C_S^{III}\, m_S^{III})\,\alpha+(A_X^{50}\, C_X^{VI}\, m_X + 50A_S^{VI}\, C_S^{VI}\, m_S^{VI})\,(1-\beta)}{(A_X^{52}\, C_X^{III}\, m_X + 52A_S^{III}\, C_S^{III}\, m_S^{III})\,\alpha+(A_X^{52}\, C_X^{VI}\, m_X + 52A_S^{VI}\, C_S^{VI}\, m_S^{VI})\,(1-\beta)} \quad \text{(S3)}$$

$$R_{53/52}^{VI} = \frac{(A_X^{53}\, C_X^{III}\, m_X + 53A_S^{III}\, C_S^{III}\, m_S^{III})\,\alpha+(A_X^{53}\, C_X^{VI}\, m_X + 53A_S^{VI}\, C_S^{VI}\, m_S^{VI})\,(1-\beta)}{(A_X^{52}\, C_X^{III}\, m_X + 52A_S^{III}\, C_S^{III}\, m_S^{III})\,\alpha+(A_X^{52}\, C_X^{VI}\, m_X + 52A_S^{VI}\, C_S^{VI}\, m_S^{VI})\,(1-\beta)} \quad \text{(S4)}$$

where:

$R_{50/52}^{III}$, $R_{53/52}^{III}$, $R_{50/52}^{VI}$,$R_{53/52}^{VI}$, ^{50}Cr(III)/^{52}Cr(III), ^{53}Cr(III)/^{52}Cr(III), ^{50}Cr(VI)/^{52}Cr(VI) and ^{53}Cr(VI)/^{52}Cr(VI) ratios measured in the blend samples (corresponding to Cr(III)-EDTA and Cr(III)-DPCO peaks, respectively)

C_X^{III}, concentration of Cr(III) in the unknown sample (µmole g^{-1})

C_X^{VI}, concentration of Cr(VI) in the unknown sample (µmole g^{-1})

C_S^{III}, concentration of Cr(III) in the ^{50}Cr(III) spike solution (µmole g^{-1})

A_X^{50}, A_X^{52}, A_X^{53}, natural abundance (%) of ^{50}Cr, ^{52}Cr, and ^{53}Cr in the sample

$50A_S^{III}$, $52A_S^{III}$, $53A_S^{III}$, abundance (%) of ^{50}Cr, ^{52}Cr and ^{53}Cr in the Cr(III) spike

$50A_S^{VI}$, $52A_S^{VI}$, $53A_S^{VI}$, abundance (%) of ^{50}Cr, ^{52}Cr and ^{53}Cr in the Cr(VI) spike

m_X, mass of the sample (g)

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

m_S^{VI}, mass of the ^{53}Cr(VI) spike solution (g)

α, fraction of Cr(III) oxidized to Cr(VI) after spiking

β, fraction of Cr(VI) reduced to Cr(III) after spiking

Based on the SS-ID model illustrated by the Eqs. (S1)-(S4), the concentration of the analytes (C_X^{III} and C_X^{VI}) as well as the corresponding interconversion factors (α and β), can be calculated by means of the Eqs (S5)-(S12) [18].

$$C_X^{III} = \frac{R_3\,B_5 + R_1\,R_4\,B_3 - R_1\,B_5 + R_2\,B_1 - R_4\,B_1 - R_3\,R_2\,B_3}{R_3\,R_2\,A_2 - R_3\,A_3 + R_1\,A_3 + A_1 R_4 - R_1\,A_2\,R_4 - R_2\,A_1} \quad \text{(S5)}$$

$$C_X^{VI} = \frac{R_3\,B_6 + R_1\,R_4\,B_4 - R_4\,B_2 + R_2\,B_2 - R_1\,B_6 - R_3\,R_2\,B_4}{R_3\,R_2\,A_2 - R_3\,A_3 + R_1\,A_3 + A_1 R_4 - R_1\,A_2\,R_4 - R_2\,A_1} \quad \text{(S6)}$$

$$\alpha = (B_2\,R_4\,A_2 - B_2\,A_3 + R_3\,B_4\,A_3 - B_6\,R_3\,A_2 + B_6\,A_1 - R_4\,B_4\,A_1)(B_1\,A_3 - B_1\,R_2\,A_2 - R_1\,B_3\,A_3 - B_5\,A_1 + $$
$$+B_5\,R_1\,A_2 + R_2\,B_3\,A_1 - R_1\,B_4\,A_3 + B_2\,A_3 - B_2\,R_2\,A_2 + R_2\,B_4\,A_1 - B_6\,A_1 + B_6\,R_1\,A_2)/K \quad \text{(S7)}$$

$$\beta = (B_1\,R_2\,A_2 - B_1\,A_3 + R_1\,B_3\,A_3 + B_5\,A_1 - B_5\,R_1\,A_2 - R_2\,B_3\,A_1)(B_1\,A_3 - R_3\,B_3\,A_3 - B_1\,R_4\,A_2 + R_4\,B_3\,A_1 + $$
$$+B_5\,R_3\,A_2 - B_5\,A_1 - B_2\,R_4\,A_2 + B_2\,A_3 - R_3\,B_4\,A_3 + B_6\,R_3\,A_2 - B_6\,A_1 + R_4\,B_4\,A_1)/K \quad \text{(S8)}$$

where:

R_1, R_2, R_3, R_4, isotope ratios: $R_{50/52}^{III}$; $R_{53/52}^{III}$; $R_{50/52}^{VI}$ and $R_{53/52}^{VI}$, respectively

A_1, A_2, A_3, natural abundance (%) for each isotope (50, 52 and 53) in the sample multiplied by the corresponding sample mass: $A_1 = 50A_X \times m_X$; $A_2 = 52A_X \times m_X$; $A_3 = 53A_X \times m_X$

B_1, B_3, B_5, isotopic abundances (%) for each isotope (50, 52 and 53) in the Cr(III) spike multiplied by N (given by Eq. S9): $B_1 = 50A_S^{III} \times N_S^{III}$; $B_3 = 52A_S^{III} \times N_S^{III}$; $B_5 = 53A_S^{III} \times N_S^{III}$

B_2, B_4, B_6, isotopic abundances (%) for each isotope (50, 52 and 53) in the Cr(VI) spike multiplied by N (given by Eq. S9): $B_2 = 50A_S^{VI} \times N_S^{VI}$; $B_4 = 52A_S^{VI} \times N_S^{VI}$; $B_6 = 53A_S^{VI} \times N_S^{VI}$

$$N_S^x = m_S^x \times C_S^x \quad \text{(S9)}$$

where:

x, Cr(III) or Cr(VI)

m_S^{III}, mass of the Cr(III) spike solution (g)

C_S^{III}, concentration of the Cr(III) spike solution (μg kg^{-1})

The K factor in Eqs. (S7)-(S8) is defined as follows (Eq. S10):

$$K = d_1(A_1 - R_1\,A_2)(A_3 - R_2\,A_2)(A_1 - R_3\,A_2)(A_3 - R_4\,A_2) \quad \text{(S10)}$$

where d_1 is given by Eq. (S11)

$$d_1 = (K_{11} - K_{21})(K_{42} - K_{32})(K_{22} - K_{12})(K_{31} - K_{41}) \quad \text{(S11)}$$

and K_{ij} factors by the equations (S12).

$$K_{11} = \frac{B_1 - R_1 B_3}{A_1 - R_1 A_2} \qquad K_{12} = \frac{B_2 - R_1 B_4}{A_1 - R_1 A_2}$$

$$K_{21} = \frac{B_5 - R_2 B_3}{A_3 - R_2 A_2} \qquad K_{22} = \frac{B_6 - R_2 B_4}{A_3 - R_2 A_2} \quad \text{(S12)}$$

$$K_{31} = \frac{B_1 - R_3 B_3}{A_1 - R_3 A_2} \qquad K_{32} = \frac{B_2 - R_3 B_4}{A_1 - R_3 A_2}$$

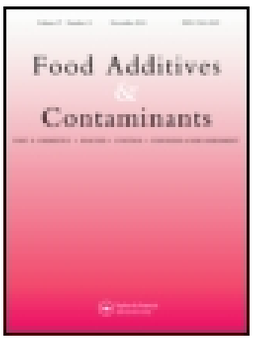
$$K_{41} = \frac{B_5 - R_4 B_3}{A_3 - R_4 A_2} \qquad K_{42} = \frac{B_6 - R_4 B_4}{A_3 - R_4 A_2}$$

Article II

Marina Saraiva, Rachida Chekri, Thierry Guérin, Jens J. Sloth and Petru Jitaru

Chromium speciation analysis in raw and cooked milk and meat samples by species specific isotope dilution and HPLC-ICP-MS

Food Additives & Contaminants: Part A (2021)



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Chromium speciation analysis in raw and cooked milk and meat samples by species-specific isotope dilution and HPLC-ICP-MS

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ABSTRACT

This study aimed at the assessment of the impact of various culinary processes on the fate of chromium (Cr) species (Cr(III) and Cr(VI)) in infant formula milk, semi-skimmed milk and bovine meat samples. The cooking procedures were boiling at 70°C/100°C (milk samples) and frying without and with oil (95°C and 120°C) (bovine meat). The levels of Cr(III) and Cr(VI) in raw and cooked samples were determined by high-performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) using double spike species-specific-isotope dilution (SS-ID). The species were extracted by sequential complexation of Cr(III) with ethylenediaminetetraacetic acid and of Cr(VI) with 1,5-diphenylcarbazide in the same analytical run by heating at 70°C for 50 min. Anion exchange chromatography using a Dionex IonPac™ AG7 column and a mobile phase consisting of 10 mM HNO_3, 2.5% MeOH and 30 mM EDTA at pH 2 was employed for species separation. The quantification limits were 0.013 and 0.049 µg kg^{-1} for Cr(III) and Cr(VI), respectively. ANOVA test used to compare the mean Cr species concentrations showed no significant differences between raw and cooked samples. The results obtained in the present study show that oxidation of Cr(III) to Cr(VI) does not occur during thermal cooking of milk and bovine meat samples. A selection of 10 samples of each type were analysed in terms of total Cr (Cr_{total}) as well as speciation (Cr(III) and Cr(VI)). Cr(VI) was not quantified in any of these samples, whereas Cr(III) levels ranged from 0.22 (infant formula milk) up to 80 µg kg^{-1} (chorizo sausage). Additionally, Cr(III) and Cr_{total} levels were comparable hence demonstrating that in the samples analysed in this study, Cr is found exclusively as Cr(III) species.

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Chromium speciation; HPLC-ICP-MS; species-specific-isotope dilution; milk/meat samples; cooking

Introduction

Chromium (Cr) is probably the most controversial of the transition elements in food chemistry due to the opposing toxicities of its main species, namely Cr(III) and Cr(VI) (Cronin 2004; IARC 2012). Whereas Cr(VI) has been known for its mutagenic and carcinogenic effects, Cr(III) was considered essential to humans due to the maintenance of the glucose tolerance factor, and lipid and protein metabolism. However, the European Food Safety Authority (EFSA) stated recently that there is no convincing evidence of beneficial effects of Cr(III) for healthy people (EFSA 2014b, 2017). Hence, speciation analysis of both Cr species in foods and drinking water is important for a better comprehension of global Cr status and its impact on human health (Arnich et al. 2012; EFSA 2014a; Yu and He 2017).

Several studies so far have reported that Cr(VI) is absent in foodstuffs due to the inherent reduction potential of organic matter, which leads to conversion of Cr(VI) to Cr(III) (Novotnik et al. 2013, 2015; EFSA 2014a; Vacchina et al. 2015; Hernandez et al. 2017, 2018; Milačič and Ščančar 2020). On the other hand, other studies have reported the presence of Cr(VI) in various raw foodstuffs such as milk, meat, bread and cereals, tea, mushrooms and rice as well as in cooked food (Figueiredo et al. 2007; Ambushe et al. 2009; Soares et al. 2010; Mandiwana et al. 2011; Pyrzynska 2017; Chen et al. 2020; Shittu et al. 2020) and notably in toasted bread (Mathebula et al. 2017). These findings have led to opposition to the results presented and arguments against the proposed presence of Cr(VI) in food (Pyrzynska 2016; Milačič and Ščančar 2018). In a recent paper, the presence of Cr(VI) in foodstuffs was proposed to be due to analytical artefacts caused by

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species interconversion during the analytical process (Milačič and Ščančar 2020).

The only analytical approach capable to accurately assess Cr(III) and Cr(VI) levels and their corresponding interconversion is based on species-specific (SS) isotope dilution (ID)-inductively coupled plasma mass spectrometry (SS-ID-ICP-MS) (Milačič and Ščančar 2020; Saraiva et al. forthcoming). This approach is also suitable for the accurate assessment of Cr species fate during preparation processes whereas conventional analytical approaches (i.e. without SS-ID) are not able to correct for the simultaneous species interconversion. SS-ID is capable of providing information in terms of species concentration and their interconversion in a single analytical run (Kingston et al. 1998; Tirez et al. 2003; Rodriguez-Gonzalez et al. 2005; Rodriguez-Gonzalez and Alonso 2019). The rather complex nature of the methodology is most likely the reason for the apparent lack of studies dealing with the assessment of the fate of Cr species during preparation of foodstuffs. Most studies so far have dealt only with the assessment of the fate of total Cr (Cr_{total}) in various samples including, e.g. fish, rice and pumpkin, vegetables, following various preparation procedures (Wang and Farid 2015; Rasmussen et al. 2017; Barbosa et al. 2018; Fidelis et al. 2018; Rittirong and Saenboonruang 2018).

The aim of the present study was to assess the levels of Cr(III) and Cr(VI) in infant formula milk, semi-skimmed milk and bovine meat as well as their potential interconversion in this type of samples after various preparation procedures, by using a novel analytical approach based on a recently developed SS-ID-HPLC-ICP-MS method (Saraiva et al. forthcoming). To our knowledge, this is the first study dealing with the systematic assessment of the fate of Cr species (III and VI) in these types of samples after being cooked.

Materials and methods

Instrumentation

Sample preparation, including the species complexation and extraction, was carried out by heating using a heating block system (DigiPREP MS, SCP Science, Courtaboeuf, France). The separation of Cr(III) and Cr(VI) was carried out by HPLC using a Dionex ICS-5000$^+$ system (ThermoFisher Scientific, Courtaboeuf, France) equipped with a Dionex IonPac™ AG7 column (2 mm internal diameter, id x 50 mm length x 10 µm particles diameter) (ThermoFisher Scientific).

Sonication water bath (Model 86483, Fischer BioBlock, Rungis, France) was used for the preparation of the solutions used for the complexation of Cr(III) and Cr(VI), ethylene diamine tetraacetic acid (EDTA) and 1,5-diphenylcarbazide (DPC).

An ICP-MS iCAP Q (ThermoFisher Scientific) equipped with a PFA standard nebuliser and a cyclonic quartz spray chamber was used for Cr species detection after their on-line HPLC separation. Kinetic energy discrimination (KED) in high sensitivity mode using helium as a collision gas was employed throughout the study. ICP-MS was directly connected with the outlet of the HPLC column via PEEK tubing (80 cm, 0.18 mm i.d).

ICP-MS measurement conditions were optimised daily by short-term stability tests run in standard and KED mode. Data were processed using Thermo Fisher Scientific Qtegra software.

For Cr_{total} determination, an ICP-MS Agilent 7700 (Agilent Technologies, Courtaboeuf, France) equipped with an Octopole Reaction System and a CETAC ASX-500 auto-sampler (CETAC Technologies, Omaha, NE, USA) was used. The optimum operating conditions of the HPLC-ICP-MS (iCAP-Q) coupling and the Agilent 7700 ICP-MS were reported in a previous study (Saraiva et al. forthcoming).

Materials, reagents and standard solutions

All solutions including the mobile phase were prepared with ultrapure water (18.2 MΩ cm, Millipore Milli-Q™, Merck Millipore, Saint Quentin in Yvelines, France).

Standard stock solutions of Cr(III) and Cr(VI) (Inorganic Ventures, Christiansburg, USA), both at 1000 mg L^{-1}, were used to prepare the calibration standards for the speciation analysis, while isotopically enriched solutions (spikes) of ^{50}Cr(III) and ^{53}Cr(VI) (100 µg mL^{-1}) (ISC Science, Oviedo, Spain) were used for SS-ID. The abundance of ^{50}Cr in the Cr(III) spike was 97.4% while that of ^{53}Cr in the Cr(VI) spike was 92.4% (as provided by the manufacturer's certificate). The natural abundances of the various Cr isotopes (50, 52 and 53) used for SS-ID calculations are those reported by the Internal

Union of Pure ad applied chemistry (IUPAC 2002; CIAAW 2019). The intermediate solutions were prepared daily by diluting the stock standard solutions with ultrapure water.

For Cr_{total} determination by ICP-MS, stock standard solutions of Cr and Sc (used as an internal standard) of 1000 mg L^{-1} (SCP Science, Quebec, Canada) were used for establishing a calibration curve. Nitric acid (67–69% m/m, NORMATOM®, VWR, Fontenay-sous-Bois, France) was used for preparation of the HPLC mobile phase.

A solution of ethylene diamine tetraacetic acid (EDTA, 99.995%, Sigma-Aldrich) was used to chelate Cr(III) whereas 1,5-diphenylcarbazide (DPC, Sigma-Aldrich) was used for the derivatisation of Cr(VI) (Saraiva et al. forthcoming).

The standard solutions used for external calibration were prepared by mixing the required amount of intermediate solution with 16 mL EDTA solution, 200 µL of DPC solution and the necessary amount of ultrapure water to reach a final volume of 20 mL. The EDTA solution used for the complexation of Cr(III) was prepared by dissolving 0.45 g of reagent into 500 mL of ultrapure water using a sonication water bath at maximum power (140%) for 15 min at 25°C. The DPC solution was prepared by dissolving 50 mg of the reagent in 10 mL of methanol (≥99.8%, AnalaR NORMAPUR® ACS, VWR, France). Then, 50 mL of ultrapure water containing 2.8 mL of H_2SO_4 (95–97%, Merck KGaA, France) was added (by continuously stirring). The final DPC solution was made up to 100 mL with ultra-pure water in a volumetric flask (Sanchez-Hachair and Hofmann 2018) and was stable for 1 month at room temperature in the dark.

All stock and intermediate standard solutions were stored in the refrigerator at 4–5°C for a maximum of 1 month.

Analytical procedures

Samples and sample preparation

The samples analysed in this study were bovine meat, infant formula and semi-skimmed milk purchased in retail shops in Maisons-Alfort (France).

Bovine meats (≅1 kg) were homogenised using a stainless steel free automatic mixer (Retsch Grindomix GM 200, Haan – Germany) with titanium blades. The liquid samples (infant formula milk and semi-skimmed milk) were homogenised for 4 h using a rotary shaker (Heidolph reax 2, Merck KGaA, Darmstadt, Germany). For both samples, after homogenisation, one part was kept in the refrigerator (≈ 5°C) while Cr(VI) was added to another part to further test the behaviour of Cr species by culinary treatment. In the latter case, the baby milk (500 mL) was spiked with 0.25 ng mL^{-1} Cr(VI), the semi-skimmed milk (500 mL) with 1.0 ng mL^{-1} of Cr(VI) and the steak beef (200 g) with 0.5 ng g^{-1} of Cr(VI). The remaining bulk samples were kept as a backup and stored at −18°C. The spike and standard solutions were stored in the dark at ≈ 5°C.

Each sample was subjected to two different cooking procedures. Temperature measurement during the cooking procedures was done with a properly cleaned probe. The internal temperature in each sample was recorded and described below. The milk samples were heated using a microwave at 70°C for 2 min as well as on a hot plate for 5 min at 100°C. The temperatures used in this study were comparable with those used in other studies (Diaconescu et al. 2013; Mehdipour et al. 2018).

The bovine meat samples were fried for 5 min without and with sunflower oil (≈ 30 mL) at 120°C (oil frying) and 95°C (dry frying), respectively. Cr_{total} level in the sunflower oil is not known but its contribution is assumed to be negligible to that in the fried meat samples. The temperatures used for heating the bovine meat samples were similar to those normally used in daily life for preparation of this type of food. The temperatures employed for heating of the milk are considerably higher compared to those typically used in daily life; this option was chosen because if no Cr(III) to Cr(VI) interconversion occurred at these extreme conditions, the same would be the case at lower temperatures.

Each sample (milk and meat) was prepared in triplicate and each of the cooked sample was analysed in duplicate. The results in terms of Cr speciation were compared to those obtained for the same raw (uncooked) matrix. Due to the absence of

Cr(VI) in the foodstuffs analysed here, the raw samples were spiked with Cr(VI) at 0.25, 1.0 and 0.5 μg kg^{-1} for formula milk, semi-skimmed milk and bovine meat, respectively. After cooking, the samples were analysed on the same day.

Subsamples of 0.3 g of milk and bovine meat were thoroughly (manually) mixed for ≅10 min (to achieve isotope equilibrium) with the required amount of ^{50}Cr(III) and ^{53}Cr(VI) spikes directly in the 50 mL polypropylene tubes and then placed in the heating block system at 70°C. Then, 16 mL of EDTA solution was added to reach a final concentration of 2.4 mM and the mixture was heated for 25 min. The solution was subsequently cooled down to approximately 30°C (≅15 min) and then 200 μL of DPC solution was added to reach a final concentration of 0.02 mM. The mixture was heated again at 70°C for 25 min. The extract was cooled down to room temperature and then filtered using 0.45 μm polyvinylidene fluoride syringe filters (Merck Millipore, Cork, Ireland) prior to the analysis (conducted on the same day).

For this study, 10 different samples from each of the sample types: semi-skimmed milk, infant formula milk and meat/meat products were purchased at a regular supermarket. The samples were subjected to Cr(III)/Cr(VI) speciation analysis by SS-ID-HPLC-ICP-MS using the extraction procedure described above (the same used for the analysis of raw and cooked samples). They were also analysed for their Cr_{total} content based on the procedure described in the following section.

Determination of total Cr by ICP-MS

Cr_{total} levels in the samples (raw and cooked) were determined by Agilent 7700 ICP-MS after microwave-assisted acid digestion, as described in detail in a previous study (Saraiva et al. forthcoming). In brief, 0.5 g of bovine meat or 2.0 g of milk were directly weighed into 80 mL quartz vessels and then thoroughly mixed overnight with 3 mL of concentrated HNO_3. Then, 3.0 mL of ultra-pure water was added before the microwave treatment. The digests were quantitatively transferred to polypropylene tubes (50 mL) and then filled up with ultrapure water after the addition of the internal standard. The analysis was carried out by ICP-MS using the external calibration approach with the concentration 0, 1, 2.5, 5, 7.5 and 10 μg kg^{-1} (and Sc as internal standard).

Speciation analysis of Cr(III) and Cr(VI) by HPLC-ICP-MS using SS-ID

Speciation analysis of Cr(III) and Cr(VI) in raw and cooked samples was carried out using HPLC-ICP-MS and double spike species-specific ID (SS-ID-HPLC-ICP-MS) as described in detail in a previous study (Saraiva et al. forthcoming). Briefly, a Dionex IonPac™ AG7 column (2 mm internal diameter, id x 50 mm length ×10 μm particles diameter) was used for separation of the Cr species. An ICP-MS iCAP Q (ThermoFisher Scientific) equipped with a PFA standard nebuliser and a cyclonic quartz spray chamber was coupled to the HPLC system for Cr species detection. The instrumental setting conditions of the HPLC-ICP-MS (iCAP-Q ICP-MS) coupling were reported in a previous study (Saraiva et al. forthcoming).

The ID approach used in this study is based on the addition of a known amount of analyte having an altered isotopic profile (*spike*) and the measurement of the resulting isotope ratio of the blend sample. In species-specific ID approaches (SS-ID), the spikes must be the actual species having an isotopic profile different than the natural ones. If simultaneously quantification of both species is to be carried out by SS-ID in a single run, each species must have a different isotopic composition of the element of interest (Cr in this case). Doing so, double spike SS-ID allows not only for simultaneous quantification of each species, but also the assessment of their mutual interconversion by providing information on the corresponding interconversion factors.

In this study, the samples were spiked with a mix of ^{53}Cr(III) and ^{50}Cr(VI) spikes and four isotope ratios, namely ^{50}Cr(III)/^{52}Cr(III), ^{53}Cr(III)/^{52}Cr(III), ^{50}Cr(VI)/^{52}Cr(VI), and ^{53}Cr(VI)/^{52}Cr(VI), were determined. The concentration of each species as well as the interconversion factors were determined by using the equations reported in detail elsewhere (USEPA 2014; Saraiva et al. forthcoming).

More details regarding the optimisation of the sample preparation step, including species extraction, are provided in a previous study (Saraiva et al. forthcoming).

Statistical analysis

Analysis of variance (one-way ANOVA) was performed to test significant differences between Cr_{total}

and Cr species (III and VI) levels in raw and cooked milk and bovine meat samples. All analyses were carried out with six replicates for Cr(III) and Cr(VI) and in triplicate for Cr_{total}. F-test and Student t-test were performed at a significance level of 0.05, using Excel software.

Results and discussion

Assessment of total Cr in raw and cooked samples

So far, most studies related to the impact of cooking on Cr have focused mainly on Cr_{total} (Table 1). The general outcome of these studies is that the concentrations of Cr_{total} in cooked foods were slightly lower compared to the raw foods.

Cr_{total} results obtained in the present study are reported in Table 2. In general, Cr_{total} levels for all samples do not differ between the different cooking procedures. For milk samples, the Cr_{total} levels in raw, microwave and hot plate heated were 2.97 ± 0.15, 2.95 ± 0.13 and 2.92 ± 0.12 μg kg^{-1}, respectively. Similarly, for semi-skimmed milk, the Cr_{total} levels measured in raw, microwave and hot plate heated were of 5.22 ± 0.11, 5.20 ± 0.13 and 5.21 ± 0.10 μg kg^{-1}, respectively.

For bovine meat, the values in raw, fried without oil and fried with oil were 5.21 ± 0.12, 5.20 ± 0.09 and 5.25 ± 0.11 μg kg^{-1}, respectively.

An ANOVA test was used to compare the mean Cr_{total} levels between and within groups. For all three sample types, no significant differences in terms of Cr_{total} for the different sample treatments were observed. These findings are comparable with those reported in similar studies for fish, vegetables, pasta and pulses (Alberti-Fidanza et al. 2002; Perugini et al. 2014; Rasmussen et al. 2017; Mehdipour et al. 2018; Rittirong and Saenboonruang 2018), in which Cr_{total} levels also did not differ between untreated and treated samples. These results showed no significant differences related to Cr_{total} in the raw and two culinary treatments performed for food samples.

Assessment of Cr(III) in raw and cooked samples by SS-ID-HPLC-ICP-MS

The samples investigated in this study (semi-skimmed milk, infant formula milk and bovine meat) were previously analysed during the method validation (Saraiva et al. forthcoming) and for all samples, the level of Cr(VI) was < LOQ. In this study, the samples were cooked in the first instance without any addition of Cr(III) or Cr(VI) to study the fate of Cr(III).

The Cr(III) mass fraction measured in the raw and cooked samples, including the ratio (%) between the levels in cooked and raw samples, is reported in Table 3. A one-way ANOVA test on the milk and bovine meat samples did not show significant differences in Cr(III) levels between raw and cooked samples.

These findings confirm the stability of Cr(III) in these types of samples, even when subjected to cooking at relatively high temperatures (ratio $Cr(III)_{cooked}/Cr(III)_{raw}$ found for the three food samples studied were between 93% and 103%).

Assessment of Cr(III) and Cr(VI) fate during cooking of milk and bovine meat samples

Very few studies reported comparative Cr speciation analysis in raw and cooked food, and they have focused on bread, cereals and edible animal oils (Table 4). This is the first study that addresses the possible interconversions of Cr species with the effect of temperature treatment in milk and bovine meat samples. A similar study to this was carried out by Mathebula et al. (2017) using samples of bread and breakfast cereals, in which the results showed a significant increase of Cr(VI) level in toasted bread compared to the raw sample. These findings are in contrast to another similar study (Kovács et al. 2007) on bread samples, which showed no Cr(III) oxidation during the heating process and a study on three types of oils, for which, also no Cr(VI) was found (Lin et al. 2016). These two latter studies are in agreement with the results obtained in our study for milk and bovine meat.

In order to assess the fate of Cr(III) and Cr(VI) during cooking of milk and meat samples, the raw samples (free of Cr(VI)) were spiked with Cr(VI) as follows: 0.25 μg kg^{-1} for infant formula milk, 0.5 μg kg^{-1} for bovine meat and 1.0 μg kg^{-1} for the semi-skimmed milk. The results in terms of Cr(III) and Cr(VI) speciation analysis in milk and bovine meat samples (raw and cooked) are summarised in Table 2. All samples were also

Table 1. Overview of the literature data on total chromium in various samples using various cooking procedures.

Sample	Cooking procedure	Sample preparation	Detection technique	Cr_{total} level (μg kg^{-1})	Reference
Leguminous vegetables – pea and bean	(A) fresh vegetables, (B) after blanching, (C) after cooking in 2% brine to consumption consistency, (D) frozen products from samples B and C, after 12 months of storage at – 30°C and then prepared for consumption, (E) frozen products from sample C were defrosted and heated in a microwave oven	Incineration and ash dissolved with HNO_3 and $HClO_4$	ICP-AES[a]	Broad bean (mg/100 g fresh matter) (A) 0.007; (B) 0.007; (C) 0.006; (D) 0.007; (E) 0.006 Broad pea (mg/100 g fresh matter) (A) 0.016; (B) 0.011; (C) 0.011; (D) 0.011; (E) 0.012 French-bean (mg/100 g fresh matter) (A) 0.017; (B) 0.016; (C) 0.015; (D) 0.014; (E) 0.015	Lisiewska et al. 2008
Pumpkin	Heated to 5 min at 100°C using gas stove	Acid digestion (HNO_3)	AAS[b]	(mg/kg) Raw: 0.9–0.1, Cooked: 0.6–0.1	Fidelis et al. 2018
Whitefish – Rutilus frisii kutum	(A) Microwave oven (2450 MHz, 10 min), (B) Grilled in the oven (180°C, 20 min), (C) Fried in sunflower oil (180°C, 4 min), (D) boiled in a stainless steel container (5 min), (E) steamed in a rice cooker (10 min), (F) brine salted for 1 h	Hot plate digestion (HNO_3)	AAS	Raw: 2.8, Cooked conditions: (A) 2.06, (B) 2.18, (C) 2.54, (D) 2.00, (E) 2.36, (F) 0.00256	Mehdipour et al. 2018
Rice	Heated using a hot plate (95°C for 2 h) (A) regular tap water, (B) de-ionised water, (C) acidic water, (D) basic water	Hot plate digestion (HNO_3)	ICP-MS	Raw: 270 Cooked conditions: (A) 300, (B) 410, (C) 320, (D) 280	Rittirong and Saenboonruang 2018
Seafood – Prawns and halibut	(A) smoked, (B) peeled prawns	Microwave digestion (HNO_3)	ICP-MS	Raw: 150, Cooked conditions: (A) 120, (B) 30	Rasmussen et al. 2017
Norway lobster	Boiled (105°C): (A) white meat, (B) brown meat	Microwave digestion (HNO_3, H_2O_2, HF)	ICP-MS	Raw: white: 150, brown: 620Boiled: white: 120, brown: 380	Perugini et al. 2014
Pasta, Meat, Fish, Vegetables and Pulses	Meat: raw vs hotplate/grilled/roasted Fish: raw *vs* roasted/Fried Vegetables: raw *vs* cooked/Fried	Microwave digestion (HNO_3)	GF-AAS[c] AAS	(mg/100 g dry weight) Pasta Raw: 54.9–103.5, Cooked: 63.1–107.7 Meat Raw: 12.2–63.0, Cooked: 11.8–66.7 Fish Raw: 44.0–88.5, Cooked: 44.7–87.9 Vegetables Raw: 41.9–62.6, Cooked: 53.4–54.8 Pulses Raw: 89.4, Cooked: 81.8	Alberti-Fidanza et al. 2002
Crayfish	Cooked for 10 min in aluminium or stainless steel saucepan containing 4 L of tap water, 0.3 L of salt, and herbs (dill).	Incineration and ash dissolved with HNO_3	GFAAS	(mg/kg fresh weight) Abdominal Raw: 0.019–0.025 Cooked: 0.018–0.088; Hepatopancreas Raw: 0.042–0.050 Cooked: 0.13–0.18	Jorhem et al. 1994
Mediterranean finfish and shellfish	Samples boiled 2–3 min in water/panfried (170 C)/grilled domestic electric oven (180°C)	Microwave digestion (HNO_3)	GF-AAS	(μg/g) Raw: 0.05–0.26, Pan-fried: 0.06–0.52, Grilled: 0.05–0.13	Kalogeropoulos et al. 2012
Vegetables	(A) raw material, (B) blanched material before freezing, (C) cooked material before freezing, (D)cooked in 2% brine after 12 months frozen storage from material blanched before freezing, (E) product defrosted and heated in a microwave oven after 12 months frozen storage from material cooked before freezing	Microwave digestion (HNO_3 and $HClO_4$)	ICP-AES	(g/100 g) (A) 7.28–18.11, (B) 6.61–16.14, (C) 8.59–17.15, (D) 7.40–16.78, (E) 9.56–18.66	Kmiecik et al. 2006
Seafood – fish, cephalopods, crustaceans and bivalves	Steamed (90 ± 3°C) for 60 min.	Microwave digestion (HNO_3)	ICP-MS	Raw: 150, Steamed: 250	Barbosa et al. 2018
Beed meat	Cooked at 80°C for 3 min. (A) Raw, (B) Conventional cooking, (C) Plate/Ohmic cooking 50 Hz, (D) Plate/Ohmic cooking 10 Hz	Incineration and ash dissolved with HNO_3	ICP-MS	(mg/120 g) (A) 0.004, (B) 0.077, (C) 1.540, (D) 0.091	Wang and Farid 2015
Milk and yoghurt	Fermentation process, draining of yoghurt, cooking and salting	Heat: 95°C/20 min and left for cooling to 40–45°C.	ICP–AES	Raw: 770, Strained yoghurt: 1230, Salted yoghurt: 430	Güler 2007

[a]Inductively coupled plasma-atomic emission spectrometry.
[b]Atomic absorption spectrometry.
[c]Graphite furnace-atomic absorption spectrometry.

Table 2. Levels (± standard deviation, SD) of Cr_{total} (by ICP-MS) in raw milk and meat samples as well as Cr(III)-Cr(VI) (SS-ID HPLC-ICP-MS) in raw and spiked samples undergoing different cooking processes (the recovery of both Cr(VI) and Cr_{total} by SS-ID is also reported).

	Mass fraction ±SD (μg kg^{-1}, n = 6)				Recovery ± SD (%, n = 6)[d]		
Sample	Cr_{total}[a] (ICP-MS)	Cr(III) (SS-ID)	Cr(VI)[b] (SS-ID)	Cr_{total}[c] (SS-ID)	Cr(VI)[b] (SS-ID)	Cr_{total} (SS-ID)	β± SD (%)[e]
Infant formula milk							
Raw	2.97 ± 0.15	2.63 ± 0.05	0.25 ± 0.02	2.88 ± 0.06	100 ± 2	97 ± 1	117 ± 10
Microwave	2.95 ± 0.13	2.62 ± 0.10	0.23 ± 0.02	2.84 ± 0.13	92 ± 8	96 ± 5	109 ± 6
Hot Plate	2.92 ± 0.12	2.79 ± 0.07	0.27 ± 0.01	2.96 ± 0.12	108 ± 4	101 ± 6	115 ± 11
Semi-skimmed milk							
Raw	5.22 ± 0.11	4.55 ± 0.04	0.92 ± 0.01	5.47 ± 0.04	92 ± 3	105 ± 0	106 ± 5
Microwave	5.20 ± 0.13	4.47 ± 0.15	0.85 ± 0.04	5.23 ± 0.06	85 ± 6	101 ± 1	118 ± 7
Hot Plate	5.21 ± 0.10	4.38 ± 0.15	0.88 ± 0.12	5.26 ± 0.11	88 ± 4	101 ± 3	112 ± 3
Steak Beef							
Raw	5.21 ± 0.12	4.49 ± 0.17	0.49 ± 0.03	4.98 ± 0.09	98 ± 10	96 ± 3	100 ± 2
Fried without oil	5.20 ± 0.09	4.62 ± 0.05	0.48 ± 0.05	5.10 ± 0.10	96 ± 11	98 ± 5	113 ± 5
Fried with oil	5.25 ± 0.11	4.78 ± 0.05	0.46 ± 0.04	5.24 ± 0.14	92 ± 10	100 ± 1	110 ± 4

[a]Cr natural + Cr(VI) spiked (n = 3).
[b]spiked.
[c]Cr(III)+Cr(VI) (SS-ID).
[d]The recovery for Cr(VI) determination (SS-ID) is calculated by comparison with the spiking level whereas for Cr_{total} (SS-ID) the recovery factor is assessed by comparison with total chromium level of the blend sample analysed by ICP-MS.
[e]Yield of Cr(VI) conversion to Cr(III) during the analytical process.

Table 3. Levels of Cr(III) in raw and cooked food as well as the fraction of Cr(III) in cooked samples compared to the raw sample obtained by SS-ID HPLC-ICP-MS.

Sample (n = 3)	Cr(III) mass fraction ± SD (μg kg^{-1})	$Cr(III)_{cooked}/Cr(III)_{raw}$ ± SD (%)
Infant formula milk		
Raw	2.72 ± 0.06	-
Microwaved	2.63 ± 0.17	97 ± 5
Hot plate	2.51 ± 0.12	93 ± 4
Semi-skimmed milk		
Raw	4.50 ± 0.04	-
Microwaved	4.43 ± 0.06	98 ± 1
Hot plate	4.34 ± 0.17	97 ± 4
Bovine meat		
Raw	4.48 ± 0.09	-
Fried without oil	4.46 ± 0.13	98 ± 4
Fried with oil	4.62 ± 0.18	103 ± 5

analysed in terms of Cr_{total} by ICP-MS (see also Table 2).

As can be seen in Table 2, excellent recovery was obtained for the analysis of both raw and cooked samples spiked with Cr(VI) at various levels. It is worth to note that all samples were spiked with Cr(VI) one week before their analysis hence these results show the stability of Cr(VI) spiked in these matrices. Nevertheless, this does not necessarily confirm the EFSA hypothesis related to the absence of Cr(VI) in foodstuffs (EFSA 2014a) because the behaviour of Cr(VI) in natural foodstuffs may be different than that of Cr(VI) artificially spiked to the same sample types.

As can also be seen in Table 2, the total reduction of Cr(VI) ($\beta \cong 100\%$) during sample preparation (extraction) of the spiked matrices was observed in all cases while no oxidation of Cr(III) occurs ($\alpha \cong 0$ in all cases, not shown here). Nevertheless, the SS-ID approach is capable of correcting the species interconversion concomitantly with their quantification. Therefore, without the capability of SS-ID, Cr(VI) eventually incorporated into foodstuffs would not be accurately assessed because of its reduction to Cr(III).

It is worth to note that ID approach is capable to ensure quantitative data if the isotopic equilibrium (sample + spike) is achieved. Nevertheless, the high comparability of Cr(III) and Cr_{total} levels obtained in our study is most probably due to the fact that Cr(III) species is easily extractable rather than the capability of SS-ID approach to provide accurate data even in case of partial extraction. This finding was confirmed in a previous study by Hernandez et al. (2018) where Cr(III) levels were highly comparable with those of Cr_{total} when applying a conventional quantification approach (external calibration). ANOVA was also used to assess the differences between the Cr(VI) levels in raw and cooked samples. For all samples and culinary procedures studied here, there were no significant differences between the groups.

An example of a chromatogram obtained for

Table 4. Overview of the literature data on chromium speciation analysis in bread and breakfast cereals and animal oils using increase of the temperature.

Sample type	Analyte	Cooking procedure	Sample preparation	Detection technique	Cr_{total} (μg kg^{-1})	Cr(VI) (μg kg^{-1})	Reference
Bread Breakfast cereals	Cr(III) Cr(VI)	Bread toasted for 3 min on an Essential 900 W	Alkaline extraction: 0.10 mol L^{-1} Na_2CO_3	HR-CS AAS[a]	Untoasted: 57.6–156.1	Untoasted: 19.3–63.9 Toasted: 33.65–76.3	Mathebula et al. 2017
Bread	Cr(III) Cr(VI)	Temperature-controlled melting furnace. The temperature was increased gradually to 900°C using 50°C/30 min heating steps.	Digestion with nitric acid and hydrogen-peroxide	GFAAS[b]	–	Cr(III) does not change to Cr(VI) during the heating process	Kovács et al. 2007
Edible animal oils	Cr(III) Cr(VI)	Oil1-buffalo/cow/cattle; Oil 2-mixed animal oil; Oil 3- fish oil	Microwave digestion with 0,4% HF	HPLC-ICP-MS	Oil 1: 838 Oil 2: 2500 Oil 3: 131	Cr(III) Oil 1: 372, Oil 2: 687, Oil 3: 81.8 Cr(VI) <LQ	Lin et al. 2016

[a]Inductively coupled plasma-atomic emission spectrometry.
[b]Graphite furnace-atomic absorption spectrometry.

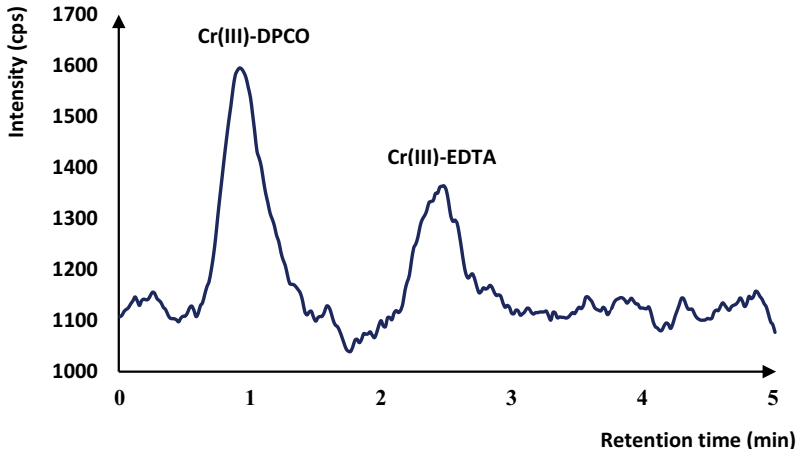

Figure 1. Chromatogram obtained for the analysis of a meat sample spiked with Cr(VI) (0.5 μg kg^{-1}) by SS-ID HPLC-ICP-MS.

the analysis of a blended bovine meat sample (mixed with Cr(III) and Cr(VI) spikes) is shown in Figure 1.

Speciation analysis of Cr(III) and Cr(VI) in different types of infant formula/semi-skimmed milk and meat/meat products

Different brands for semi-skimmed and infant formula milk, as well as meat and meat products, were analysed in this study for speciation of Cr(III) and Cr(VI) and the results are reported in Table 5. Cr(VI) was not detected (< LOD = 0.015 μg kg^{-1}) in any of the samples investigated.

All samples were also analysed in terms of Cr_{total} by ICP-MS. For most of them, Cr_{total} was < LOQ (3.0 μg kg^{-1} for milk and 10 μg kg^{-1} for meat) except for three brands of infant formula milk (≅ 5.0 μg kg^{-1}), almond drink (≅ 4.0 μg kg^{-1}) and five meat samples. In the latter case, Cr_{total} levels were significantly higher compared to infant formula milk, with levels ranging from ≅ 10 μg kg^{-1} (bacon strips) up to ≅ 52 μg kg^{-1} (pork sausage) and ≅ 82 μg kg^{-1} (Spanish chorizo sausage). An important feature in terms of Cr_{total} in meat is that the lowest levels were found in fresh meat (e.g. duck liver, veal and turkey cutlet and pork ribs) while the highest levels were measured in meat products (pork sausage and Spanish chorizo sausage). The

Table 5. Levels (± standard deviation, SD) of Cr_{total} (ICP-MS) and Cr(III) (SS-ID-HPLC-ICP-MS) measured in a selection of semi-skimmed milk, infant formula milk, meat/meat products.

	Mass fraction ± SD (μg kg^{-1}, n = 2)	
Sample [a]	Cr_{total}	Cr(III)
Semi-skimmed milk		
Brand 1	< LOD	0.68 ± 0.07
Brand 2	< LOD	0.74 ± 0.12
Brand 3	< LOD	0.57 ± 0.03
Brand 4	< LOD	0.48 ± 0.01
Brand 5	< LOD	0.66 ± 0.04
Brand 6	< LOD	0.67 ± 0.09
Brand 7	< LOD	1.05 ± 0.04
Almond drink	4.41 ± 1.30	4.38 ± 0.09
Brand 9	≥LOD < LOQ	0.69 ± 0.04
Raw	<LOD	1.93 ± 0.33
Infant formula milk		
Brand 1	7.25 ± 1.01	7.61 ± 0.84
Brand 2	≥ LOD < LOQ	2.25 ± 0.45
Brand 3	≥ LOD < LOQ	2.06 ± 0.04
Brand 4	< LOQ	2.18 ± 0.09
Brand 5	5.30 ± 0.92	5.45 ± 0.40
Brand 6	5.41 ± 1.31	5.42 ± 0.01
Brand 7	≥ LOD < LOQ	2.42 ± 0.10
Brand 8	≥ LOD < LOQ	2.90 ± 0.08
Brand 9	< LOD	1.29 ± 0.03
Brand 10	< LOD	0.22 ± 0.02
Meat and meat products		
Chicken cutlet	25.21 ± 3.61	23.41 ± 2.84
Turkey cutlet	≥ LOD < LOQ	6.12 ± 0.05
Pork rib	< LOD	4.13 ± 0.05
Beef Grilled Pavé	19.12 ± 2.44	18.11 ± 0.13
Duck liver	< LOD	3.35 ± 0.02
Ham slice	≥LOD < LOQ	7.27 ± 0.22
Spanish chorizo sausage	82.01 ± 5.63	79.72 ± 2.29
Bacon strips	10.11 ± 3.04	9.91 ± 0.17
Pork sausage (100%)	52.46 ± 2.82	53.33 ± 0.49
Cutlet veal	< LOD	1.40 ± 0.04

[a]information on the brands is not reported.
Milk samples: LOQ = 3 μg kg^{-1}; LOD = 1.5 μg kg^{-1}, for 2 g of the samples.
Meat samples: LOQ = 10 μg kg^{-1}; LOD = 5 μg kg^{-1} (sample mass = 0.6 g).

source of Cr in these meat products is not known but most probably their contamination occurs during the contact with the stainless steel utensils (knives, grinders, cookware, etc.) or from the ingredients employed in their preparation (EFSA 2014a).

Conclusions

The present study focused primarily on the assessment of the behaviour of Cr(III) and Cr(VI) species during two different thermal culinary treatments of milk and bovine meat samples. For this purpose, a primary analysis method based on double spike SS-ID in combination with HPLC-ICP-MS was employed for their quantification. This analytical approach allows the speciation analysis of Cr(III) and Cr(VI) at ultra-trace levels in a single run, and also simultaneously addresses and corrects for any species interconversion during the analytical process, particularly during the extraction step. It must be highlighted that Cr(VI) spiked in the milk and meat samples were entirely reduced to Cr(III) during the analytical process but the SS-ID approach allowed for accurate quantification of this species due to its capacity to correct for the species interconversion. ANOVA tests were conducted and showed that there were no significant differences between the raw samples and the cooked (milk and beef) ones, regardless of the culinary treatments employed. This confirms that no oxidation of Cr(III) to Cr(VI) occurs for these food samples during their thermal treatment.

A selection of 10 samples of each type (infant and semi-skimmed milk and meat/meat products) were also analysed for their total Cr as well for Cr(III) and Cr(VI) levels. Cr(VI) was not found in any of the samples analysed here hence supporting the EFSA statement related to the absence of Cr(VI) in foods (EFSA 2014a). While for the milk (semi-skimmed and infant formula), Cr(III) concentration ranged between 0.22 and 7.61 μg kg^{-1}, considerably higher levels of Cr(III) were found in meat samples (1.40–79.7 μg kg^{-1}). Actually, the lowest levels of Cr(III) were found in fresh/non processed meat (e.g. duck liver, cutlet veal to, etc.) while the maximum levels were measured in processed meat (pork sausage at 53.3 μg kg^{-1} and Spanish chorizo at 79.7 μg kg^{-1}). In all cases, the levels of Cr(III) were compatible with those of Cr_{total}, which reinforce the EFSA's statement in terms of the presence of Cr in foodstuffs exclusively as Cr(III) species.

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Article III

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Speciation analysis of Cr(III) and Cr(VI) in bread and breakfast cereals using a novel analytical approach based on species-specific isotope dilution and HPLC-ICP-MS

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Abstract

This study reports the development and validation of a novel analytical approach for simultaneously (single run) 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 extraction was achieved by sequential complexation of genuine Cr(III) with ethylenediaminetetraacetic acid (EDTA) and of Cr(III) originating from Cr(VI) with 1,5-diphenylcarbazone (DPCO) in the same analytical run. The HPLC separation of Cr(III)-EDTA and Cr(III)-DPCO complexes species was carried out by anion exchange HPLC; excellent baseline separation was achieved 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 months. The method quantification limits were 0.014 μg kg^{-1} for Cr(III) and 0.047 μg 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 to 4.4 % for Cr(III) and from 0.6 to 7.9% for Cr(VI). Similarly, the coefficient of variation in terms of intermediate reproducibility (CV_R) ranged from 1.3 to 4.4% for Cr(III) and from 2.0 to 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 quantified in any of these samples while Cr(III) levels ranged between 5.2 and 176.3 μg kg^{-1} for bread samples and 23.8 and 350.3 μg kg^{-1} for breakfast cereals. These results are comparable with the levels of total Cr analysed in the same samples by ICP-MS. The method presented here with combined use 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.

Keywords: Total chromium; chromium speciation; species-specific isotope dilution; HPLC-ICP-MS; bread; breakfast cereals

1. Introduction

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

Cr(VI) has been recognized for several decades as being carcinogenic and mutagenic, whereas Cr(III) was for a long time considered as a beneficial element for human health (Pyrzynska, 2016). However, the European Food Safety Authority (EFSA) stated that there is no convincing evidence of beneficial effects of Cr(III) for healthy people (EFSA, 2014a). Moreover, EFSA stated that Cr exist in food exclusively as Cr(III), due to the reduction feature of the foodstuffs matrix. Several other studies showed that in food with a high content of organic matter Cr(VI) is rapidly reduced (Nordberg et al. 2007, Novotnik et al., 2013). Nevertheless, several studies reported the presence of Cr(VI) in foodstuffs (Lameiras et al., 1998, Soares et al., 2000, Ambushe et al., 2009, Elci et al., 2010, Mathebula et al., 2017, Chen et al., 2020) hence questioning the reducing power of the organic matter and antioxidants in the food matrix. It appears 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 selective Cr(VI) extraction and analysis without chromatographic separation (Milačič and Ščančar, 2020). It is worth to highlight that this controversy can be addressed only by using truly selective analysis methods, which are also 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 in tandem with inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) (Hamilton, et al., 2018; Pyrzynska, 2016; Milačič and Ščančar, 2020; Saraiva et al., 2021)The most critical step of a Cr speciation analysis method is the species extraction, especially due to their important chemical behaviour. Cr(VI) extraction is most commonly performed in an alkaline medium due to the stability of Cr(VI) in such medium. On the other hand, Cr(III) is stable at low pH and is easily oxidized under strong alkaline conditions in the absence of organic matter (Kovács et al., 2007; Hosseini et al., 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 derivatisation reactions commonly used. For this purpose, ethylenediaminetetraacetic acid (EDTA) (in largely used to complex Cr(III),whereas complexing of Cr(VI) is not possible (Soares et al., 2009; Arnaud and Annette, 2018; Lin et al., 2016; Séby and Vacchina 2018). In

most of the cases, Cr(VI) is selectively reduced to Cr(III) and the latter species is complexed by EDTA in a different step (but the same analytical run) to differentiate it from the genuine Cr(III) (Saraiva et al., 2021a-b). This approach, combined with the SS-ID and HPLC-ICP-MS is currently the state of art analytical approach for accurate speciation analysis of Cr at trace and ultra-trace levels.

The aim of this study was to the development 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 of an approach developed previously 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 application to ultra-trace speciation analysis of Cr(III) and Cr(VI) in a selection of bread and breakfast cereals sample for which the accuracy is assessed by spike recovery experiments.

2. Materials and methods

2.1 Instrumentation

An closed microwave digestion system (Anton Paar, Multiwave 3000, 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 agents.

At ANSES (France), total Cr was measured by using an ICP-MS Agilent 7700 (France) and the working conditions are summarized in Table 1. At DTU Food (Denmark), total Cr was measured by using an iCAP TQ ICP-MS (Denmark) and the working conditions are summarized also in Table 1.

The ICP-MS was equipped with a PFA standard nebulizer and a cyclonic quartz spray chamber and was optimized daily by short-term performance tests run in both standard and KED modes.

The separation of Cr(III) and Cr(VI) species was carried out by using a Dionex ICS-6000 HPLC system (ThermoFisher Scientific, Waltham, MA, USA) 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 0.18 mm i.d PEEK tubing. The optimized ICP-MS and HPLC operating conditions are summarized in Table 1.

Table 1. Optimum ICP-MS parameters for total Cr determination and Cr speciation analysis by SS-ID HPLC-ICP-MS

	HPLC parameters	
Column	Dionex IonPac™ AG7 (2 mm x 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	
	Thermo iCAP-TQ	**ICP-MS 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	^{52}Cr	^{52}Cr

2.2 Reagents and standard solutions

Ultrapure water (18.2 MΩ cm, prepared by Milli-Q™ Integral 5 Elix Technology, Merck Millipore, Darmstadt, Germany) were used throughout the experiment. A solution of Ethylene diamine tetraacetic acid (EDTA, 99.995%, Sigma-Aldrich) was used to chelate Cr(III) to [Cr(III)-EDTA]-complex and consequently the use of 1,5 - Diphenylcarbazide (DPC, Sigma-Aldrich) for chelate Cr(VI) to [Cr(VI)-DPC]-complex.

Standard stock solutions containing of Cr(III) and Cr (VI) at 1000 mg L^{-1} (SCP Science, Quebec, Canada) were used for the external calibration.

^{50}Cr(III) (97.4%) and ^{53}Cr(VI) (92.4%) spikes solutions at 100 mg L^{-1} (ISC Science, Oviedo, Spain) were used throughout for SS-ID. The natural abundances of the various Cr isotopes used for SS-ID calculations are those reported by commission on isotopic abundances and atomic weights (CIAAW 2019).

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

EDTA solution was prepared by dissolving 0.45g EDTA to a final volume of 500 mL in ultrapure water using ultrasounds for 15 min at the maximum power (140%) at 25ºC. DPC solution was prepared by dissolving 0.05 g of DPC in 10 mL of methanol (≥ 99.8%, AnalaR NORMAPUR® ACS, VWR, 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.

2.3 Food samples

Twenty-two samples of various types of bread and breakfast cereals were purchased from local supermarkets in in Maisons-Alfort, France while the breakfast cereals were purchased in Birkerød, Denmark (see Table 2). It is worth to note that the bread samples were made with different flours such as rye, malt, spelt, wheat and 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 (NIST, Gaithersburg, USA) was also analyzed using the same procedure as food analysis for the internal quality control.

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

Samples	**Place of purchase – retail shop**
Bread:	
Danish Rye	Birkerød (Denmark)
Malt flour	
"Burger" bread	
Spelt flour	
"Pita" bread	
Baguette	Maisons-Alfort (France)
Sandwich	
Wheat flour	
Bread toasted	
Bread toasted complete	
"French" bread	
Breakfast cereals:	
Cacao cereals	
All bran	
Bran flakes bio	
Corn flakes	
Weetabix 100% whole	
Weetabix Fruit + Nuts	Birkerød (Denmark)
Cacao cereals	
Granola bio	
Granola gluten free	
Fruity muesli	
Toasted muesli	

2.4 Determination of total Cr

Cr_{total} levels were determined by ICP-MS following digestion using concentrated HNO_3 (67% v/v) in a closed microwave digestion system. 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 HNO_3. Before the microwave treatment, 3 mL of ultra-pure water was added. The digests were quantitatively transferred to polypropylene tubes (50 mL) and then diluted to 50 mL with ultrapure water after the addition of the internal standards (^{45}Sc, ^{89}Y, ^{103}Rh). The analysis was carried out by ICP-MS using external calibration in the range 1.0 to 10 µg L^{-1} (1, 2.5, 5, 7.5 and 10). After dilution with ultrapure water, the digests were analysed by ICP-MS according to the operating conditions given in Table 1.

2.5 Determination of Cr species

Briefly, a subsample of 0.3 g was thoroughly mixed with the required amount of $^{50}Cr(III)$ and $^{53}Cr(VI)$ spikes directly in 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 ºC during 60 minutes. 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; the final volume was made up to 20 mL with ultrapure water. After addition of DPC solution, the mixture was heated again at 90 ºC during 25 min. The extract was cooled down to room temperature (≈ 30 ℃ for 15 min) and then filtered using 0.45 µm polyvinylidene fluoride syringe filters (Merck Millipore, Ireland) 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 analysed daily (before the real samples) in order to assess the overall instrumental performance and the stability of the $^{50}Cr(III)$ and $^{53}Cr(VI)$ spikes used for SS-ID.

More details regarding the optimization of the sample preparation procedure is provided in a previous study (Saraiva et al., 2021).

2.6 Method validation

One of the main challenges related to Cr speciation analysis is the assessment of the accuracy of the analytical methodology. Food based CRMs are difficult to produce due to potential Cr species instability in such matrices (Oliveira, 2012). Currently no commercially foodstuffs based CRM with assigned values for Cr species exists. Therefore, the common approach used to assess the method accuracy is 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 (IUPAC, 2002; NF V03-110, 2010;). For this purpose, three bread sub-samples were spiked with Cr(VI) at three levels (5, 10 and 15 µg kg^{-1}) and each blend was analysed in duplicate on 5 different days to assess the accuracy, the repeatability and the

intermediate reproducibility. The validity domain was defined between the method limit of quantification (LOQ) and the upper tested concentration (15 µg kg^{-1}).

3. Results and discussion

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

The procedure for the extraction of Cr(III) and Cr(VI) was based on previously reported dealing with Cr speciation in milk and meat samples (Saraiva et al., 2021). It is worth to note that the conditions for extraction of Cr(III) and Cr(VI) in milk and meat 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 (Milačič and Ščančar, 2020; Hernandez et al, 2017).

Consequently, in this study, the extraction temperature and time was specifically optimized bread analysis. It is worth to note that when the extraction protocol developed for milk and meat is applied to bread analysis, most of the Cr was apparently identified as Cr(VI) (Table 3). This is most probably because Cr(III) was not released during the first step of the extraction (EDTA chelation), hence the genuine Cr(III) was actually not measured. Cr(III) was then released during the second step of the extraction (DPC derivatisation) and consequently falsely identified as Cr(VI). An increased temperature and times during the first extraction step was therefore necessary to release the genuine Cr(III) from the matrix and complex it with EDTA. At increased temperatures and times, a decrease in the apparent Cr(VI) level was also observed.

Table 3. Levels of Cr_{total} (by ICP-MS) and Cr species, including their sum (SSID-HPLC-ICP-MS) in "Campagne" bread (made of wheat flour) using different extraction temperature and time.

		Mass fraction + STDEV (µg kg^{-1}, n=2)				
EDTA step	**DPC step**	**Cr_{total}**	**Cr(III)**	**Cr(VI)**	**Cr(III)+Cr(VI)**	**Cr(III)+Cr(VI)/ Cr_{total} (%)**
70 °C-25 min	70 °C-25 min	26.6 ± 3.8	1.8 ± 0.29	28.5 ± 2.79	30.3 ± 2.4	114 ± 5
70 °C-60 min	70 °C-60 min		15.5 ± 0.75	6.6 ± 0.95	22.4 ± 1.9	84 ± 2
90 °C-25 min	90 °C-25 min		19.6 ± 0.34	< 0.05 (LOQ)	19.6 ± 1.8	74 ± 3
90 °C-60 min	90 °C-25 min		25.8 ± 0.40	< 0.05 (LOQ)	25.8 ± 1.5	97 ± 1
90 °C-80 min	90 °C-25 min		27.7 ± 0.42	< 0.05 (LOQ)	27.7 ± 2.0	104 ± 2
90 °C-120 min	90 °C-25 min		28.9 ± 0.10	< 0.05 (LOQ)	28.9 ± 2.1	109 ± 6

The experiments showed that a temperature of 90 °C and an extraction time of at least 60 min is necessary for the first extraction 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 time for the second extraction step (DPC derivatisation), a set of extraction experiments using a spiked bread sample extracted during with different times (5-30 min) at fixed temperature (90 °C) was carried out (Table 4).

Table 4. Data for the determination of Cr(VI) in a spiked bread sample (1.0 µg kg^{-1}) at different extraction times

DPC step (90°C)	$Cr(VI)^{s}_{piked}$ (µg kg^{-1})[a]	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

mean ± SD (n=2)

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

In order to verify that the optimized extraction conditions provided quantitative extraction of Cr(III), two different types of bread with different levels of Cr(III) were also spiked with different levels of Cr(III) from 5 to 15 µg kg^{-1} and analysed with the optimized procedure (Table 5).

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)

Spike levels of Cr(III) (µg.kg^{-1})	0	5	10	15
Bread 1				
Genuine Cr(III) level (µg kg^{-1})[a]	130 ± 1.6	137 ± 3.8	153 ± 1.3	158 ± 2.5
Recovery factor (%) [a,b]	97 ± 1.2[a]	99 ± 2.7	106 ± 0.9	105 ± 1.2
RSD (%)	1.2	2.7	0.9	1.6
Bread 2				
Cr(III) (µg kg^{-1}) [a]	26.8 ± 1.6	31.8 ± 0.9	41.2 ± 2.1	44.7 ± 0.7
Recovery factor (%) [a,b]	101 ± 2.2[a]	101 ± 3.0	113 ± 5.7	107 ± 1.7
RSD (%)	2.2	2.9	5.1	1.6

[a] mean ±SD (n=2)

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

In all cases the results showed good recoveries (97-113 %) for the different levels of spiking which hence confirmed that these extraction conditions were optimal for the extraction of both Cr 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 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.

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] (µg kg^{-1})	Bias (%)	CV_r (%)	CV_R (%)	LOD[b] (ng kg^{-1})	LOQ[b] (ng kg^{-1})
Cr(III)						
"Campagne" bread with wheat flour	26.6	0.30	4.4	4.4		
Bread with spelt flour	37.8	-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%)	219.3	0.01	1.3	1.3		
Cr(VI)						
Bread + 5 µg kg^{-1}	5.1	0.03	0.6	3.3		
Bread + 10 µg kg^{-1}	9.9	0.14	7.9	8.9	14	47
Bread + 15 µg 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

The relative repeatability was similar to other studies (Vacchina et al., 2015), where values in the range from 1 to 10 % are typically reported.

The LOQ obtained were 14 ng kg^{-1} and 47 ng kg^{-1} for Cr(III) and Cr(VI), respectively. It is worth to 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 species in the bread and breakfast cereal samples are presented in Table 7.

The values for Cr_{total} varied between LOQ (10 µg kg^{-1}) and 202.4 µg kg^{-1} for bread samples and 24.4 and 360.2 µg 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 µg kg^{-1}). For breakfast cereals, the levels found are agreement with reported by Farre and Lagarda, 1986 (118-162 µg kg^{-1}) and 316 µg kg^{-1} reported by Roussel et al., 2007 in breakfast cereals with cacao.

The levels for Cr(III) varied between 5.2 and 176.3 µg kg^{-1} for bread samples and 23.8 and 350.3 µg kg^{-1} for breakfast cereals. In all samples Cr(VI) was not detected and the Cr(III) results where in good agreement with the Cr_{total} results (87 to 102% in bread samples and 90 to 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).

Table 7. Mean total Cr and Cr species levels in bread and breakfast cereals obtained by ICP-MS and SS-ID HPLC-ICP-MS, respectively.

	Mass fraction ± SD (µg kg^{-1}, n=2)			**Cr(III)/Cr$_{total}$** [a] (%)
	Cr$_{total}$	**Cr(III)**	**Cr(VI)**	
Bread samples				
Bread with rye flour	85.7 ± 5.0	87.7 ± 4.5		102
Bread with malt flour	66.8 ± 2.3	67.2 ± 3.3		101
"Burger" with wheat flour + seeds	170.1 ± 2.2	170.1 ± 5.6		100
Bread with spelt flour	202.4 ± 3.4	176.3 ± 6.1		87
“Pita” with wheat flour	76.3 ± 4 .7	76.9 ± 1.5		101
"Baguette" with wheat flour	134.7 ± 19.1	130.6 ± 8.0	< 0.047	97
"Sandwich" with wheat flour	44.7 ± 6.3	43.8 ± 3.2		98
"Ball" with wheat flour	<LOQ [a]	5.6 ± 0.4		-
Toasted bread with wheat flour	<LOQ [a]	5.2 ± 0.1		-
Toasted bread with complete wheat flour	<LOQ [a]	9.6 ± 0.4		-
"Campagne" bread with wheat flour	26.6 ± 3.8	26.8 ± 0.6		101
Breakfast cereals				
Cacao (20.1%) – Brand I	360.2 ± 50.9	350.3 ± 5.5		97
All bran (100% whole grains)	67.2 ± 2.7	63.3 ± 5.2		94
Bran flakes bio	25.8 ± 0.1	24.8 ± 0.6		96
Corn flakes	24.4 ± 1.2	25.8 ± 0.1		106
Weetabix 100% whole grains	25.1 ± 1.0	25.1 ± 1.5		100
Weetabix Fruit + Nuts	61.8 ± 1.9	62.8 ± 2.0	< 0.047	102
Cacao (16.5%) – Brand II	219.3 ± 6.8	238.0 ± 1.4		109
Granola bio	41.5 ± 1.7	39.8 ± 3.1		96
Granola gluten free	26.0 ± 0.0	27.4 ± 0.7		105
Fruity muesli	26.3 ± 1.3	23.8 ± 1.6		90
Toasted muesli	40.8 ± 1.4	39.7 ± 0.3		97

[a] LOQ = 10 µg kg^{-1}

There was no Cr(VI) in any bread and breakfast cereals analyzed, agreeing with other authors (Kovács et al., 2007; Novotnik et al., 2013; Vacchina et al., 2015; Hernandez et al., 2017; Milačič and Ščančar, 2020).

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

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

Several studies on Cr speciation have reported the presence of Cr(VI) in cereals. One particular example, reported by Soares et al., (2010), is the presence of Cr(VI) in bread. In their study, bread samples were investigated using an alkaline extraction followed by quantification using electrothermal atomic absorption method (ETAAS) and it was reported that Cr(VI) represented 12% of the total Cr concentration (5.65 ± 5.44 µg kg^{-1} for white bread and 6.82 ± 4.88 µg kg^{-1} for whole grain bread). More recently, Mathebula et al. (2017) reported Cr(VI) in bread samples from South Africa using selective extraction and high resolution continuum source atomic absorption spectrometry (HR-CS-AAS). The results showed that 33-73% of total Cr (58.2-

156.1 µg kg^{-1}) in bread was present as Cr(VI) (20.4-470.4 µg kg^{-1}). In both of these cases, an *off-line* analytical approach was used aiming at selective extraction of Cr(VI) and followed by the determination of total Cr by atomic spectrometry. It is therefore highly plausible that the application of analytical methods that are not specific for Cr(VI) analysis, such as ETAAS, as well as the use of an *off-line* methods of analysis produce erroneous results regarding the presence of Cr(VI) in common food samples (Milačič and Ščančar, 2020). Such *off-line* approaches are, however, not able to take potential species-interconversions into account and correct for these (Milačič and Ščančar, 2020). Consequently, *on-line* analytical procedures with hyphenated separation and detection (e.g. HPLC-ICP-MS) should be preferred in combination with the use of SS-ID (using Cr(III) and Cr(VI) spikes, each species being enriched in a different isotope). Such approaches allow the analyst to detect and correct for any species transformation during the analytical procedure leading to reliable speciation results. SS-ID-HPLC-ICPMS was used by Novotnik et al (2013), who studied chromium speciation in a range of tea infusions and bread samples and concluded that Cr(VI) was not stable in these matrix types due to the presence of organic matter and antioxidant and questioned the reports on the presence of Cr(VI) in food.

In order to further investigate Cr(VI) stability in bread samples, a series of spiking experiments were conducted. A selected bread sample was spiked with different concentrations of first Cr(III) and subsequently the experiment was repeated with spiking with Cr(VI) at different concentrations. The outcome of the experiments is shown in Fig. 1. In the case of Cr(III), the spiked amounts were quantitatively recovered (104% ± 5, n=4) and in all cases Cr(VI) was not detected. When spiking with Cr(VI), the spikes were quantitative recovered as Cr(III) for all spiking levels (98% ± 7, n=4) and no Cr(VI) was detected. The result support the hypothesis that Cr(VI) cannot be stable in a bread matrix and all Cr is present as Cr(III) form.

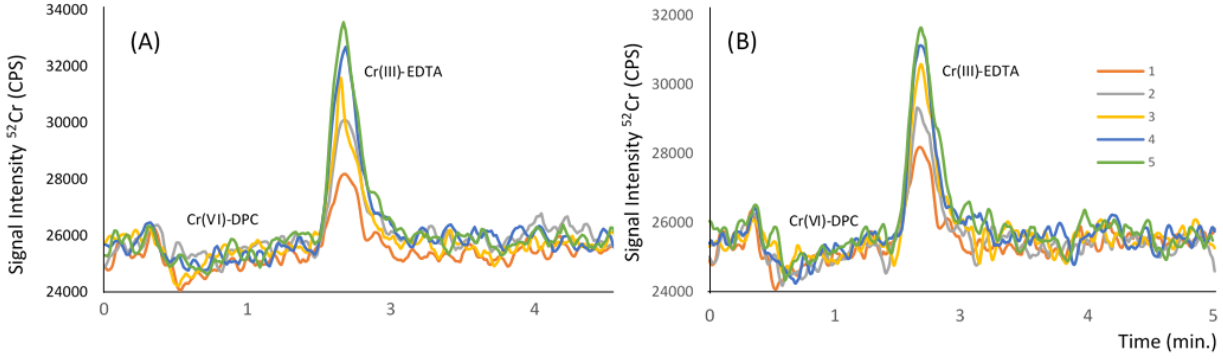

Figure 1. Chromatograms of bread spiked with Cr(III) (A) and Cr(VI) (B), with differents levels: (1) No spiked – natural levels of Cr(III) ; (2) spiked with 5 µg kg^{-1}; (3) spiked with 10 µg kg^{-1}; (4) spiked with 15 µg kg^{-1}; (5) spiked with 20 µg kg^{-1}

The experimental work conducted in the present study supports the findings by Novotnik et al (2013) and demonstrate 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.

4. Conclusion

The present study included an optimised procedure for extraction of Cr species (Cr(III)+Cr(VI)) in bread and breakfast cereals prior to determination by species-specific isotope-dilution (SS-ID) HPLC-ICP-MS. The optimised procedure was applied to a range of bread (made with different types of flour) and breakfast cereals (with different flour compositions, with and without: fruits, nuts and cacao).

The extraction procedure defined in previous studies for other matrices (milk and meat) has not become viable for this type of more complex matrices such as bread and breakfast cereals. Hence it was necessary to increase the time and temperature in water bath extraction of 70 ^{o}C for 25 min to 90 ^{o}C for 60 min in the EDTA complexation step with Cr(III) and in the Cr(VI) complex, the change was only in increasing the temperature to 90 °C maintaining the 25 min. These changes that had to be made may be due to the fact that Cr(III) is more strongly bound in cereals matrices and therefore its release is more difficult at lower temperatures.

A selection of 22 samples (bread and breakfast cereals) were analysed for their contents of total Cr as well as for Cr(III) and Cr(VI) speciation. Cr(VI) was not found in any of the samples analysed hence supporting the EFSA statement related to the absence of Cr(VI) in foods. The results support the hypothesis that presence of Cr(VI) in bread is not possible and all Cr is present as Cr(III) form as stated by EFSA in 2014, due to the Cr(VI) reducing power of organic samples.

It must be highlighted that Cr(VI) spiked in bread were entirely reduced to Cr(III) during the analytical process but the SS-ID approach allowed accurate quantification due to its capacity to correct for any species interconversion.

The specific chemistry of Cr in food matrices must be taken into account when choosing the most appropriate analytical procedure, so that it can provide accurate and reliable results on Cr speciation. The most powerful and reliable analytical tool for specifying Cr in food is the association of species-specific isotopic dilution with an online method of analysis such as HPLC-ICP-MS.

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Article IV

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

In preparation to be submitted to *Journal of Agricultural and Food Chemistry*

Abstract

This study reports for the first time speciation analysis of Cr(III) and Cr(VI) in rice samples using a novel analytical approach based on species specific-isotope dilution (SS-ID) and high performance liquid chromatography coupled to inductively coupled mass-spectrometry (HPLC-ICP-MS) and using selective single run species complexation/derivatisation. This approach allow accurate quantification of Cr species at ultra-trace levels (limit of quantification of 0.013 μg kg^{-1} for Cr(III) and 0.049 μg kg^{-1} for Cr(VI)) with excellent precision; the coefficient of variation in terms of intermediate reproducibility (one month time span assessment) ranged from 1.3 to 4.4% for Cr(III) and from 2.0 to 8.9% for Cr(VI), respectively Cr(VI) was not quantified in any of the rice samples analysed here while the Cr(III) levels ranged between 0.59 (whole grain rice) up to 104 μg kg^{-1} (brown rice). All samples were also analysed for their total Cr content (by ICP-MS solely), which was in all cases compatible with the Cr(III) levels measured in the same samples. In order to assess the stability of Cr(III) and Cr(VI) in the rice, a sample was spiked with Cr(III) and Cr(VI) (individually) at different levels (5, 10, 15 and 20 μg kg^{-1}). The spiked samples were analysed after 2h by SS-ID HPLC-ICP-MS, which is capable to accurately quantify Cr(III) and Cr(VI) in a single run and simultaneously correct the eventual species interconversion during the analytical process. The results showed a complete reduction of Cr(VI) to Cr(III) while Cr(III) spiked to the rice remained stable at all spiking levels. This finding aims to clarify the current controversy related to the presence of Cr(VI) and hence supporting EFSA's statement regarding the absence of Cr(VI) from foodstuffs.

Keywords: Cr(III)-Cr(VI)speciation analysis, species-specific isotope dilution; HPLC-ICP-MS; rice

1. Introduction

Soil is the main source of nutrition for plants and has a fundamental role in the Earth's crust. However, due to intense industrialization and urbanization in the last century, the soil has become the main sink of many potentially toxic elements (PTEs) (Ali et al., 2015; Mamatha et al. 2014). Cultivation of plants such as rice on contaminated soils may lead in the accumulation of PTEs in their edible parts, hence entering in the food chain thereby posing risks to human health (Qureshi et al., 2016; Fan et al., 2017).

The rice is the major food crop for more than 3.5 billion people worldwide (Xiao et al, 2021) with estimates the global rice production 2020/2021 will be 500 million tons and global rice consumption is forecast at a record 496 million tons (USDA. 2020). Actually, Asia accounts for more than 90% of the global rice production (Mackill et al., 2012). FAO data (not estimates) reveal that China, India, Indonesia, Bangladesh, Viet Nam, Thailand and Myanmar were the top seven rice-producing countries in the world in 2019, with the total production of 596 Mt of rice, around 80% of world rice production (755 473 800 tonnes) (FAOSTAT, 2019).

Soil pollution with PTE, including chromium (Cr) is a global problem due to discharges of industrial waste or hazardous waste from the electroplating, tanning and ore refining industries (Francisco et al., 2002), questioning the health of the local or general consumer due to the export of this rice (Xiao et al., 2013).

Cr is one of the most toxic elements reported in rice apart from arsenic (As), cadmium (Cd) and mercury (Hg) and lead (Pb) (Huang et al., 2013), with values of 270-410 µg kg^{-1} (Rittirong et al., 2018), 40.6-408 µg kg^{-1} (Chen et al., 2020), 170-250 µg kg^{-1} (Dokpikul et al., 2018) and 384-906 µg kg^{-1} (Naseri et al., 2014).

Cr presence in soils an consequently in rice can have both natural and/or anthropogenic origin, the latter sources being industrial electroplating, metal finishing, leather tanning and pigment production (Kotaś and Stasicka, 2000, Yu-Ling and Shiuh-Jen, 2001, Séby et al., 2003).

The most stable oxidation states of Cr are Cr(III) and Cr(VI), with very different toxicity and mobility. Cr(III) is considered as an essential nutrient and trace levels of Cr(III) is essential to mammals for the maintenance of glucose tolerance factor, and lipid and protein metabolism (Ghaedi et al., 2006; Lewicki et al., 2014). Nevertheless, the European Food Safety Authority (EFSA) stated relatively recently that there is no convincing evidence of beneficial effects of Cr(III) for healthy people. In contrast to Cr(III), Cr(VI) is mobile, bio-available, mutagenic and carcinogenic by inhalation (Pyrzynska, 2014; EFSA, 2014).

An adequate knowledge of the chemical and physiological functions and also of the toxic effects of Cr is necessary for an accurate assessment of its distinct species. Hence, speciation analysis of Cr in foods is important (Ambushe et al., 2009; Yu and He, 2017; Li et al., 2015; Fernández et al., 2018; Zhu et al., 2019). Previous studies in terms of Cr speciation in foodstuffs showed relatively high levels in bread and breakfast cereals (Saraiva et al., 2021; Mathebula et al., 2017; Hernandez et al., 2017; Vacchina et al., 2015). Recent

studies showed also the presence of Cr species in rice, including Cr(VI) (Chen et al., 2020), whereas other authors reported the absence of Cr(VI) in rice (Dokpikul et al., 2018).

Therefore, due its very large consumption of rice worldwide, it is important to assess the accurate distribution of Cr species in rice. Nevertheless, accurate speciation analysis of Cr (Cr(III) and Cr(VI)) in foodstuffs, including cereals is still an analytical challenge, mainly because of the low levels of these species in such sample but also because of the inherent Cr chemistry. Actually, Cr(III) and Cr(VI) are relatively easily interconverted, which commonly lead to analytical artefact and false reporting of Cr(VI) detection in foodstuffs. The only analytical approach capable to simultaneously quantify Cr(III) and Cr(VI) in food samples while correcting for the species interconversion is species specific isotope dilution (SS-ID) in combination liquid chromatography (HPLC) coupled to inductively coupled plasma-mass spectrometry (ICP-MS) (Saraiva 2021a,b).

It is worth to note that due to the reducing features of the food matrices, it is accepted nowadays that in food Cr is present exclusively as Cr(III) (EFSA 2014) and hence Cr(VI) reported in such type of samples is an analytical artefact (Milačič and Ščančar, 2020; Hamilton et al., 2018; Ščančar and Milačič, 2014; Novotnik et al., 2013). However, studies related to Cr speciation analysis in rice are very scarce and hence a definitive conclusion regarding the absence of Cr(VI) in such type of food needs still to be confirmed.

This study aims at the assessment of Cr_{total} levels as well as of Cr(III) and Cr(VI) levels in various type of rice samples using a novel analytical approach based on SS-ID HPLC-ICP-MS and selective species complexation (Saraiva et al., 2021a). A study regarding the behaviour of Cr species spiked to rice was also carried out in order to assess the stability of these species in the rice matrix and hence to answer the controversy related to the presence of Cr(VI) in foodstuffs, including rice.

2. Materials and methods

2.1. Instrumentation

Cr species complexation and extraction was carried out by heating using a heating block system (DigiPREP MS, SCP Science, Courtaboeuf, France). The separation of Cr(III) and Cr(VI) was carried out by HPLC using a Dionex ICS-5000$^+$ system (ThermoFisher Scientific, Courtaboeuf, France) equipped with a Dionex IonPac™ AG7 column (2 mm internal diameter, id x 50 mm length x 10 µm particles diameter) (ThermoFisher Scientific).

A sonication water bath (Model 86483, Fischer BioBlock, Rungis, France) was used for the preparation of the solutions used for the complexation of Cr(III) and Cr(VI), ethylene diamine tetraacetic acid (EDTA) and 1,5-Diphenylcarbazide (DPC).

An ICP-MS iCAP TQ (ThermoFisher Scientific) equipped with a PFA standard nebulizer and a cyclonic quartz spray chamber was used for determination of total Cr (Cr_{total}) and the on-line detection of Cr species separated by HPLC. Kinetic energy discrimination (KED) in high sensitivity mode using helium (He) as a collision gas was employed throughout the study. The HPLC column was directly connected to the ICP-MS the via a 0.18 mm i.d PEEK tubing (80 cm).

ICP-MS measurement conditions were optimized daily according to the guidelines of the instrument producer. Data were processed using the ThermoFisher Scientific Qtegra software.

The optimum operating conditions of the HPLC and ICP-MS (iCAP-TQ) are reported in a previous study (Saraiva et al, 2021).

2.2. Reagents and standard solutions

All solutions including the mobile phase were prepared with ultrapure water (18.2 MΩ cm, Millipore Milli-Q™, Merck Millipore Integral, USA).

Standard stock solutions of Cr(III) and Cr(VI) (Inorganic Ventures, Christiansburg, USA), both at 1000 mg L^{-1}, were used to prepare the calibration standards for the speciation analysis, while isotopically enriched solutions (spikes) of ^{50}Cr(III) and ^{53}Cr(VI) (100 µg mL^{-1}) (ISC Science, Oviedo, Spain) were used for SS-ID. The abundance of ^{50}Cr in the Cr(III) spike was 97.4% while that of ^{53}Cr in the Cr(VI) spike was 92.4% (as provided by the manufacturer's certificate). The natural abundances of the various Cr isotopes (50, 52 and 53) used for SS-ID calculations are those reported by the Internal Union of Pure ad applied chemistry (IUPAC) (CIAAW, 2019). The intermediate solutions were prepared daily by diluting the stock standard solutions with ultrapure water.

For Cr_{total} determination by ICP-MS, stock standard solutions of Cr and Sc (internal standard) of 1000 mg L^{-1} (SCP Science, Quebec, Canada) were used for establishing a calibration curve. Nitric acid (67-69% m/m, NORMATOM®, VWR, Søborg, Denmark) and methanol (for HPLC, ≥ 99.9%, Sigma-Aldrich, Søborg, Denmark) were used for preparation of the HPLC mobile phase.

A solution of ethylene diamine tetraacetic acid (EDTA, 99.995%, Sigma-Aldrich) was used to chelate Cr(III) and 1,5-Diphenylcarbazide (DPC, Sigma-Aldrich) for Cr(VI) derivatisation.

The EDTA solution used for the complexation of Cr(III) was prepared by dissolving 0.45 g of reagent into 500 mL of ultrapure water using a sonication water bath at maximum power (140%) for 15 minutes at 25 °C. The DPC solution was prepared by dissolving 50 mg of the reagent in 10 mL of methanol. Then, 50 mL of ultrapure water containing 2.8 mL of H_2SO_4 (95-97%, Merck KGaA, France) was added (by continuously stirring). The final DPC solution was made up to 100 mL with ultra-pure water in a volumetric flask (Sanchez-Hachair and Hofmann, 2018) and was stable for 1 month at room temperature in the dark (Arnaud, 2018).

All stock and intermediate standard solutions were stored in the refrigerator at 4-5°C for a maximum of one month.

2.3. Analytical procedures

2.3.1 Samples and sample preparation

Ten different rice samples purchased from a supermarket in Birkerød (Denmark) were analysed in this study for Cr_{total} and Cr(III)-Cr(VI) speciation. The samples were homogenized to a fine powder using a stainless steel free automatic mixer (Retsch Grindomix GM 200, Haan-Germany) with titanium blades. The brief description of the samples analyzed in this study is provided in Table 1.

Table 1. Rice samples used for quantification of total Cr and Cr speciation

Sample	Rice type	Color	Origin	Other info
Risotto	Arborio	white	Italy	short-grain, starchy variety
Sushi	Calrose	white	Japan	medium-grain
Basmati	Basmati	white	India	very long and slim grains
Black	Black	black or purple	Southeast Asia	whole-grain rice that is quite dark in color; medium-grain
Red	Red	red	Italy	long grains similar to jasmine rice
Brown	Brown	brown	South Asia	organic; chewy texture and nutty flavour. long grain brown rice
Jasmin	Jasmine	white	Asian	organic; long-grain, processed as white rice (milled and polished)
Arborio	Arborio	white	Italy	almost round grain; creamy consistency; Pearled Barley
Parboiled	Parboiled	brown/yellow	Not stated	organic; Parboiling happens before rice is milled, that is before the inedible outer husk is removed to yield brown rice but before brown rice is refined to make white rice.
Whole grain	Brown	brown	China	unprocessed and contains all parts of the grain. As a result, it is usually brown and contains high levels of fibre and nutrients.

A certificate reference material (CRM) SRM® 8436 - Durum wheat flour (NIST, Gaithersburg, USA) was used as internal quality control for Cr_{total} measurements.

Each rice sample was prepared in duplicate for both Cr_{total} determination and Cr speciation analysis.

2.3.2. Determination of total Cr by ICP-MS

The quantification of Cr_{total} levels in rice samples were carried out by Thermo ICP-MS after microwave-assisted acid digestion with HNO_3, as described in details in a previous study (Saraiva et al., 2021). In brief, 0.3 g of rice was directly weighed into 80 mL quartz vessels and 3 mL of concentrated HNO_3 and 3 mL of ultra-pure water was added before the microwave treatment. The digests were quantitatively transferred to polypropylene tubes (50 mL) and then filled up with ultrapure water after the addition of the solution of Sc used as internal standard. The quantification was carried out by using the external calibration in the range 0-1010 µg L^{-1}.

In order to ensure the internal quality control, CRM (RM® 8436 - Durum wheat flour) was analysed daily in parallel with the rice samples. Cr_{total} obtained for the analysis in duplicate in three different of this CRM was 20.4 ± 2.0 µg kg^{-1} was found, which was in good agreement with the certified value (22.1 ± 0.1) hence ensuring the accuracy of the analyses of rice for Cr_{total}.

2.3.3. Speciation analysis of Cr(III) and Cr(VI) by SS-ID HPLC-ICP-MS

Speciation analysis of Cr(III) and Cr(VI) in rice samples was carried out using SS-ID HPLC-ICP-MS as described in details in a previous study (Saraiva et al., 2021). Briefly, a subsamples of 0.3 g of rice were thoroughly mixed (to achieve isotopes equilibrium) with the required amount of ^{50}Cr(III) and ^{53}Cr(VI) spikes directly in the 50 mL polypropylene tubes and then placed in the heating block system at 90°C. Then, 16 mL of EDTA solution was added and the mixture was heated for 60 min. The solution was subsequently cooled down (30ºC, ≅15 minutes) and then 200 µL of DPC solution was added. The mixture was heated again at 90°C for 25 min. The extract was cooled down to room temperature and then filtered using 0.45 µm polyvinylidene fluoride syringe filters prior to the analysis (carried out on the same day). To quantify Cr(III) and Cr(VI) and simultaneously assess the interconversion factors, four isotope ratios, namely ^{50}Cr(III)/^{52}Cr(III), ^{53}Cr(III)/^{52}Cr(III), ^{50}Cr(VI)/^{52}Cr(VI), and ^{53}Cr(VI)/^{52}Cr(VI), were determined based on the HPLC peaks corresponding to Cr(III) and Cr(V) species.

3. Results and discussion

The studies related to Cr_{total} determination in different types of rice showed no relationship between the degree of processing (polishing, etc.) and the total Cr levels. Actually, as can be seen in Table 2 which presents an overview of Cr_{total} and Cr(III)/Cr(VI) levels in different rice types and origins, it appears that in the white (polished) rice, Cr_{total} are generally higher than those in brown rice (Chen et al., 2020). It is also worth to note that some authors also found Cr(VI) in rive from Taiwan, which is in contradiction with the generally accepted fact that Cr(VI) is lacking from foodstuffs.

Table 2. Overview of total Cr and Cr(III)/Cr(VI) levels in different rice types and origins

Rice type	Origin	Mass fraction (µg kg^{-1})		Reference
		Cr_{total}	Cr(VI)	
White **	Thailand	270-410	n.d	Rittirong et al., 2018
Polished **	Taiwan	40.6-408	5.93-142	Chen et al., 2020
Brown *		57.5	12.4	
Cereal based in rice *		115	22.8	
Jasmine **	Thailand	180-220	< LOD	Dokpikul et al., 2018
White **		180-230		
Black **		230-250		
Brown **		170-210		
Riceberry **		190-230		
Polished **	Shiraz-Iran	384-906	n.d	Naseri et al., 2014

* one exemplar of rice was used;
** three exemplars of rice were used;
n.d: no data available

3.2. Assessment of total Cr in rice samples

In this study, total Cr was measured in a selection of 10 types of rice and the data are reported in Table 3.

Table 3. Total Cr levels in rice samples measured by ICP-MS

Sample	Mass fraction ± SD (µg kg^{-1}, n=2)
Whole grains	0.5 ± 0.1
Arborio	3.9 ± 0.2
Parboiled	6.5 ± 0.5
Risotto	6.5 ± 0.4
Jasmin	8.1 ± 0.3
Sushi	8.8 ± 0.5
Dark	11.1 ± 0.7
Red	14.0 ± 0.6
Basmati	17.3 ± 0.9
Brown	102.2 ± 3.9

As can be seen, globally, Cr_{total} levels in the rice analysed in this study are lower compared to those reported by other authors (Table 3). Additionally, in our study, a considerably higher level of Cr_{total} was measured in the brown rice, whereas the lowest level was found in rice with whole grains.

These results are difficult to explain because the less the industrial processing (as the case of the whole grain sample), the higher the levels of Cr_{total}. This (lower levels with the degree of processing) is confirmed by the levels found in the rice with more consistent industrial processing such arborio (3.9 ± 0.2 µg kg^{-1}) and parboiled (6.5 ± 0.5 µg kg^{-1}).

3.3. Speciation analysis of Cr(III) and Cr(VI) in different types of rice samples

Table 4 reports the results in terms of speciation analysis of Cr(III)and Cr(VI) in ten rice samples by SS-ID HPLC-ICP-MS.

As can be seen from Table 4, the Cr(III) levels range between 0.59 and 104 μg kg^{-1} and they are highly compatibles with the Cr_{total} measured in the same samples. It is also worth to note that Cr(VI) levels in the rice analysed here were below the limit of detection (LOD).

These findings are in agreement with the EFSA statement that all Cr present in foodstuffs is in the form of Cr(III) (EFSA, 2014) but also with previous studies dealing with Cr speciation in rice (Dokpikul et al., 2018) and milk, bovine meat, bread and breakfast cereals (Saraiva et al. 2021a,b).

Table 4. Levels of Cr(III) and Cr(VI) in rice samples measured by SS-ID HPLC-ICP-MS and comparison with total Cr levels

Sample	Mass fraction ± SD (μg kg^{-1}, n=2)		
	Cr(III)	**Cr(VI)**	**Recovery (%)[a]**
Whole grains	0.6 ± 0.1		110
Arborio	4.2 ± 0.1		108
Risotto	5.9 ± 0.1		92
Parboiled	6.6 ± 0.1		102
Jasmin	8.2 ± 0.2	< LOD	101
Sushi	9.7 ± 0.1		110
Black	10.9 ± 0.1		103
Red	14.3 ± 1.4		102
Basmati	16.9 ± 0.1		98
Brown	103.9 ± 3.7		102

[a] $Cr(III)/Cr_{total}$ (%)

This studies and those mentioned previously are in contradiction with the study by Chen et al. (2020), where Cr(VI) was measured in rice at relatively high levels (5.93 up to 142 μg kg^{-1}). The Cr(VI) data reported by Chen et al. (2020) is most probably due to an analytical artefact taking into account that these authors employed an off-lien method relying on separate extraction of Cr(VI) followed by the measurement of Cr_{total} content in the extract by HPLC-ICP-MS. Although a separation step is carried out, this approach is not per se capable to assess species interconversion during the analytical process (Hamilton et al., 2018; Milacic and Ščančar, 2020).

Actually, SS-ID HPLC-ICP-MS is the only analytical approach suitable for simultaneous speciation analysis of Cr(III) and Cr(VI) in foodstuffs due to its capability to quantify the species and to correct for their interconversion during the same analytical run.

3.4. Study of the behaviour of Cr species in the rice matrix

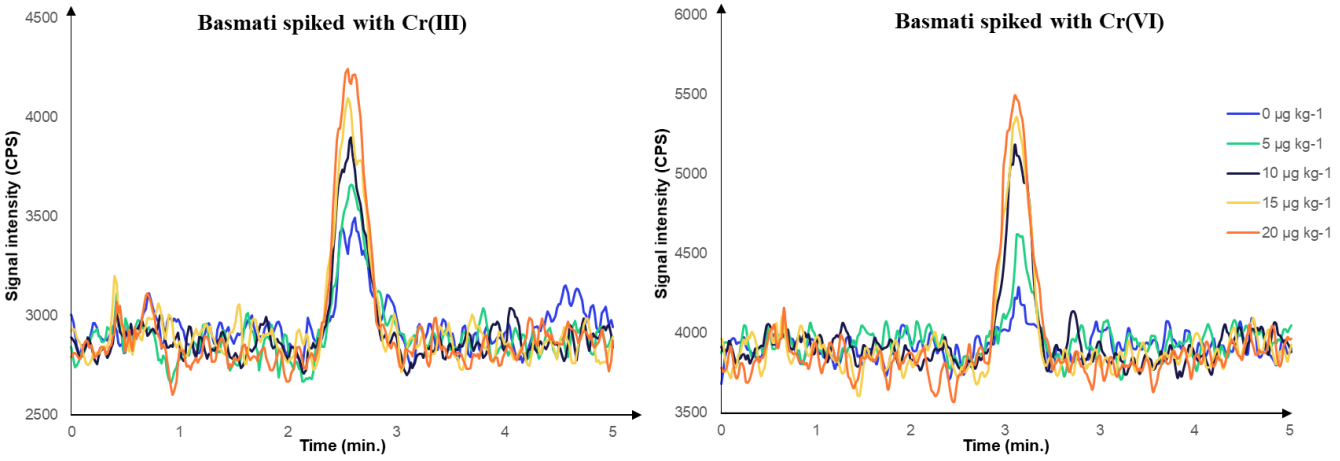
In order to assess the fate of Cr(III) and Cr(VI) in the rice matrix, these species were spiked individually to a sample of rice (basmati) and the blend was analysed by SS-ID HPLC-ICP-MS; the results are presented in Table 5.

Table 5. Levels of Cr(III) and Cr(VI) measured by SS-ID HPLC-ICP-MS in (basmati) rice spiked with these species at different levels.

	Mass fraction (µg kg^{-1})					
Spiking level (µg kg^{-1})	**Expected**		**Measured**		**Recovery (%)** [a]	
Cr(III)	**Cr(III)**	**Cr(VI)**	**Cr(III)**	**Cr(VI)**	**Cr(III)**	**Cr(VI)**
0	16.9	< LOQ	16.9 ± 0.2	< LOQ	-	-
5	21.9		22.4 ± 0.2		102	
10	26.9		29.4 ± 0.1		109	
15	31.9		35.3 ± 0.2		111	
20	36.9		39.5 ± 1.1		107	
Cr(VI)						
0	16.9	0	16.8 ± 0.4	< LOQ	99	-
5		5	22.9 ± 0.1		-	
10		10	27.3 ± 0.1		-	
15		15	33.5 ± 1.0		-	
20		20	35.5 ± 0.7		-	

[a] ratio of the measured Cr(III) level to the theoretical (expected) one

The results in Table 5 confirm that Cr(III) is highly stable in the rice matrix (recoveries between 102-111%) were obtained for this species at the spiking levels investigated here). In turn, Cr(VI) spiked to the rice (basmati) sample was completely reduced to Cr(III) at all spiking levels, as can be seen in Fig. 1.



(a) (b)

Figure 1. Chromatograms showing the results of the analysis of basmati rice spiked with different levels of Cr(III) (a) and Cr(VI) (b)

These results are also confirmed by the fact that factor describing Cr(III) oxidation to Cr(VI) (α) during the analytical process is 0 in all case while the one describing Cr(VI) reduction to Cr(III) (β) was maximum ($\cong$100%) in all cases.

These findings confirms the prevailing assumption that Cr(VI) is not stable in food matrices, including rice samples, due to the reducing features of such matrices (Novotnik et al., 2013; Vacchina et al., 2015; Hamilton et al., 2018; Milacic and Ščančar, 2020). This was accurately assessed by using a primary method of analysis such as SS-ID in combination with HPLC-ICP-MS that corrects the interconversions occurring during the analytical procedure hence preventing reporting erroneous data caused by the formation of analytical artefacts.

4. Conclusion

This study reports for the first time speciation analysis of chromium (Cr(III) and Cr(VI)) in rice by using a primary method of analysis such as species specific isotope dilution in combination with HPLC-ICP-MS. This approach allowed the determination of both Cr species at ultra-trace levels in a single run, and also simultaneously addresses and corrects for any species interconversion during the sample pre-treatment.

Cr(III) levels in the rice samples analysed here were highly variable depending on the rice type, whereas Cr(VI) was not found in any of the samples, most probably due to the instability of this species in the food matrices having a reducing character. This was also confirmed in our study by the analysis of a sample spiked with Cr(III) and Cr(VI) individually (at different levels) which showed a complete conversion of Cr(VI) to Cr(III), whereas Cr(III) was perfectly stable in the rice matrix. This study supports EFSA's statement that Cr(VI) is absent in foodstuffs and hence the entire Cr content ids in Cr(III) form.

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