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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<sup>-1</sup> HNO<sub>3</sub> + 2.5 % (v/v) MeOH + 0.30 mol L<sup>-1</sup> 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<sup>-1</sup> for Cr(III) and 0.049 µg kg<sup>-1</sup> 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<sub>r</sub>) 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<sub>R</sub>) 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<sup>-1</sup>, 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.

**Keywords:** Chromium speciation; species specific-isotope dilution; HPLC-ICP-MS; foodstuffs

## 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].

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 ( $18.2 \text{ M}\Omega \text{ cm}^{-1}$ , 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,  $\geq 99 \%$ , Sigma-Aldrich) was used as a derivatisation agent for Cr(VI) determination.  $\text{HNO}_3$  (67% v/v, Suprapur) used as an eluent was purchased from VWR (Fontenay sous-Bois, France).

Standard stock solutions ( $1000 \text{ mg L}^{-1}$ ) 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  $^{50}\text{Cr(III)}$  and  $^{53}\text{Cr(VI)}$  ( $100 \mu\text{g mL}^{-1}$ ) (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 ( $0.9 \text{ g L}^{-1}$ ) solution, 200  $\mu\text{L}$  of DPC ( $0.5 \text{ g L}^{-1}$ ) 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<sup>-1</sup> (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<sup>-1</sup> was prepared by dissolving 0.45 g of reagent into 500 mL of ultrapure water and the mixture was sonicated for 15 minutes at 25 °C (sonication bath Model 86483, Fischer BioBlock, Rungis, France) at maximum power. DPC solution at 0.5 g L<sup>-1</sup> 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<sub>2</sub>SO<sub>4</sub> (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 1 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<sup>+</sup> 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 optimised 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<sub>total</sub> 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 ( $\cong$  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  $\approx$  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<sup>-1</sup> Cr(VI), the semi-skimmed milk (500 mL) with 1 ng mL<sup>-1</sup> of Cr(VI) and the steak beef (200 g) with 0.5 ng g<sup>-1</sup> 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  $\approx$  5 °C.

#### 2.3.2. Determination of total chromium by ICP-MS

Cr<sub>total</sub> levels were determined by ICP-MS using an accredited method by employing samples digestion using concentrated HNO<sub>3</sub> (67% v/v) in a closed microwave digestion system on the basis of the procedure described in a previous study [37]. In brief, 0.5 g of 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<sub>3</sub>; 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<sup>-1</sup> (1, 2.5, 5, 7.5 and 10) were analysed to obtain the calibration curve.

### 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  $2.4 \times 10^{-3}$  mol L<sup>-1</sup> (or 2.4 mM) and the mixture was heated at 70 °C during 25 min. The solution was cooled down ( $\cong$ 15 minutes) 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, <sup>50</sup>Cr(III) and <sup>53</sup>Cr(VI) spikes were added and the blend solution was thoroughly mixed to achieve isotopes equilibration. The abundance of <sup>50</sup>Cr in the Cr(III) spike was 97.4% while that of <sup>53</sup>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 International Union of Pure and Applied Chemistry (IUPAC) [38].

Finally, it must be emphasized that a standard mixture of Cr(III) (5.0 ng L<sup>-1</sup>) and Cr(VI) (2.5 ng L<sup>-1</sup>) was analysed daily in order to assess the overall instrumental accuracy and the stability of <sup>50</sup>Cr(III) and <sup>53</sup>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 ( $\geq 5$ ) corresponding to natural (CRM, if available) or spiked matrices. Further, a tolerance interval ( $\beta_{TI}$ , Eq. 1) is calculated for each level;  $\beta_{TI}$  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:

$\beta_{TI}$ , 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 of the 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):

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

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  $\beta$ -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 ( $\lambda$ ) is set according to the criteria expected of repeatability and intermediate precision. Based on the accuracy profile approach, a method is validated when the  $\beta$ -expectation tolerance interval for a given result is comprised within the acceptability limit ( $\lambda$ ).

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):

$$U = k \times u_c = k \times \frac{CV_R \times \bar{X}}{100 \times \sqrt{n}} \quad (5)$$

where:

k, coverage factor (k= 2 for p=95%);  $\bar{X}$ = 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. Optimisation 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,  $\text{CrO}_4^{2-}$ ) was derivatized by reaction with DPC. Both chemical reactions are shown in Fig. 1a-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  $\text{Cr}^{3+}$  complexation with EDTA leads to an inversion of the charge of this species. Similarly, the reaction of Cr(VI) (in  $\text{CrO}_4^{2-}$  form) with DPC (Fig. 1b) involves firstly the reduction of Cr(VI) to Cr(III) and subsequently the complexation of the resulting  $\text{Cr}^{3+}$  with dyphenylcarbazone (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 ion exchange. 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) 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.

### 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  $5 \mu\text{g kg}^{-1}$  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 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 of both 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 optimised by the analysis of a mixture of Cr(III) and Cr(VI) (at 5 µg kg<sup>-1</sup>) 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  $\cong$ 100%) was obtained at 25 min for each species.

### 3.1.3. Optimisation 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<sup>-1</sup> by SS-ID HPLC-ICP-MS. For a sample amount of 0.2 g and a final extract solution of 20 g, these levels correspond to 5 mg kg<sup>-1</sup> in a real sample, which reflects the worst case scenario case of foodstuffs contamination with Cr species.

The optimisation 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 to be used for Cr(VI) derivatisation was optimised 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<sup>-1</sup> were 2.40 and 0.02 mM, respectively and they were used throughout the study.

## *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] hence, 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<sub>3</sub> was optimized in the range from 0.2 (0.6 mol L<sup>-1</sup> HNO<sub>3</sub>) up to 4.5 (0.01 mmol L<sup>-1</sup> HNO<sub>3</sub>) by the analysis of a standard mixture of Cr(III) and Cr(VI) at 5 µg kg<sup>-1</sup>. 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<sup>-1</sup> and the most efficient separation ensuring quantitative complexation of Cr(III) with EDTA was obtained at 0.30 mmol L<sup>-1</sup> (recovery of 105% and 97% for Cr(III)-EDTA and Cr(III)-DPCO, respectively).

Further optimisation 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  $\geq 1.5$ .

Overall, the optimum HPLC mobile phase consisted of a mixture of HNO<sub>3</sub> (10 mmol L<sup>-1</sup>), EDTA (0.30 mmol L<sup>-1</sup>) and methanol (2.5%, v/v) at pH=2.

Finally, the separation temperature was optimized in the range between 20-50 °C (20, 25, 30, 35, 40, 45 and 50 °C). The optimum resolution factor ( $\geq 1.5$ ) 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  $5 \mu\text{g kg}^{-1}$  by SS-ID HPLC-ICP-MS under optimised conditions.

### 3.3. Optimization of species specific isotope dilution

To carry out SS-ID, the samples were spiked with known amounts of  $^{50}\text{Cr(III)}$  and  $^{53}\text{Cr(VI)}$  spikes to achieve isotope ratios ( $^{50}\text{Cr}/^{52}\text{Cr}$  and  $^{53}\text{Cr}/^{52}\text{Cr}$ ) in the blend samples  $\sim 1.0$ . 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).

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

where:

$M_S^{50}$ , mass of  $^{50}\text{Cr(III)}$  spike solution (g)

$C^{III}$ , expected concentration of Cr(III) in the sample ( $\mu\text{g kg}^{-1}$ )

$m_x$ , sample mass (g)

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

$C_S^{50}$ , concentration of the  $^{50}\text{Cr(III)}$  spike solution ( $\mu\text{g kg}^{-1}$ )

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  $^{50}\text{Cr(III)}/^{52}\text{Cr(III)}$ ,  $^{53}\text{Cr(III)}/^{52}\text{Cr(III)}$ ,  $^{50}\text{Cr(VI)}/^{52}\text{Cr(VI)}$ , and  $^{53}\text{Cr(VI)}/^{52}\text{Cr(VI)}$ , respectively. The concentration of each species as well as the species interconversion factors ( $\alpha$  and  $\beta$ ) 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  $^{50}\text{Cr}/^{52}\text{Cr}$  and  $^{53}\text{Cr}/^{52}\text{Cr}$  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  $1/2 \times \text{LOQ}$ ,  $\text{LOQ}$  and  $2 \times \text{LOQ}$ , respectively; the other 3 levels corresponded to spiked samples (no CRM is currently available).

LOQs was initially estimated based on the IUPAC criterion ( $10 \times \sigma_0$ ), where  $\sigma_0$  is the standard deviation determined from the measurements of 21 blank samples under repeatability conditions. The LOQs were  $0.030 \mu\text{g L}^{-1}$  and  $0.049 \mu\text{g L}^{-1}$  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  $\beta$  was set to 85 %, which means that the risk of future expected results falling outside these limits is below 15%. The acceptance limits ( $\lambda$ ) were set to  $\pm 30\%$ , which corresponds to intermediate precision coefficient of variation estimated at  $\cong 10\%$  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 Figs. 5a-b.

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

For Cr(VI) determination,  $\beta$ -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  $0.817 \mu\text{g L}^{-1}$ .

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 summarised 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 7-14% 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  $CV_r$  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 ( $\lambda$ ) and the tolerance limits ( $\beta$ ). 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<sup>-1</sup>. 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 ( $\alpha$  and  $\beta$ ) 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,  $\alpha$  and  $\beta$  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<sub>tot</sub> was also determined in these samples and both the speciation and Cr<sub>tot</sub> 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 analyzed here, whereas Cr(III) levels ranged between 2.3 and 4.7  $\mu\text{g kg}^{-1}$ . Additionally, Cr(III) levels are compatibles with Cr<sub>tot</sub>, 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  $\mu\text{g kg}^{-1}$  (semi-skimmed milk) were also carried out (Table 5).

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<sub>tot</sub> 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.

#### **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 3-5  $\mu\text{g kg}^{-1}$ . 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.

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## Figures captions

**Figure 1.** Chemical reactions of Cr(III) with EDTA (a) and of Cr(VI) with DPC (b).

**Figure 2.** Optimisation of the pH for the complexation/derivatisation of Cr(III) with EDTA and Cr(VI) with DPC by the analysis of a standard mixture ( $5 \mu\text{g kg}^{-1}$ ) by SS-ID HPLC-ICP-MS.

**Figure 3.** Optimisation of the complexation/derivatisation time of Cr(III) with EDTA and Cr(VI) with DPC by the analysis of a standard mixture ( $5 \mu\text{g kg}^{-1}$ ) by SS-ID HPLC-ICP-MS.

**Figure 4.** Chromatogram obtained by the analysis of a standard mixture of Cr(III) and Cr(VI) at  $5 \mu\text{g kg}^{-1}$  by SS-ID HPLC-ICP-MS.

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

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

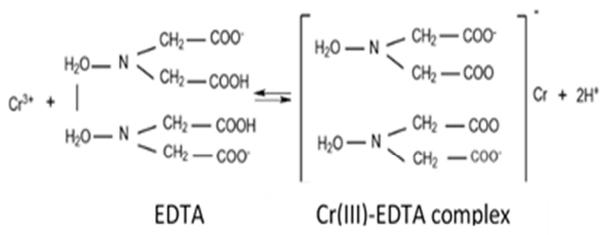
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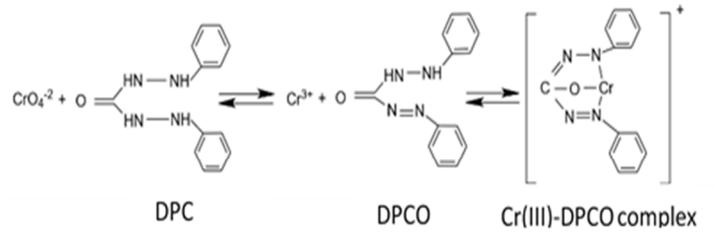
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(a)



(b)

**Figure 1**

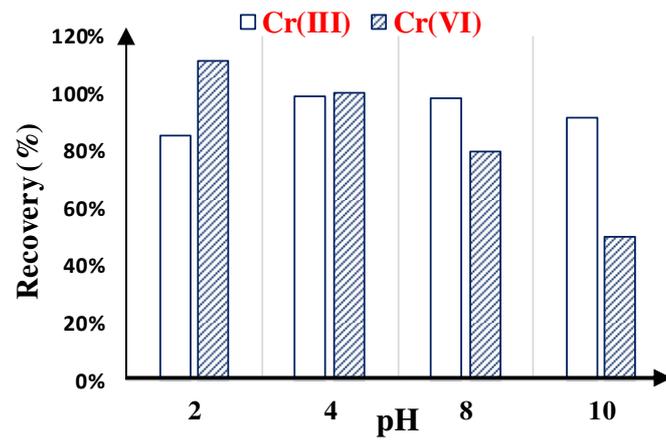
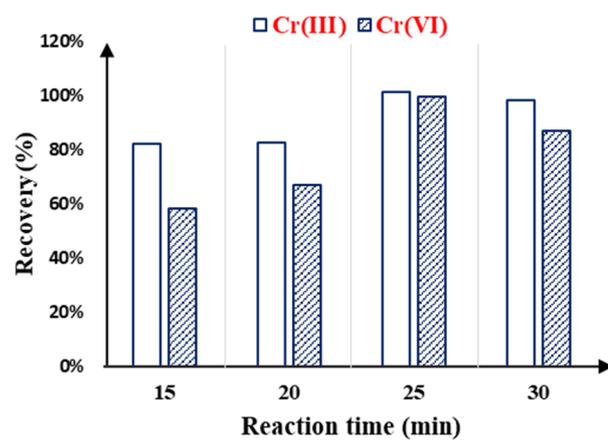


Figure 2



**Figure 3**

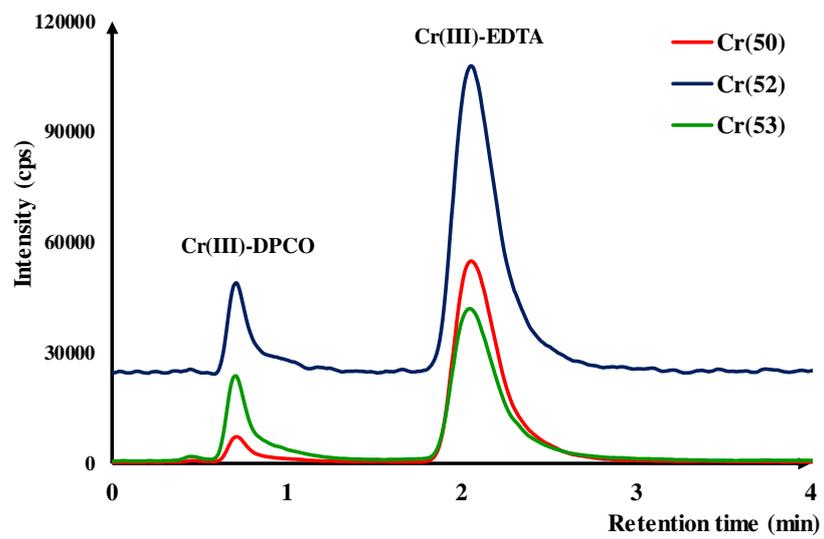


Figure 4

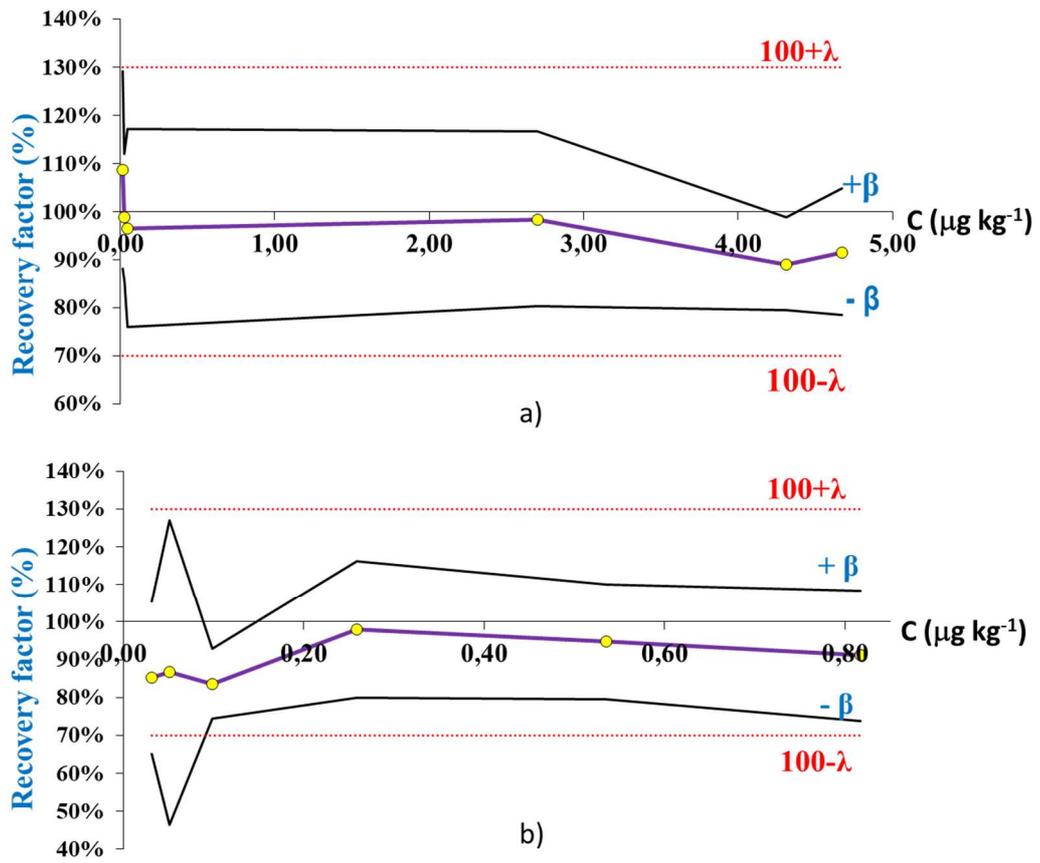


Figure 5

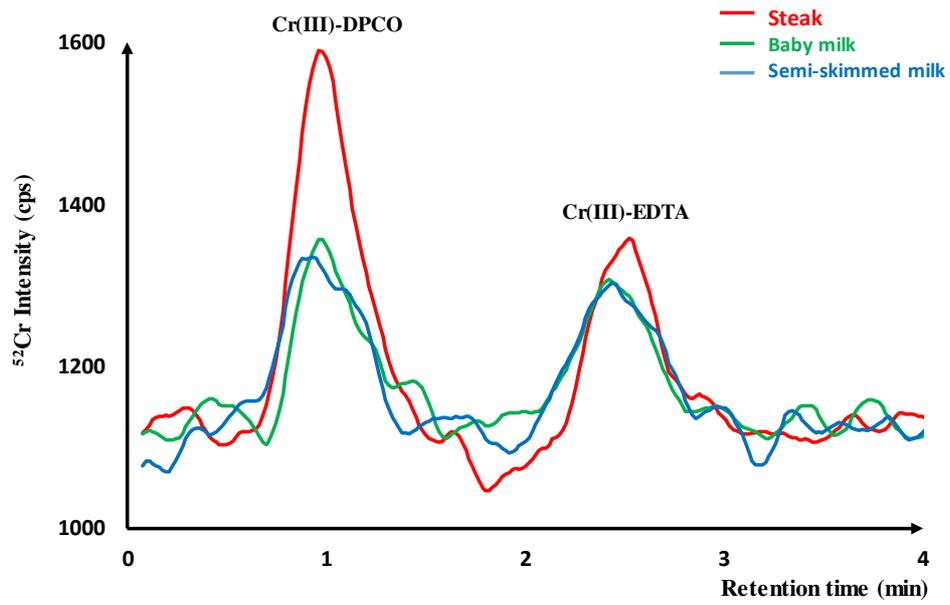


Figure 6

**Table 1**

Optimum ICP-MS and HPLC instrumental parameters for Cr speciation analysis and Cr<sub>total</sub> determination.

Parameter	ICP-MS	
	Thermo iCAP Q (Cr speciation)	Agilent 7700 (Cr <sub>total</sub> )
Plasma gas flow (Ar)	15 L min <sup>-1</sup>	15 L min <sup>-1</sup>
Auxiliary gas flow (Ar)	≅ 0.80 L min <sup>-1</sup>	≅ 0.99 L min <sup>-1</sup>
Nebuliser gas flow (Ar)	≅ 0.92 L min <sup>-1</sup>	≅ 0.95-1.00 L min <sup>-1</sup>
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 <sup>-1</sup>	≅ 4.3 mL min <sup>-1</sup>
Plasma power	1550 W	1500 W
Dwell time	250 ms	100 ms
Monitored isotopes	<sup>50</sup> Cr, <sup>52</sup> Cr, <sup>53</sup> Cr, <sup>13</sup> C	<sup>52</sup> Cr
<b>HPLC separation</b>		
Column	Dionex IonPac™ AG7: 2 mm x 50 mm, 10 μm	
Mobile phase	10 <sup>-2</sup> mol L <sup>-1</sup> HNO <sub>3</sub> + 2.5 % (v/v) MeOH + 0.30 mol L <sup>-1</sup> EDTA (pH = 2.1)	
Injection volume	20 μL	
Flow rate	0.20 mL min <sup>-1</sup>	
Elution mode	Isocratic	
Injector temperature	5 ± 3°C	
Column temperature	30 ± 1°C	
Total acquisition duration	5 min	

**Table 2**

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

Sample	Level <sup>a</sup> ( $\mu\text{g kg}^{-1}$ )	Bias (%)	CV <sub>r</sub>		CV <sub>R</sub>		LOD <sup>d</sup> ( $\mu\text{g kg}^{-1}$ )	LOQ <sup>d</sup> ( $\mu\text{g kg}^{-1}$ )
			Level 1 <sup>b</sup>	Level 2 <sup>c</sup>	Level 1 <sup>b</sup>	Level 2 <sup>c</sup>		
<b>Cr(III)</b>								
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		
<b>Cr(VI)</b>								
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		

<sup>a</sup> genuine level for Cr(III) and spiking level for Cr(VI); <sup>b</sup> Level < 2 $\times$ LOQ; <sup>c</sup> Level  $\geq$ 2 $\times$ LOQ; <sup>d</sup> sample weight of 0.3 g

**Table 3**

Average values  $\pm$ standard deviation (n=12<sup>a</sup>) obtained for the inter-conversion factors ( $\alpha$  and  $\beta$ ) for Cr(III) and Cr(VI) speciation analysis in synthetic (spike) 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.

<b>Sample</b>	<b>Level<sup>b</sup> (<math>\mu\text{g kg}^{-1}</math>)</b>	<b><math>\alpha</math> (%)</b>	<b><math>\beta</math> (%)</b>
Standard solution at LOQ level <sup>c</sup>	<sup>b</sup>	25 $\pm$ 18	55 $\pm$ 17
Baby milk	2.70	0	111 $\pm$ 10
Semi-skimmed milk	4.31	0	112 $\pm$ 9
Steak beef	4.67	0	110 $\pm$ 6

<sup>a</sup> analyses carried out in duplicate in 6 different days during 2 months

<sup>b</sup> genuine level for Cr(III) and spiking level for Cr(VI)

<sup>c</sup> 0.030  $\mu\text{g L}^{-1}$  and 0.049  $\mu\text{g L}^{-1}$  for Cr(III) and Cr(VI), respectively.

**Table 4**

Levels of  $C_{\text{tot}}$  (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 $\pm U^a$ ( $\mu\text{g kg}^{-1}$ )		
	$C_{\text{tot}}^b$	Cr(III) <sup>c</sup>	Cr(VI) <sup>c</sup>
Baby milk	2.30 $\pm$ 0.33	2.64 $\pm$ 0.26	< LOQ (0.049)
Semi-skimmed milk	4.40 $\pm$ 0.62	4.45 $\pm$ 0.27	< LOQ (0.049)
Steak beef	4.65 $\pm$ 0.66	4.73 $\pm$ 0.34	< LOQ (0.049)

<sup>a</sup> expanded uncertainty (k=2); <sup>b</sup> n=2; <sup>c</sup> n=5

**Table 5**

Levels of Cr<sub>tot</sub> (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).<sup>a</sup>

Sample	Level ± U <sup>b</sup> (µg kg <sup>-1</sup> , n=5)					
	Cr <sub>tot</sub> <sup>c</sup>	Cr(III)	Cr(VI)	R_Cr(VI) <sup>d</sup> (%)	Cr(III)+ Cr(VI) <sup>e</sup>	Cr <sub>tot_speciation</sub> / Cr <sub>tot_ICP-MS</sub> (%)
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

<sup>a</sup> Cr(VI) spiking levels: baby milk 0.25 µg kg<sup>-1</sup>; steak beef 0.5 µg kg<sup>-1</sup>; semi-skimmed milk: 1.0 µg kg<sup>-1</sup>

<sup>b</sup> expanded uncertainty (k=2)

<sup>c</sup> n=2

<sup>d</sup> recovery factor for Cr(VI) determination after spiking

<sup>e</sup> sum of Cr(III) and Cr(VI) levels obtained by speciation analysis

