Quantification of total chromium and hexavalent chromium in UHT milk by ETAAS

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Procedures for the quantification of total chromium and hexavalent chromium in UHT milk samples are presented. Total chromium was determined directly in milk with the addition of a surfactant and a mixture of Pd and Mg as a chemical modifier. For the selective separation of hexavalent chromium, the sample pre-treatment consisted in precipitation of proteins and elution of the supernatant through a Chromabond NH_2 column. The metal was eluted with nitric acid. Both total chromium and hexavalent chromium were evaluated by atomic absorption spectrometry with electrothermal atomization using the same instrumental conditions. The detection limits were 0.2 and 0.15 $\mu g\ l^{-1}$ for total chromium and hexavalent chromium, respectively. The linearity ranges under the optimized conditions were 0.2–20 and 0.15–50 $\mu g\ l^{-1}$. For total chromium the precision was 4.9 and 5.7% for the analytical and the over-all procedure, respectively, and for hexavalent chromium 4.3 and 4.9%, respectively. The validation of both procedures was performed by the standard additions method and the recoveries were higher than 93% in all cases. For total chromium, a certified reference material was also used to validate the methodology. The methods were applied to the determination of total chromium and hexavalent chromium in 60 UHT milk samples.

Introduction

Depending on their oxidation state, certain metals can induce adverse actions in biological entities with different toxicity expressions. In some cases they may even promote biological functions or toxicological actions, depending on the metal species. Chromium characterizes this duality very well, because in its two major oxidation states, it is either considered to be a bioelement in the trivalent form or has mutagenic properties in the hexavalent form. Another important biological aspect is that whereas trivalent chromium is poorly absorbed at the gastro-intestinal level, the hexavalent form is extensively absorbed.[1]

The link between human exposure to chromium dust in contaminated industrial environments and lung cancer has been established over the past few years. This occupational situation has constituted, in fact, the principal concern regarding hexavalent chromium exposure.[2] More recently, attention has been given to contamination from industrial wastes of natural and sea-water, soils and, ultimately, food. Consequently, it is very important to evaluate the levels of hexavalent chromium in representative components of the diet as a result of this environmental contamination and or technological procedures.

Speciation of chromium has rarely been performed in foodstuffs, in contrast to water and soils in which most available techniques have already been implemented. Basically, three main procedures for the selective quantification of hexavalent chromium in water have been adoped, *i.e.*, a selective chelation/extraction technique, coprecipitation and column separation. Furthermore, these sample pre-treatments allow concentration of the species, improving the sensitivity of the analytical methods. Using the chelation/extraction technique, Subramanian[3] selectively extracted, with isobutyl methyl ketone (IBMK) Cr^{VI} and simultaneously Cr^{III} plus Cr^{VI}, after chelation with different concentrations of ammonium pyrrolidinedithiocarbamate (APDC), from natural and drinking water samples. Mullins[4] separated Cr^{III} and Cr^{VI} in natural waters by coprecipitation with hydrated iron^{III} oxide, converted total dissolved chromium to Cr^{VI} and extracted with APDC into IBMK. Coprecipitation of Cr^{III} in sea-water with $Ga(OH)_3$ was performed, followed by reduction of Cr^{VI} to Cr^{III} and coprecipitation of Cr^{III} and Cr^{VI} was evaluated by difference.[5] The complexation of Cr^{III} and Cr^{VI} with trifluoroacetylacetone and the different volatilities of the complexes formed were used to determine Cr^{VI} in river water samples.[6]

Undoubtely, the use of columns with several packing materials to separate the two main chromium species is the most common procedure. Preconcentration on ion-exchange resin columns were adopted for chromium(III)/(VI) speciation in fresh waters,[7] underground water and waste water,[8] and water samples from chrome-plating baths.[9] The effectiveness of cellulose with phosphonic acid exchange groups for the preconcentration of Cr^{III} and cellulose with quaternary amine groups for the preconcentration of Cr^{VI} was examined in comparison with conventional ion-exchange resins for speciation analysis in natural water.[10] A method for the field determination of Cr^{VI} in tap and ground water was developed using anion-exchange resin beads or anion-exchange membrane filters.[11]

Preconcentration and separation of Cr^{III} and Cr^{VI} in synthetic aqueous solutions was performed using an alumina column, eluting selectively the two species as a function of pH.[12]

A combination of procedures making use of chelation of species and subsequent separation on columns with several materials was tried with success. Thus, the determination of Cr^{III} and Cr^{VI} in sea-water was achieved by complexing Cr^{III} with quinolin-8-ol and subsequent adsorption on a macroporous resin; reduction of Cr^{VI} to Cr^{III} and applying the same procedure allowed the evaluation of Cr^{VI} by difference.[13] In natural waters Cr^{VI} was preconcentrated selectively on a C_{18} bonded silica column using sodium diethyldithiocarbamate as the chelating agent. The Cr^{VI} chelate was eluted with ethanol.[14] Selective chelation of Cr^{III} with EDTA allows the simultaneous enrichment of Cr^{III} and Cr^{VI} on an anion-exchange resin for speciation analysis in aqueous solutions.[15] Three different analytical techniques, a 1,5-diphenylcarbazide spectrophotometric

method, chelating ion-exchange chromatography (Chelex 100) and ion-pairing RP-HPLC separation were evaluated for the determination of Cr^{VI} in soil extracts.[16]

The quantification of total chromium in milk in both its liquid[17–19] and powder forms[17,20,21] has been performed. However, to our knowledge, the selective quantification of the hexavalent form in this matrix has not been carried out. As milk constitutes an important fraction of the human diet the aim of this work was to develop a method which would allow the selective quantification of hexavalent chromium in such a matrix.

Total chromium was quantified by ETAAS directly in the milk after addition of a surfactant and Pd–Mg as chemical modifier. The quantitative and selective extraction of hexavalent chromium was achieved with a Chromabond NH_2 ion-exchange column after precipitation of proteins and pouring of the supernatant into the columns. The hexavalent chromium selectively linked was eluted with nitric acid and quantification was performed by ETAAS using the same instrumental conditions as for total chromium.

After validation of the implemented methodologies, the quantification of total chromium and hexavalent chromium was performed in 60 UHT milk samples acquired in the local market.

Experimental

Apparatus

Metal quantifications were carried out with a Perkin-Elmer (Norwalk, CT, USA) HGA-700 furnace installed in a Model 1100B spectrometer with deuterium arc background correction, equipped with an AS-60 autosampler and an Epson EX-800 printer.

The analyses were performed using Perkin-Elmer HGA tubes with an integrated platform. A single-element hollow-cathode lamp (Perkin-Elmer Model AAPC 303-6039) was operated at 25 mA, and all data were taken at 357.9 nm. The slit width was 0.7 nm and argon was used as the purge gas, with an internal flow rate of 300 ml min^{-1}. Readings on the spectrometer were taken using the peak area mode.

Materials and reagents

To avoid contamination, all poly(tetrafluoroethylene) (PTFE) materials, pipettes, micropipette tips, autosampler cups and calibrated flasks were immersed for 24 h in freshly prepared 15% v/v HNO_3. Subsquently they were rinsed thoroughly with doubly de-ionized water and dried in a dust-free environment before use.

All acids were of Suprapur grade (Merck, Darmstadt, Germany).

Chromium(III) standard solutions were prepared daily from a 1000 μg ml^{-1} solution (Titrisol, Merck). An aqueous solution of 1000 μg ml^{-1} chromium(VI) was prepared by dissolving 2.829 g of potassium dichromate (AnalaR, BDH, Poole, Dorset, UK) in 1 l of de-ionized water; from this, other dilute working standard solutions were prepared daily.

The chemical modifier used was a mixture of palladium nitrate (Merck), and magnesium nitrate (Merck), prepared by mixing equal volumes of solutions containing 3 and 2 g l^{-1}, respectively, in 15% v/v HNO_3.

A Triton X-100 (Sigma, St. Louis, MO, USA) aqueous solution was prepared at an appropriate concentration and added to all samples and standards used for the determination of total chromium to achieve a final 1% (m/v) concentration in the surfactant.

A certified reference material, SRM 1549 Non-Fat Milk Powder (NIST, Gaithersburg, MD, USA), containing 0.0026 ± 0.0007 μg g^{-1} Cr was used to validate the method described for total chromium.

Chromabond NH_2/3 ml/500 mg columns, (*i.e.*, aminopropyl phase with a 3 ml volume and 500 mg of sorbent) were obtained from Macherey–Nagel (Düren, Germany). These columns were used to separate and concentrate hexavalent chromium selectively both in standards and in milk samples prior to quantification.

Samples

Sixty UHT milk samples of different commercial brands were acquired at random in the local market. Some of these samples were also used to validate the techniques. Total chromium and hexavalent chromium contents were evaluated by applying the implemented methods.

Sample pre-treatment

Total chromium. Milk (1.5 ml) was mixed with 10% Triton X-100 (200 μl) and diluted with de-ionized water to a volume of 2 ml.

Hexavalent chromium. Milk (40 ml) was mixed with 5 ml of 2% sodium acetate (pH 3.5). The sample was vortex mixed and centrifuged at 3000 rpm for 30 min. The supernatant was transferred to another tube, 0.001% alizarin (5 ml) was added and the mixture was agitated for 2 min. Thereafter, 40% sodium acetate was added up to pH 4.9. This solution was homogeneized and poured through the previously conditioned column.

Column conditioning was achieved with two column volumes of 1 M nitric acid followed by two column volumes of distilled water.

Selective retention of Cr^{VI} in the column was quantitatively achieved by passing the supernatant through the column at a flow rate of 4 ml min^{-1}. After adsorption of the sample the column contents were dried under vacuum and the Cr^{VI} was eluted with two column volumes of 2 M HNO_3.

Analytical conditions

Both total chromium and hexavalent chromium were quantified by ETAAS. The instrumental conditions were the same for both cases and are summarized in Table 1.

Results and discussion

Optimization of sample pre-treatment

Total chromium. Almost all of the relevant literature refers to prior digestion of the milk for quantification of total chromium.[17–20] However, in a study carried out on milk and milk powder for the development of methods to quantify several metals, including chromium,[22] this element was quantified directly in the milk or in the slurry after addition of Triton X-100. In order to compare which of the two procedures gives the best results, we tested both using two aliquots of the same milk sample. Briefly, for the digestion preparation, 2 ml of homogenized milk were measured into a PTFE flask, HCl (1 ml) and HNO_3 (2 ml) were added, the flask was heated on a stove overnight and the contents were diluted with doubly de-ionized water to 10 ml. The other milk aliquot (1.5 ml) was

mixed with 10% Triton X-100 (200 μl) and diluted with de-ionized water to 2 ml. Total chromium was measured in both acid solutions. The results were similar and, consequently, the direct procedure was adopted as it was simpler, more economical and free of contamination.

Hexavalent chromium. As the prevalent form of chromium in food is the trivalent state, the crucial step in the measurement of hexavalent chromium consists in the selective and quantitative separation of this species from a complex matrix without disturbing the natural equilibrium between the different species present. Moreover, owing to its expected low levels, it was important to preconcentrate the metal present in the hexavalent form in order to improve the sensitivity of the method.

To our knowledge, no methods for chromium speciation in milk have been published. Our previous experience with the selective separation of Cr^{VI} in solid matrices[23,24] was not transposable to milk. In literature, almost all the procedures for chromium speciation have been applied to environmental samples, namely water and soils. Thus, to extract Cr^{VI} selectively from total milk we first tried the conventional chelation/extraction procedures, and adapted a procedure established by others to extract Cr^{VI} selectively from water.[3] Owing to the emulsions generated when total milk was agitated with organic solvents, the first step consisted of protein precipitation. In the first trial, milk was fortified with hexavalent chromium, proteins were precipitated with acetic acid–sodium acetate buffer (pH 3.5), the supernatant was separated and hexavalent chromium complexation was tried with APDC followed by extraction with IBMK. Other chelants were also assayed, namely ethylenediaminetetraacetic acid, sodium diethyldithiocarbamate, quinolin-8-ol, and a mixture of APDC with sodium diethyldithiocarbamate. In addition to IBMK, chelate extraction was also tried with different organic solvents, namely hexane, light petroleum and chloroform. None of the chelation/extraction systems tried provided efficient recoveries, which were normally less than 25%.

As it is expected that Cr^{VI} is predominantly present in the anionic forms hydrogenchromate and dichromate,[25] an anion exchanger was tried. Thus, a solid-phase extraction method using a silica column modified with NH_2 was tried and, as observed from the validation of the method, this procedure provided a complete and selective extraction of hexavalent chromium from milk supernatant.

Table 1 Instrumental conditions and furnace programmme for the measurement of total chromium and hexavalent chromium

Parameter	Value	
Wavelength/nm	353.7	
Drying temperature/°C	80	200
Ramp/s	15	15
Hold/s	20	20
Ashing temperature/°C	1200	
Ramp/s	30	
Hold/s	30	
Atomization temperature/°C	2400	
Ramp/s	0	
Hold/s	4	
Chemical modifier	3 g l^{-1} $Pd(NO_3)_2$ + 2 g l^{-1} $Mg(NO_3)_2$	
Injection volume of sample/modifier/μl[a]	15/10	
Inert gas	Argon	
Flow rate/ml min^{-1}	300	
Background correction	Deuterium arc	
HGA tubes	With integrated platform	
Gas stop flow	Atomization step	
Measurement mode	Integrated absorbance	

[a] The autosampler was programmed to pipette sequentially 10 μl of the modifier solution and 15 μl of the milk sample/acid eluate/standard solution and dispense them together on the platform.

Analytical conditions and validation of the methodology

The furnace programme and analytical conditions for quantification of chromium are summarized in Table 1. The optimized furnace temperatures and the hold times for all the steps were established with standard acid solutions of chromium, hexavalent chromium, total milk and column eluate containing only hexavalent chromium selectively separated from milk. The best reading conditions, namely atomization and ashing temperatures, were the same for the different samples studied and, therefore, total chromium and hexavalent chromium could be determined with the same spectrometer conditions and the sample absorbance readings could be interpolated directly on the calibration graph.

Calibration against acidified standard solutions was performed for total chromium and hexavalent chromium and the calibration graph was linear over the range 0.15–50.0 μg l^{-1}.

To calculate the detection limits, 20 determinations were carried out on each 0.2% acid solution of chromium nitrate and potassium dichromate for total chromium and hexavalent chromium, respectively. The values were calculated as the concentration corresponding to three times the standard deviation (*s*) of the background noise and were 0.15 μg l^{-1} for both.

The precision of the analytical method was evaluated by measuring the absorbance signals for the same milk sample 20 times for total chromium. For hexavalent chromium the absorbance signals were measured 20 times in the same eluted supernatant of milk. The results obtained were 4.9 and 4.3%, respectively. For evaluation of the precision of the over-all procedure, readings for 20 different samples (total milk and eluted milk supernatant) were taken and the values obtained were 5.7 and 4.9%, respectively.

Recovery studies of the metal were performed for both procedures. For total chromium, aliquots of milk were fortified with three different concentrations of metal standard solutions, mixed with surfactant and the metal was quantified using the established conditions. For hexavalent chromium, the fortified milk was submitted to the over-all pre-treatment (protein precipitation, supernatant separation and elution through the columns) and the metal was quantified in the eluate. The results are presented in Table 2. For hexavalent chromium in milk there was no available reference material. For total chromium, the accuracy of the method was also validated by analysing a certified reference material (NIST SRM 1549). For this purpose, from the powdered milk reference material, 12 aliquots of reconstituted milk were prepared with de-ionized water, mixed with Triton X-100 and absorbance readings were

Table 2 Results obtained in the spike recovery study

Parameter	Total chromium			Hexavalent chromium		
Concentration[a]/(μg l^{-1}) (n = 10)	2.5	5.0	10.0	2.5	5.0	10.0
Recovery ± s (%)	95 ± 4	95 ± 3	97 ± 3	93 ± 3	95 ± 3	94 ± 3

[a] Concentration of metal standard solution added to total milk prior to the application of the different pre-treatment procedures.

Table 5 Total chromium and hexavalent chromium contents (μg l^{-1}) (range and mean values) in 60 milk samples obtained in the local market

Species	Skim milk	Simple half-fat milk	Supplemented half-fat milk	Whole milk
Total chromium	0.95	1.33	0.95	2.70
	(< 0.63–1.77)	(0.65–2.74)	(0.65–1.36)	(1.42–5.70)
Cr^{VI}	< 0.15	0.48	0.40	0.68
	(< 0.15–0.15)	(0.15–0.74)	(0.24–0.60)	(0.20–1.20)

Table 3 Principal constituents of milk[a] used to prepare the simulated matrix used in the interference studies

Constituent	Concentration/g per 100 g	Constituent	Concentration/g per 100 g
Chloride	0.156	Cobalt	0.008
Calcium	0.126	Iron	0.0001
Potassium	0.175	Copper	0.00007
Sodium	0.045	Lactose	5.0
Phosphates	0.075	Citrates	0.176
Magnesium	0.012	Casein	2.5
Manganese	0.005		

[a] From Ref. 26.

Table 4 Deviations from expected values for total chromium and hexavalent chromium obtained in the interference studies

Species	Concentration added/μg l^{-1}	Concentration found[a]/μg l^{-1}	Deviation from expected value (%)
Total chromium	2.5	2.4	4.0
	5.0	4.8	4.0
	10.0	9.8	2.0
Cr^{VI}	2.5	2.3	8.0
	5.0	4.7	6.0
	10.0	9.1	9.0

[a] The results represent the mean values of 10 assays.

obtained using the established conditions. The certified value was 0.0026 ± 0.0007 μg g^{-1} and the value found by applying the implemented methodology was 0.0029 ± 0.0003 μg g^{-1}. The standard additions method was also applied to these certified milk samples for 2.5, 5.0 and 10.0 μg l^{-1} of total chromium and the recoveries were better than 97%.

The stability of hexavalent chromium in milk was evaluated by spiking several portions with three different concentrations of hexavalent chromium standard solutions (10, 25 and 50 μg l^{-1}). After standing for 12, 24 and 48 h the spiked samples were submitted to the over-all procedure and Cr^{VI} was quantified. The recoveries were better than 90% in all instances.

Interference studies

A simulated milk matrix was prepared by mixing the principal organic and inorganic constituents of milk listed in Table 3.[26] Total chromium and hexavalent chromium were then added at three different concentrations (2.5, 5.0 and 10.0 μg l^{-1} for both) to three aliquots of this synthetic milk matrix. The implemented methodologies were applied for the determination of total chromium and hexavalent chromium and the absorbance readings were interpolated on the calibration graph. The deviations from the expected values are presented in Table 4. As can be observed, the deviations found are acceptable and we can conclude that there was no noticeable interference from the principal constituents of total milk.

Application of the implemented methods

The implemented methods were applied to quantify total chromium and hexavalent chromium in 60 UHT milk samples (skim milk, simple half-fat milk, supplemented half-fat milk and whole milk) acquired in the local market. The results are presented in Table 5. For total chromium the values found (< 0.63–5.7 μg l^{-1}) are in agreement with the values obtained by Muzzarelli *et al.*[17] (0.2–3.6 μg l^{-1}and Larsen and Rasmussen[21] (0.5–2.2 μg l^{-1}). More recently, Coni *et al.*[19] quantified total chromium in raw milk and the values found ranged between 0.042 and 0.208 μg g^{-1} dry mass. Considering that the solid extract of raw milk is 130 g l^{-1},[27] and converting the results found by these authors into our units, we found levels ranging from 5.5 to 27.0 μg l^{-1}, which are much higher than those referred to above. This can be explained by the differences in sampling; we analysed commercial brands of UHT milk which are supposed to represent the national milk production with a fat level technologically controlled, whereas Coni *et al.* reported total Cr in raw milk from five specific Italian farms.

In the present study the mean values found for the monitored samples were 0.95, 1.26 and 2.7 μg l^{-1} in skim, half-fat (simple and supplemented together) and whole milk, respectively. We are not able to state whether a relationship exists between the decrease in total chromium level and the decrease in the level of fat in milk.

As has already been mentioned, we have no knowledge of methodologies to quantify Cr^{VI} in milk and, consequently, this is the first time that this species is evaluated in such a food. The values found (< 0.15–1.20 μg l^{-1}) are, in terms of mean values (< 0.15–0.68 μg l^{-1}), about 2–4 times lower than those for total chromium. As hexavalent chromium is well absorbed through the gastrointestinal tract and is a genotoxic species, we cannot foresee a risk from such levels in milk.

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