CHROMIUM CONTENT IN DIFFERENT KINDS OF SPANISH INFANT FORMULAE AND ESTIMATION OF DIETARY INTAKE BY INFANTS FED ON RECONSTITUTED POWDER FORMULAE

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Short title: Chromium content of Spanish Infant Formulae

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Abstract

The essentiality of chromium in humans is well documented. Trivalent chromium, main chemistry form found in foods, is essential for maintaining normal glucose metabolism. Because of analytical difficulties, several literature reports of chromium content of foods, especially for the lower levels, show large variability and they should be interpreted with caution for a valid interpretation of reliable results. A Zeeman background correction transversely-heated graphite furnace atomic absorption spectrometry was used to determine the chromium content of 104 different infant formulae (cow's milk and soy protein based) marked in Spain following an acid attack sample preparation procedure in a closed, pressurized and microwave digestion unit.

Mean and range chromium values, regarding types and main protein-based infant formulae are presented. Additionally, the influence of the type of container used, the impact of industrial process from different manufacturers and the physical state (powder and liquid formulae) on chromium levels is also discussed. In general, the infant formulae contain a higher chromium concentration than that found in human milk (reference range: 0.20 – 8.18 $\mu g\ l^{-1}$), particularly in case of hypoallergenic (18.16 ± 7.89 $\mu g\ l^{-1}$), lactose-free (11.37 ± 3.07 $\mu g\ l^{-1}$), preterm (11.48 ± 3.15 $\mu g\ l^{-1}$) and soya (10.43 ± 4.05 $\mu g\ l^{-1}$) formulae. The maximum theoretical estimated intake of infant fed on studied formulae was lower than the upper limit safety for trivalent chromium of 1 mg kg^{-1} (14 $\mu g\ Kg^{-1}$ b.w. day^{-1}) recommended by the experts of Council for Responsible Nutrition (CRN ULS, 2004), amounting to about 10 %, 15-18 % and 26 % for standard (adapted and follow-up) and toddler; soya, lactose-free and preterm; and hypoallergenic formulae, respectively.

Therefore, manufacturers are called for continued effort to routinely monitor chromium levels, mainly for specialised and preterm formulae, and at the same time, might consider the inclusion of labelling value for chromium at least in these complex formulations.

Keywords: Chromium, Infant formula, Graphite furnace atomic absorption spectrometry (GF-AAS), Drinking water, Daily Intake.

Introduction

The essentiality of chromium in humans is well documented. Trivalent chromium, main chemistry form found un foods, is essential for maintaining normal glucose metabolism. Signs and symptoms of deficiency have been shown in infants suffering from kwashiorkor or protein-energy malnutrition, found improvement in impaired glucose tolerance after chromium supplementation (Anderson 1989). Nevertheless no literature report has described the response to chromium supplementation in infant fed breast milk or infant formula presently in use. Probably because those infants have not become obviously chromium deficient, being chromium intake likely adequate for pre- or term infants (Zlotkin 2003).

Human milk feeding is the preferred nutritional model for infants during the first four to six months of life. Its composition, including micronutrients, is therefore used as a reference for infant formula manufacturing, due to the fact that infants fed on breast milk do not generally show trace element deficiencies. Upon cautious inspection of the data and references from literature, it is thus possible to suggest a reference chromium range of 0.20 – 8.18 $\mu g\ l^{-1}$ for human milk (Table I). Moreover, the chromium concentration does not show significant variation (Deelstra et al. 1988) or exhibits slight rising tend (not significant) during the lactation period (Wappelhorst et al. 2002).

[Insert table I about here]

A reliable assessment of chromium intake is essential to avoid the potential risk of deficiency or, far from it, to notice the principal sings of adverse health and toxic effects for infants. In this respect, the estimation of the theoretical chromium intake by infants exclusively fed on infant formulae and the comparison with the recommended values of Adequate Intake (AI) and Tolerable Upper Intake Level (UL), seems to be advisable (FNB, 2001).

International paediatric organizations found insufficient data on which to recommend a minimum or maximum chromium content of standard infant formulae (Raiten et al. 1998). It was noted that as this micronutrient is ubiquitous in nature a formula based on usual food ingredients does not need any chromium addition. However, the Australia New Zealand Food Authority agreed with no need for the addition or supplementation of chromium in adapted and follow-up formulas but suggests an amount of no less than 0.36

μg 100 KJ^{-1}, (10.0 μg l^{-1}) and no more than 2.0 μg 100 KJ^{-1}, (56.7 μg l^{-1}) for an infant formula product for specific dietary use, as preterm, specialised or soya formulae, listing the chromium sulphate as exclusive additional permitted chemistry form (ANZFA 2002, 2005).

Furthermore progress in the understanding of chromium nutritional chemistry has been frustratingly slow, hampered by serious analytical difficulties encountered in the accurate measurement of very low chromium concentrations in biological and food samples. Several literature reports of chromium content of foods, especially for the lower levels, show large variability and they should be analysed with caution for a valid interpretation of reliable results (Miller-Ihli 1996).

Today many of analytical problems found have been overcome by the development of closed microwave attack and highly sensitive analytical instruments. Electrothermal atomization atomic absorption spectrometry provided with Zeeman background correction is recognized to be suitable for accurate chromium determination in food digests (Tinggi et al. 1997). Regardless chromium analysis at low levels is still considered a challenge to the technical skill of the analyst (Kubadda et al. 2003), in view of the fact that contamination of samples can relatively easily occur, although all kind of precautions were taken, a strict analytical quality assurance programme must be adopted to obtain reliable results (Krachler et al. 2000).

The aim of the present survey was twofold. First, the quantification of chromium levels in the majority of infant formulae sold commercially in Spain is presented. Additionally, potential sources of exogenous (processing, manufacturing and packaging practices) and endogenous (raw material) origin on chromium levels is also discussed. It might be suggested that elevated chromium levels were the result of the process of manufacture such as different studies have reported about the probability that much of chromium associated with processing foods is exogenous chromium introduced during processing (Miller-Ihli 1996, Reilly 2002, 2004). The processing industry uses almost exclusively stainless containing 13 – 30 % chromium in its processing equipment material. Thus, contamination in infant formula with chromium should be expected.

And finally, the determination of total chromium is appropriate to evaluate the essential chromium food composition data. In this nutritional context, the obtained information is of

paramount importance for the reliable assessment of daily chromium intake of infants fed on studied infant formulae. Therefore, the estimation of theoretical chromium intake from both, infant formulae and additional contribution of tap water to reconstitution of powder formulae; and its comparison with recommended values of Adequate Intake (AI) and Tolerable Upper Intake Level (UL) is advisable, since data on the potential adverse effects of chromium intake are not conclusive (FNB 2001) and, thus caution on fortification practices is requested to manufactures of infant formulae.

Material and Methods

Infant formula and water samples

104 infant formulae samples of different commercial brands, included both powder (n = 97) and ready to use (n = 7) formulae, were acquired from specialised markets and pharmacies in Pamplona (Spain). The infant formulae studied were either milk- (n = 97) or soy- (n = 7) based types. Different types of cow's milk-based formulae were classified in: Preterm formula (n = 7, product specially formulated to satisfy particular needs of infants born prematurely or of low birth weight), Adapted formula (formula which satisfies the nutritional requirements of infants from the first day to 4-6 months of age; *Type 1*: formula adapted following the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommendations (n = 21) (Koletzko et al. 2005) and *Functional 1*: formula adapted with special additives or new ingredients (n = 13), Follow-up formula (formula which constitutes the principal liquid source of nourishment in a progressively diversified diet for infants aged from six months of age; *Type 2*: formula adapted following the ESPGHAN recommendations (n = 20) and *Functional 2*: formula adapted with special additives or new ingredients (n = 10), Toddler formula (formula similar to Follow-up but specially designed for infants from 1 to 3 years age old, n = 6), Hypoallergenic formula (specialised formula which are normally based in hydrolysed proteins, n = 10), Lactose-free formula (Formula designed as principal source of food for lactose intolerant infants, n = 10).

Drinking water samples were collected from eighty eight sampling points, selected to be representatives of the Community of Navarra (Spain), according to a population census provided by the local office of the National Statistical Institute. A strict protocol was established to carry out tap water sampling. Tap water samples were collected in acid-washed low density polyethylene containers. All samples were acidified with sub-boiling

nitric acid until approximately pH = 2, frozen and kept at – 20 ºC until required for analysis.

Reagents and solutions

All chemicals used were of highest purity available and all the material were nitric acid-washed (5 %, v/v) for at least 6 days and given them a thorough rinse with ultrapure water.

Standard and sample solutions were prepared with ultrapure deionised water type Milli-Q (resistivity 18 MΩ · cm). Concentrated 65 % nitric acid (suprapur grade, Merck, Darmstadt, Germany) was additionally purified by sub-boiling distillation (Hans Kürner, Rossenheim, Germany). Working standard solution were prepared in subboiling nitric acid to match the acid concentration in digestion solutions from a stock 1000 mg l^{-1} standard solution supplied by Merck. A solution of magnesium nitrate (0.150 g $Mg(NO_3)_2 \cdot H_2O$ - suprapur Merck- were diluted in 100 ml with ultrapure water) was used as matrix chemical modifier.

The Standard Reference Material 1549 – non-fat milk powder – (National Institute of Standards and Technology, Gaithersburg, USA) was used for validation of the methodology.

Analytical procedures

Special care was devoted to minimize the risk of adventitious contamination when handling. Infant formulae were opened in the clean room under flow laminar bench using talc-free gloves (Rotiprotect® Carl Roth, Karlsruhe, Germany). All steps of sample preparation procedure were carried out in a laboratory equipped for trace element analysis and following a strict handling rules.

A infant formula sample amount of 0.3 – 0.5 g accurately weighted (powder formula) or 3 ml of ready to use formula were placed into high pressure teflon digestion vessel and treated with 8 ml of sub-boiling nitric acid in a microwave digestion system (Ethos Plus, Millestone s.r.l., Sorisole, Italy). The optimised microwave digestion programme applied included two steps: 25-170 ºC for 10 min. and 170 ºC for 10 min., both at 1000 W, followed immediately by ventilation at room temperature. Digested samples were diluted to 10 ml in a volumetric flask with ultrapure water and finally, transferred to pre-cleaned polypropylene tubes. Solutions were kept stored frozen at - 20 ºC until analysis. All infant formula samples were digested in triplicate.

Chromium concentrations were determined in sample digestions by Zeeman background correction graphite furnace atomic absorption spectrometry (ZGF-AAS, Perkin Elmer Aanalyst 800, Norwalk, CT, USA), under the optimized operating conditions shown in table II. Transversely-heated graphite tubes with end caps supplied by Perkin Elmer were used. A single element hollow cathode lamp (Perkin Elmer) was operated at 25 mA. Samples and standards were atomised and all data were taken at 357.9 nm with a slit width of 0.7 nm. Argon of 99.9999 % purity at 250 ml min^{-1} flow was used as internal gas. Readings on the spectrometer were taken using the peak area mode.

[Insert table II about here]

Injections (10 μl sample and 10 μl matrix modifier) were made in triplicate. Measurements were accomplished by direct calibration using aqueous standards (0-10 μg l^{-1}) made up each day by dilution from stock standard solution with enough subboiling nitric acid to a final acid concentration similar to digested samples. The analytic sensitivity expressed as the characteristic mass was calculated to be 4.8 pg.

Quality assurance

An strict analytical quality control programme was employed during the study.

Limit of detection and quantification: Blank reagent values were monitored throughout the survey and subtracted from the measured sample concentration to calculate the final result in infant formulae samples. Detection limit (LOD) in the acid digest solution was calculated according to the definition and criteria established by IUPAC ($X_b + 3\sigma_b$) as the average of three times the standard deviation of the reagent blank, setting at 0.15 μg l^{-1} (n = 6) equivalent to 0.51 μg l^{-1} when expressed in terms of infant formula, calculated on the basis of the analytical sample weight (0.4 g), volume of dilution (10 mL) and the mean percentage of reconstitution (13.5 g of powder formula per 100 mL of water) for powder formulae or analytical sample (3mL) and dilution (10 mL) volume in the case of ready-to-use formulae. In the same way,. the limit of quantification (LOQ = $X_b + 10\sigma_b$) was set at 0.18 μg l^{-1} (n = 6) that corresponds to 0.62 μg l^{-1} (expressed in terms of infant formula wet weight). Blank reagents in tap water analysis, consisting of ultrapure deionised water and reagents, were subjected to a similar sample preparation, storage and mixing with buffer solution, setting the LOD at 0.08 μg l^{-1}.

Accuracy: The accuracy of the analytical chromium method was verified by analyzing the standard reference material SRM 1549 (non-fat milk powder), prior to the analysis of unknown infant formulae samples. 1g. of reference material was digested in the microwave

apparatus and diluted up to 10 mL with ultrapure water. The level of concentration determined in the acid solutions (mean ± standard deviation value: 0.240 ± 0.009 μg l^{-1}), close to the LOQ, provides the reliability of the analytical method. The found value (mean ± standard deviation at 95 % confidence interval: 2.40 ± 0.06 ng g^{-1}, n = 12) show acceptable good agreement with SRM chromium certified value (2.6 ± 0.7 ng g^{-1}). Moreover, analytical recoveries of spiked chromium at different amounts (2, 4 and 8 ng) before digestion in SRM 1549 samples were satisfactory (95.5 – 104.1 %). Recovery assays were carried out at different weights (0.75 and 1.0 g) of SRM 1549 samples, obtaining similar results. This test is particularly sensitive at low concentration to reveal analytical errors and it could be considered as an evaluation of accuracy, complementary to recovery studies (Dabeka and Ihnat 1996).

Precision: The instrumental precision estimated from six consecutive measures of the same infant formula (10.18 ± 0.09 μg l^{-1}) was 0.8 %. The relative standard deviation of the method determined in the intra-assay precision (aliquots of samples measured during the same assay session) for the blank reagent (14.0 %), SRM 1549 samples (3.6 %) and internal aqueous control (2.4 %) were comparable with those calculated for the inter-assay precision (aliquots of the same sample measured in different days), 21.6 %, 7.3 % and 4.8 %, respectively.

Quality control: An internal aqueous quality control (1.04 ± 0.02 μg l^{-1}, range = 0.93 – 1.14, n = 36) was run concurrently with blank reagent and SRM 1549 digestion, throughout the course of the analysis and always previously measured to each batch of samples, in order to satisfy the criteria established in the quality program (lower and upper action limits: 0.85 and 1.15 μg l^{-1}, respectively) and to provide on – going quality control information.

Statistical Analysis

All statistical analyses were run using SPSS v.11.0 for Windows program. The Kolmogorov-Smirnov statistic was used to assess the normality of the data distributions. Different groups of infant formula samples (classified as types or predominant protein content) were compared through non-parametric Kruskal-Wallis and Mann-Whitney U-test or Wilcoxon Signed Rank test for independient or paired groups, with statistical significance set al $p < 0.05$).

Results and Discussion

Chromium levels in infant formula

Table III summarizes the chromium concentrations for each of the different types of infant formula studied. The results are expressed as μg l^{-1} according to the manufacturer's dilution (Adapted: 12.80 – 15.9 %, Follow-up: 12.7 – 16 %; Toddler: 14.0 – 16.0 %, Preterm: 14.7 – 15.9, Hypoallergenic: 12.8 – 15.2, Lactose-free: 12.6 – 14.0 %, Soya: 12.7 – 13.5 %) instructions, owing to the fact that usually levels of trace elements in formulae are expressed in literature on a ready-to-use basis.

In a first approach, the complete distribution of chromium content in studied infant formula shows a positive skew (skewness value 0.89) with scores clustered to the left at the low values and a positive kurtosis value (0.32) indicating a rather peaked distribution (clustered in the centre). In spite of the result of the test of normality suggests a violation of the assumption of normality, fact quite common in larger samples (Pallant 2003), the inspection of the normal probability plot (Normal Q-Q plot) and the assessment of normality in the different distributions of distinct types of infant formula allow the use of mean (± standard deviation at 95 % confidence interval) and the range as useful information concerning the statistical description of data. Median is also included in table III as informative value and, at the same time, the most representative parameter in those distributions with short number of samples.

[Insert table III about here]

Globally, considering all 104 formulae together, chromium concentration determined (9.60 ± 1.13 μg l^{-1}, range 2.06 – 46.50) is higher than that found in mature human milk. Chromium values in breast milk reported from other researchers exhibit large differences. Mainly, older data tended to be high due to the lack of precautions to avoid contamination of the sample and the inadequate analytical quality assurance scheme adopted to obtain reliable results.

In this respect, as might be expected due to analytical difficulties, a wide variability in infant formula chromium content reported in literature is highlighted in table IV. Determined chromium values are in good agreement with the findings of most authors in formulae from different countries. However, values reported in some surveys (Biego et al. 1998, MAFF 1999) are considerably higher probably due to the limited number of samples or insufficient control of contamination in sample handling.

[Insert table IV about here]

Undoubtedly, the differences in the chromium contents in different commercial formulae studied are of special relevance. In this respect, the box plots are useful to compare the

different types of infant formula (Figure 1) and, simultaneously a non-parametric statistical analysis due to the short number of formulae ($n < 12$) in six of 9 studied groups, by means of Kruskall-Wallis test was carried out, establishing significant differences ($p < 0.001$).

Therefore, it is possible to try to establish a relation of dependence based on chromium content in accordance with the type of infant formulae, the complexity of manufacturing process or even better, the predominant protein source used in infant formula manufacture.

[Insert figure 1 about here]

The higher chromium levels provided by hypoallergenic (18.16 ± 7.89 μg l^{-1}), lactose-free (11.37 ± 3.07 μg l^{-1}), preterm (11.48 ± 3.15 μg l^{-1}) and soya (10.43 ± 4.05 μg l^{-1}) formulae greatly exceed those values found in adapted (type 1: 6.29 ± 1.21 μg l^{-1}, functional 1: 7.77 ± 2.22 μg l^{-1}) formulae (U-Mann Whitney test; Type 1 – HA: $p < 0.001$, Type 1 – Preterm: $p = 0.004$, Type 1 – Lactose-free: $p = 0.002$, Type 1 – Soya, $p = 0.007$; Functional 1 – HA: $p < 0.001$, Functional 1 – Preterm: $p = 0.040$, Functional 1 – Lactose-free: $p = 0.050$). Follow-up (type 2: 8.73 ± 2.17 μg l^{-1}, functional 2: 8.35 ± 1.55 μg l^{-1}) and toddler (9.68 ± 6.71 μg l^{-1}) formulae contain an intermediate level (U-Mann Whitney test; Type 2 – HA: $p = 0.001$; Functional 2 – HA: $p = 0.001$).

Nowdays, manufacturers of infant formulae currently in use do not report any chromium content on the label, probably because no supplemental chromium is added or/and fortification practices is still exercised with caution by manufacturers of infant formulae. Thereby another factor should exists which explain the large variability of chromium observed in infant formulae. In this respect, our findings could clarify the sources or factors with special influence on chromium content in infant formulae.

Firstly, the endogenous chromium content is contributed by raw material (cow's milk or isolated soy protein). Generally, cow's milk contains low and wide variable levels of chromium attributed to a different kind of feed consumed by these animals and to their different chromium content (Bratakos et al. 2002). In contrast, soy beans been characterized as a good source of chromium though its content is variable owing to soil composition and agricultural practices (Jorhem and Sundström 1993). Thereby, higher levels found in soya formulae could point out to the intrinsic chromium naturally present in soy protein.

Following, it is known that chromium in valence state III, has a strong tendency to form coordinate and chelate compounds and interacts with protein molecules in order to make up strong and stable cross-links (Bratakos et al. 2002). Therefore, it is not surprising that

studied infant formulae provide a very different content according to the predominant protein source (milk fraction or main protein type) used in the manufacture. A statistical review of the data (Kruskall-Wallis test) in table V, establishes significant differences ($p < 0.001$) in infant formulae analysed when are grouped focusing on the main protein. Apart from the formula based on free-aminoacids, the highest chromium content is founded in those formulae based on hydrolysed protein (whey hydrolysed: 13.03 ± 2.82 μg l^{-1} and casein hydrolysed: 15.03 ± 5.52 μg l^{-1}); followed by a second group integrated by casein and soy based formulae with 11.37 ± 3.04 and 10.43 ± 4.05 μg l^{-1}, respectively; and, lastly, those formulae that include exclusively cow's milk protein (whole-milk based: 9.12 ± 8.54, skim-milk based: 8.20 ± 1.27 and whey based: 6.41 ± 1.08 μg l^{-1}), which degree of chromium enrichment is owing to the cow's milk fraction. This fact is highlighted in the standard (adapted and follow-up) formulae, as can be observed in table VI (e.g. in type 2 of follow-up formulae, whole-milk based: 12.85 ± 5.55; skim-milk based 7.74 ± 2.28 and whey based: 5.96 ± 2.24 μg l^{-1}). Undoubtedly, the intrinsic chromium is likely the most source of this element in the formulae with lower technical complexity of manufacture.

[Insert tables V and VI about here]

On the other hand, migration from equipment and fittings may explain the high chromium contents found in soya, lactose-free and preterm formulae. In addition, the highest levels of chromium in casein and whey hydrolysed formulae are a direct consequence of the aggressive protein hydrolysis treatment which involves the use of chemicals and additives. Moreover, other source of contamination must be considered in these formulae, the addition of several ingredients as calcium and phosphate salts, vitamins, other minerals and trace elements.

Another potential source of chromium contamination to take account in infant formulae is the packaging in metal cans. The pasivation treatment in which chromium compounds is used to increase the lacquer adherence and resistance to oxidation of surface, can contribute significantly to the level of pick-up by canned infant formula (Reilly 2002, 2004). Chromium levels for both powder and ready to use formulae are summarised in table VII. It was not possible to establish statistical differences (Wilcoxon's test, $p > 0.05$) between the pairs of infant formula market in both aggregation form. However, powder formulae showed a tendency to be higher chromium content ha in ready to use formulae. Furthermore, general chromium levels in canned formula were consistently higher than those commercialised in aluminium bags (results not shown) for each types of infant formulae studied.

[Insert table VII about here]

In conclusion, the chromium concentration of the studied infant formulae come from both origin endogenous and exogenous, although the relatively high levels found in preterm, soya and specialised formulae are most likely the result of leached from contact with stainless steel during manufacturing or packaging rather than fortification practices, given that chromium concentrations is not listed in none infant formula analysed being currently considered as a voluntary element for nutritional information labelling.

Daily dietary chromium intake

The daily intake of this element has been estimated using the chromium concentration mean value determined in the different types of formulae according to feeding tables and dose recommended by manufacturers, and taking into consideration the different stages of infancy (0-2 weeks: 600 mL, 3-4 weeks: 900 mL, 2 month: 1050 mL, 3 month: 1100 mL; 4-5 month: 1250 mL, 6 month: 1000 mL, 7-12 months: 960 mL, 2 year: 560 mL, 3 year: 560 mL), under the assumption that most infants observe similar feeding regimens and only formulae. Anyway, it is necessary to underline the fact that most of infants fed on follow-up formulae also receive beikost (complementary feeding) nutrients from four-five months old which may increase the chromium intake from other food sources better than formulae.

The AI was set by the National Research Council based on chromium intake of infants principally fed on human milk, due to no functional criteria of chromium status reflect their dietary intake. These requirements have been established as 0.2 μg day^{-1} (29 ng $kg^{-1}day^{-1}$), 5.5 μg day^{-1} (611 ng $kg^{-1}day^{-1}$), and 11 μg day^{-1} (1222 ng $kg^{-1}day^{-1}$) for infants 0-6 months, 7-12 months and 1-3 years old, respectively (FNB, 2001).

The daily intake values calculated of chromium from the newborn period to the third year of life are show in table VIII. The chromium supplied by different infant formulae is clearly higher, approximately 45 and 75-100 times with standard and specialised (hypoallergenic and lactose-free) formulae respectively, than that provided by human milk. All studied formulae provided amounts of chromium intake higher than recommended AI.

[Insert table VIII about here]

Unfortunately, the differences in bioavailability of chromium from breast milk and infant formulas are not known (Goldhaber 2003, FNB, 2001). However, chromium absorption via the gastrointestinal tract has been inversely related to dietary intake (Anderson and Kozlovsky 1985), depending upon chemical form, oxidation state and other factors (lack or presence of ascorbic acid, carbohydrates, phytate…) (Iyengar 1989). Chromium absorption is approximately 2 % at daily dietary intakes of 10 μg, reaching 0.5 % when this is increased at least 40 μg. Even though taking into account the percentage of absorption estimated, the studied formulae provide amounts close to the AI.

Preterm infants are a special group of risk that may be susceptible to chromium deficiency. In this sense, the Canadian Paediatric Society (CPS 1995) has proposed a chromium requirement for low birth weight and premature infants (52-98 ng $kg^{-1}day^{-1}$) distinct from those for full-term infants. Figure 2 compares the daily chromium intake of infants fed on preterm formulae and human milk (Spanish and Reference range) with this recommended CPS-AI value. These formulae provide a chromium intake slightly greater than standard formulae and greatly exceeds, approximately 55 times, that supplied by both lower level human milk and recommended adequate intake.

[Insert figure 2 about here]

Finally, as mentioned above, several dietary components, different chemical components and trace elements deficiency state could influence chromium absorption in both sense enhancing or decreasing. Therefore, taking into account the markedly amount of chromium present in infant formulae analysed, it seems to be demanded to determine more definitively the safety of infant formulae. It is true that no data or reports have shown categorical adverse effects of chromium III in human nutrition, also it was suggested that tolerable limit for chromium is quite high with an extraordinary wide margin of safety. Thus, the Council for Responsible Nutrition (CRN) set its Upper Limit Safety (ULS) at 1 mg day^{-1} (any form of Cr III) for adults (Hathcock 2004) based on a large number of trials and official reviews (FNB 2004, EVM 2003), that extrapolated to infants according to standard weight tables, reaches a maximum intake of 0.014 mg $kg^{-1}day^{-1}$ (0-2 weeks: 55 mg day^{-1}, 3-4 weeks: 63 mg day^{-1}, 2 month: 76 mg day^{-1}, 3 month: 87 mg day^{-1}; 4-5 month: 105 mg day^{-1}, 6 month: 112 mg day^{-1}, 7-12 months: 126 mg day^{-1}, 2 year: 182 mg day^{-1}, 3 year: 224 mg day^{-1}).

Luckily, the maximum theoretical estimated intake of infant fed on studied formulae was lower than the ULS (figure 3). Standard (adapted and follow-up formulae) and toddler formulae contribute the lowest chromium intake (about 10 % ULS); soya, lactose-free and

preterm formulae gave an intermediate intake (15-18 % ULS) and hypoallergenic formulae provided the highest intake (26 % ULS).

[Insert figure 3 about here]

Thereby, taking into account the additional margin of safety established by upper limit, it seems appropriate to estimate the maximum chromium concentration in a hypothetical case of an infant formula to exceed the ULS. Following the recommended feeding of manufacturers, the limit of chromium in infant formula should range from 68 to 91 μg l^{-1}. Fortunately, none of infant formula studied reach so great level of chromium concentration. Nevertheless, it should be kept in mind that the level of this element as hexavalent chromium is unknown and, both, increased absorption of this chemistry form and low stomach acidity might represent a particular vulnerability situation for infants. Its quantification could be useful in the risk assessment (Soares 2000).

Adventitious chromium contribution of drinking water

Total chromium concentrations in drinking water are usually less than 2 μg l^{-1}, although levels as high as 120 μg l^{-1} have been reported in several countries (WHO 2003). The WHO guidelines (WHO 1996) has considered the level of 50 μg l^{-1}, as a provisional guideline values, which is considered to be unlikely to give rise to significant risk to health. At this level, tap water appears to be an important source of chromium, contributing substantially to daily intake of infants.

The chromium content found in drinking water samples collected in 88 representative sampling points from the Community of Navarra, was 0.74 ± 0.19 μg l^{-1} (range 0.18 – 4.65 μg l^{-1}). This median chromium value was used to calculate, based on feeding tbles for infant formula reconstitution, te complementary contribution from tap water to daily chromium intake of infants fed on powder infant formula. The theoretical chromium intake, including minimum and maximum values, is shown in table VIII. Comparatively, the influence of chromium level in drinking water on the final concentration of reconstituted powder infant formulae is limited. Nevertheless, it is representative that the supplied chromium by tap water might find by itself the AI during the first six months of life (approximately 2 - 4 times of the considered adequate intake) and at the same time, it match the amount provided by Spanish human milk in the feeding of preterm infants (figure 2).

Conclusions

The findings of this survey demonstrate that chromium levels analysed in infant formula are markedly greater than those found in the reference range from human milk or those level (0.17 μg l^{-1}) calculated from the AI value to be necessarily provide by an infant formula to fulfil the recommendation. Therefore, the measurement of total chromium routinely by manufacturers and its inclusion in labelling information list will be of interest, especially in those complex infant formulae designed for special uses. In this respect, viewed from a research standpoint, speciation surveys (Bratter et al. 1998) are required in the future to research both, exogenous or intrinsic chromium source of origin and valence state of chromium released from stainless steel surfaces (Lameiras et al. 1998) with the objective of establish the total harmlessness and safety of infant formulae.

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Table I. Chromium content (μg l^{-1}) of mature human milk from different countries

Country	*Reference*	*Cr (μg L^{-1})*	*n*	*Range*
Austria	Krachler et al. 2000	24.3	27	< 0.8 – 163
Belgium	Deelstra et al. 1988	0.14 ± 0.054	10	0.10 -0.23
Finland	Kumpulainen et al., 1983	0.39 ± 0.15	20	0.20 – 0.53
Philippines	WHO/IAEA 1989	3.46	10	0.97 – 6.13
Germany	Wappelhorst et al. 2002	10.8	19	3.1 – 19.4
Guatemala	WHO/IAEA 1989	1.17	9	0.34 – 2.52
Hungary	WHO/IAEA 1989	0.78	11	0.23 – 2.24
Italy	Aquilio et al. 1996	1.2 ± 0.5	110	-
	Coni et al. 1990	9	9	2 -59
	Muzzarelli et al. 1983	-	8	0.5 -3.6
Nigeria	WHO/IAEA 1989	4.35	4	3.44 – 8.18
Spain	Cocho et al. 1992	1.56 ± 0.78	21	0.27 – 3.00
	Rivero et al. 2001	0.8 ± 0.3*	1	-
Sweden	WHO/IAEA 1989	1.48	6	0.61 – 4.34
United Kingdom	MAFF 1996	-	4	< 16 - 40
USA	Casey and Hambidge 1984	0.27 ± 0.11	49	0.06 – 1.56
	Anderson et al. 1993	0.18 ± 0.02	17	-
	Mohamedshah et al. 1998	0.22	20	0.09-0.46
Japan	Gunshin et al. 1985	6.5	26	1.40 – 21.0
Zaire	WHO/IAEA 1989	1.07	11	< LOD – 3.99

*deviation standard from five replicates

Table II. Graphite furnace program for the determination of chromium in infant formulae.

Step	***Temperature*** *(ºC)*	***Ramp*** *(s)*	***Hold*** *(s)*	***Argon flow*** *(ml min^{-1})*	***Read on***
Drying	130	15	40	250	-
Charring	1400	10	20	250	-
Atomization	2300	0	5	0	Yes
Cleaning	2500	1	5	250	-
Cooling	20	-	-	250	-

Table III. Chromium concentrations in different types of infant formulae investigated (µg l^{-1}).

Infant formulae	*n*	*Median*	*Media ± SD**	*Range*
Adapted Formula	34	6.66	6.86 ± 1.09	2.62-16.15
Type 1	21	5.89	6.29 ± 1.21	2.62-14.09
Functional 1	13	6.75	7.77 ± 2.22	2.87-16.15
Follow-up Formula	30	8.05	8.60 ± 1.47	2.06-20.33
Type 2	20	8.24	8.73 ± 2.17	2.06-20.33
Functional 2	10	7.85	8.35 ± 1.55	5.96-13.10
Toddler Formula	6	8.12	9.68 ± 6.71	2.91-21.10
Preterm Formula	7	12.94	11.48 ± 3.15	4.83-14.61
Hypoallergenic F.	10	15.02	18.16 ± 7.89	8.22-46.50
Lactose-free F.	10	11.67	11.37 ± 3.07	5.29-18.28
Soya Formula	7	9.55	10.43 ± 4.05	5.82-19.15
Total	104	8.15	9.60 ± 1.13	2.06-46.50

*SD at 95 % interval

Table IV. Chromium levels found from different types of infant formulae (μg l^{-1}).

Country	*Reference*	*Cr (μg L^{-1})*	*n*	*Description*
Austria	Krachler et al. 2000	< 4.0 – 14.5	4	Adapted formula
Belgium	Deelstra et al. 1988	6.2 ± 2.2	7	Adapted formula
		12.1	1	Soy-based
		14.2 ± 7.3	6	Special formula
France	Biego et al. 1998	45 ± 8	5	Milk-based
		26 ± 5	4	Soy-based
Italy	Muzzarelli et al. 1983	1.9 ± 1.8	7	Adapted formula
		3.9 ± 0.6	2	Lactose-free
		4.2	1	Soy-based
Nigeria	Ikem et al. 2002	6 ± 3	2	Powder Milk-based
Spain	Cocho et al. 1992	5.19 ± 3.26	17	Adapted formula
		3.83 ± 3.29	7	Follow-up
		4.19 ± 1.42	3	Preterm
		13.92 ± 4.46	3	Soy-based
		12.03 ± 7.58	8	Special formula
		21.12 ± 8.67	9	Metabolic formula
	Viñas et al. 1997	10.4*	1	-
		5.3*	1	-
		3.9*	1	-
United Kingdom	Food Standards Agency 2003	8.9*	18	Milk-based
		10.8*	3	Soy-based
		40	3	Ready to use
	Ikem et al. 2002	5 ± 5	4	Powder milk-based
		15 ± 4	6	Ready to use
	MAFF 1999	27.0 ± 6.7*	16	Powder milk-based
		15.0	4	Ready to use
	MAFF 1996	< 16 – 20	5	Powder Whey-bas.
		20	5	Liq. Whey-based
		< 16 – 20	5	Powder Casein-bas.
		< 18	5	Liq. Casein-bas.
		20 – 30	4	Follow-up
		< 16 – 20	4	Soy-based
	MAFF 1995	2.16 – 99.22	51	Powder Milk-based
USA	SHS 2005	11.2*	-	Free aa
	Ikem et al. 2002	7 ± 9	3	Powder Milk-based
		11 ± 7	2	Powder Soy-based
	Patterson et al. 1985	7.5	1	Preterm formula
		18	1	Preterm formula

Deelstra et al. 1988	3.3 – 19.6	11	Milk-based
	12.1	1	Soy-based
	5.7	1	Lactose-free
	7.5-8.1	2	HA
	18.0-23.4	3	Therapeutic F.
Abbot 1998	7.7	-	Milk-based
	20 – 40	-	Soy-based
	142 – 174	-	Casein Hydrol. F.
	8 – 22	-	Preterm

*Recalculated chromium concentration and expressed in µg/L

Table V. Chromium content in infant formulae according to the main protein contained (µg l^{-1}).

Infant formula	*n*	*Median*	*Media ± SD*	*Range*
Whey-based	23	6.66	6.41 ± 1.08 [a]	2.62-13.53
Skim-milk-based	41	7.54	8.20 ± 1.27 [a, b]	2.06-21.10
Whole-milk-based	4	7.25	9.12 ± 8.54 [a, b, c]	5.21-16.77
Soy-based	7	9.55	10.43 ± 4.05 [b, c, d]	5.82-19.15
Casein-based	10	11.67	11.37 ± 3.04 [c, d, e]	5.29-18.28
Whey hydrolysed	13	12.16	13.03 ± 2.82 [c, d, e]	7.03-20.68
Casein hydrolysed	5	14.08	15.03 ± 5.52 [e]	9.74-22.01
Free aminoacids	1	-	46.50 [f]	-

[a, b, c, d, e, f] Results with the same superscript letter were not significantly different ($p > 0.05$)

Table VI. Chromium content in infant formulae according to the different types and main protein contained (µg l^{-1}).

Infant formulae	*n*	*Median*	*Media* ± SD	*Range*
Adapted Formula				
Type 1				
Whole-milk-based	1	-	5.21	-
Skim-milk-based	5	5.23	5.86 ± 1.94	4.75-8.54
Whey-based	12	5.98	5.53 ± 1.29	2.62-8.65
Whey hydrolysed	3	10.18	10.43 ± 6.50	7.03-14.09
Functional 1				
Whole-milk-based	1	-	5.58	-
Skim-milk-based	7	6.66	8.48 ± 4.07	4.10-16.15
Whey-based	4	7.02	6.22 ± 3.64	2.87-7.97
Whey hydrolysed	1	-	11.22	-
Follow-up Formula				
Type 2				
Whole-milk-based	2	12.85	12.85 ± 5.55*	8.92-16.77
Skim-milk-based	13	808	7.74 ± 2.28	2.06-14.55
Whey-based	3	6.41	5.96 ± 2.24	4.92-6.54
Whey hydrolysed	2	15.20	15.20 ± 7.26*	10.06-20.33
Functional 2				
Skim-milk-based	7	8.02	8.39 ± 2.37	5.96-13.10
Whey-based	3	7.67	8.26 ± 3.08	7.43-9.69
Toddler Formula				
Skim-milk-based	6	8.12	9.68 ± 6.71	2.91-21.10
Preterm Formula				
Skim-milk-based	3	10.87	10.10 ± 4.93*	4.83-14.61
Whey-based	1	-	13.53	-
Casein hydrolysed	2	11.81	11.81 ± 2.92*	9.74-13.87
Whey hydrolysed	1	-	12.94	-
Hipoallergenic Formula				
Casein hydrolysed	3	15.47	17.19 ± 4.23*	14.08-22.01
Whey hydrolysed	6	13.36	13.92 ± 5.67	8.22-20.68
Free aminoacids	1	-	46.50	-
Lactose-free F.				
Casein-based	10	11.67	11.37 ± 3.07	5.29-18.28
Soya Formula				
Soy-based	7	9.55	10.43 ± 4.05	5.82-19.15

* Standard deviation value

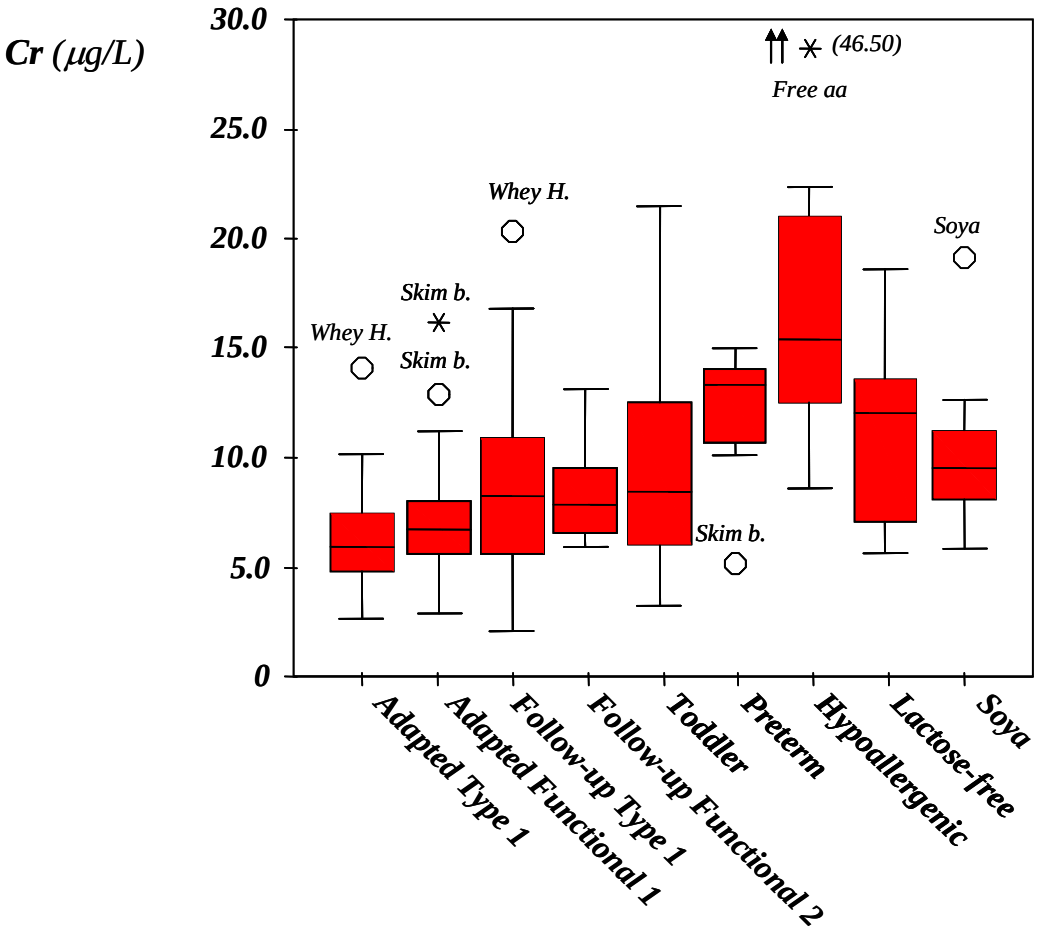
Table VII. Chromium levels (μg l^{-1}) from different container types of powder or liquid formulae.

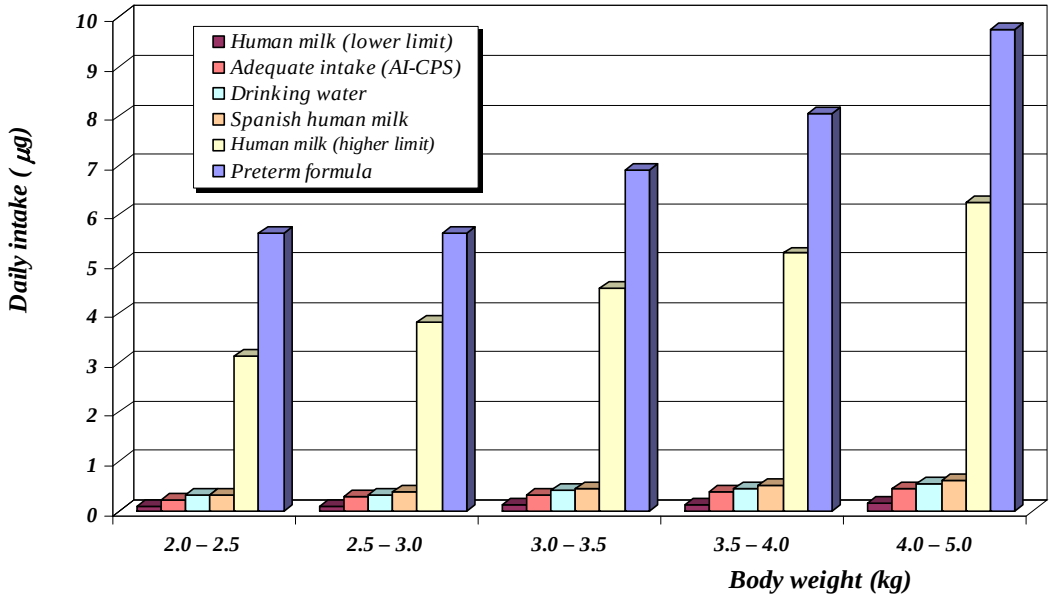
	n	***Median***	***Media*± SD**	***Range***
Powder F.	97	8.22	9.83 ± 1.19	2.06-46.50
Canned F.	61	8.39	10.00 ± 1.69	2.06-46.50
Al. bag F.	36	7.82	9.55 ± 1.56	2.87-22.01
Ready-to-use	7	7.43	6.35 ± 2.90	2.70-10.87
Tetra brick	4	5.82	6.30 ± 5.78	2.70-10.87
Glass bottle	2	8.18	8.18 ± 1.05*	7.44-8.92
Plastic bottle	1	-	2.91	-

* Standard deviation value

Table VIII. Daily intakes of chromium for infants fed on infant formulae and drinking water used in the reconstitution of powder formulae (µg day^{-1}).

Age	*Adapted*	*Follow-up*	*Toddler*	*Lactose-free*	*HA*	*Soya*	*Drinking water*
0 - 2 weeks	4.1 ± 0.7			6.8 ± 1.8	10.9 ± 4.7	6.3 ± 2.4	0.40 ± 0.10
3 - 4 weeks	6.2 ± 1.0			10.3 ± 2.8	16.3 ± 7.1	9.4 ± 3.6	0.53 ± 0.14
2 month	7.2 ± 1.1			11.9 ± 3.2	19.1 ± 8.3	11.0 ± 4.3	0.62 ± 0.16
3 month	7.5 ± 1.2			12.5 ± 3.4	20.0 ± 8.7	11.5 ± 4.5	0.67 ±0.17
4 - 5 months	8.6 ± 1.4	10.8 ± 1.8		14.2 ± 3.9	22.7 ± 9.9	13.0 ± 5.1	0.78 ± 0.20
6 month	6.9 ± 1.1	8.6 ± 1.5		11.4 ± 3.1	18.2 ± 7.9	10.4 ± 4.0	0.78 ± 0.20
7 - 12 months		8.3 ± 1.4	9.3 ± 6.4				0.60 ± 0.15
2 year			5.4 ± 3.8				0.36 ± 0.09
3 year			5.4 ± 3.8				0.36 ±0.09


Cr (µg/L)
30.0
25.0
20.0
15.0
10.0
5.0
0
(46.50)
Free aa
Whey H.
Skim b.
Whey H.
Skim b.
Soya
Skim b.
Adapted Type 1
Adapted Functional 1
Follow-up Type 1
Follow-up Functional 2
Toddler
Preterm
Hypoallergenic
Lactose-free
Soya


Human milk (lower limit)
Adequate intake (AI-CPS)
Drinking water
Spanish human milk
Human milk (higher limit)
Preterm formula
Daily intake (µg)
10
9
8
7
6
5
4
3
2
1
0
2.0 – 2.5
2.5 – 3.0
3.0 – 3.5
3.5 – 4.0
4.0 – 5.0
Body weight (kg)

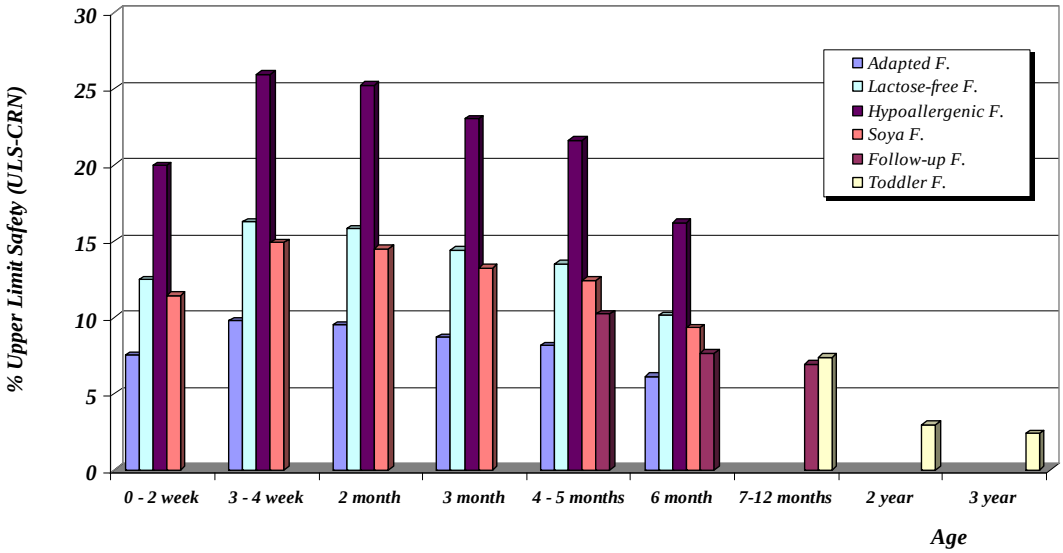

% Upper Limit Safety (ULS-CRN)
30
25
20
15
10
5
0
Adapted F.
Lactose-free F.
Hypoallergenic F.
Soya F.
Follow-up F.
Toddler F.
0 - 2 week
3 - 4 week
2 month
3 month
4 - 5 months
6 month
7-12 months
2 year
3 year
Age

Figure 1. Box plots of determined chromium content in different types of infant formulae studied (μg l^{-1})

Figure 2. Daily dietary chromium intake (μg day^{-1}) for infant fed on premature infant formulae, human milk (Spanish and Reference levels) and drinking water used in powder formula reconstitution.

Figure 3. Percentages of Upper Limit Safety for chromium estimated from infant formulae.