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Determination of chromium in urine samples by complexation–supercritical fluid extraction and liquid or gas chromatography

Veronica Arancibia*, Mauricio Valderrama, Karina Silva, Tania Tapia

Analytical Department, Faculty of Chemistry, Pontifical Catholic University of Chile, Av. Vicuna Mackenna 4860, Macul, Casilla 306, Correo 22, Santiago de Chile, Chile

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Abstract

A method was developed for the determination of chromium as the $Cr(acac)_3$ complex in urine using SFE and chromatography. Quantitative extractions were achieved when the experiments were carried out under 3000 p.s.i. of pressure, at a temperature of 120 °C, with 2.0 ml of methanol, 30 min of static extraction and 5 min of dynamic extraction. The chromium was quantified by GC–FID detection. The calibration graph of $Cr(acac)_3$ solutions was linear between 0.50 and 43.0 μg ml^{-1} of chromium (DL 0.18 μg ml^{-1}, $R=0.9994$). The same extracts were quantified by HPLC-340 nm detection. The calibration curve of the $Cr(acac)_3$ solutions was linear over a range of 0.013 to 60.0 μg ml^{-1} (DL 0.02 μg ml^{-1}, $R=0.9999$). This method was applied to urine samples from 60 diabetic patients and 21 healthy volunteers. Chromium concentration ranges were 2.5–29.5 μg l^{-1} for the diabetics and 5.9–12.3 μg l^{-1} for the normal subjects.

Keywords: Complexation; Supercritical fluid extraction; Chromium

1. Introduction

Chromium (Cr^{3+}) is an essential micronutrient for humans and it is involved in important biochemical processes such as glucose tolerance metabolism and the action of insulin. Its main role is to form a glucose tolerance factor (GTF), which acts as a cofactor of insulin in all insulin-dependent systems [1–4].

The human biological fluid with the highest Cr concentration is urine, and there is increasing interest in the determination of the Cr content in this fluid, as it can give information with which to assess the chromium nutritional status and recent uptake in humans [5,6].

Chromium levels reported in human urine vary widely; published values range from 0.32 to 12 μg l^{-1} [7]. This could be attributed to difficulties in sample treatment. In biological systems the pH is close to neutral, and under that condition Cr^{3+} forms low solubility polymeric hydroxylated complexes that are very difficult to break (*olation*), hindering atomization. The samples should be analyzed as quickly as possible after collection [8].

The most widely used techniques for chromium determination in biological materials include graphite furnace atomic absorption spectrometry (GFAAS) [7,9,10], flame atomic absorption spectrophotometry

*Corresponding author. Tel.: +56-2-686-4723; fax: +56-2-686-4744.
E-mail address: darancim@puc.cl (V. Arancibia).

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(FAAS) [11,12], electron-capture detection gas chromatography (GC–ECD) [13,14], and X-ray fluorescence spectroscopy [15].

Supercritical fluid extraction (SFE) has gained great acceptance as an alternative sample preparation and concentration technique. It is a versatile technique for the extraction and separation of mainly organic-type chemical mixtures. Direct extraction of metal ions with supercritical CO_2 is not possible because they are insoluble in supercritical fluids. In the GC, HPLC and SFE techniques it is necessary to convert the metal ions into neutral metal complexes, which have higher formation constants. On the other hand, suitable polarity or solubility in supercritical fluids and fast chelation kinetics are very important for achieving in situ complexation. High volatility, thermal stability and mainly adequate shielding of the metal atom to avoid unwanted column interactions are necessary in GC [16–21].

In this study, acetylacetone was used as chelating agent for chromium. The acetylacetonates were the first ligands studied in inorganic GC because of their ease of formation, thermal stability, volatility and solubility in many organic solvents. For the same reasons these complexes are also particularly suitable for HPLC separations. Metallic ions such as Al(III) and Cr(III) form coordinatively saturated neutral complexes which chromatograph well. Acetylacetonates of metals such as Cu(II) and Ni(II), which are not coordinatively saturated, however, tend to solvate, polymerize or undergo undesirable adsorption in column chromatography or in the GC system. The major breakthrough that transformed metal chelate GC so that it would give useful analytical results was the introduction of fluorinated beta-diketone ligands, which form complexes of greater volatility and thermal stability. However, GC and HPLC demand significant sample treatment and pre- or on-column derivatization [22–26].

The purpose of this work was to use the SFE to avoid treatment of complex samples and to reduce or eliminate the use of acids and organic solvents. It minimizes waste disposal, flammability, and toxicity. Also, SFE reduces the number of steps for analysis. On the other hand, the complex is formed in situ in the extractor. The methanolic extract obtained is injected into a chromatographic column.

The first step was to reach the optimum conditions for performing the quantitative extraction of Cr^{3+} as a complex. To that end, ''in situ'' formation of $Cr(acac)_3$ was carried out and the method for determining this ion in urine from diabetic and normal subjects was applied. GC–flame ionization detection and HPLC–UV detection were adequate to complement off-line SFE.

2. Experimental

2.1. Reagents

A standard Cr^{3+} stock solution of 84 μg ml^{-1} was prepared from $CrCl_3$ (Merck, Darmstadt, Germany) and quantified by atomic absorption; 2,4-pentanedione (acetylacetone, acac) was obtained from Aldrich (Milwaukee, WI, USA). The tris(pentane-2,4-dionato)chromium(III) ($Cr(acac)_3$) complex was prepared by mixing aqueous solutions of metal ion and ligand in a stoichiometric M:L ratio of 1:4. The solution was buffered at pH 4.5. The solid product was filtered, washed with cold water and recrystallized from ethanol. Purity was confirmed by comparison with the reported melting point. The compound was stored in vacuum. HPLC-grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water from a Millipore (Milford, MA, USA) Milli-Q system was used in the preparation of the aqueous solutions. Cellulose filter paper (Whatman 42, purchased from Merck) was used for liquid samples. SFE-grade carbon dioxide was obtained from Indura (Santiago, Chile).

2.2. Apparatus

2.2.1. Supercritical fluid extraction (SFE)

All the experiments were performed in an SFE-400 extractor (Supelco, Bellefonte, PA, USA). A 10-ml stainless steel vessel obtained from Supelco was used as the extraction vessel. Fused-silica tubing (12–15 cm×50 μm I.D.) was used as a restrictor. The flow-rate of the extraction fluid was ~0.5 ml min^{-1} (as a liquid).

2.2.2. Gas chromatography

A Perkin-Elmer Model Autosystem 9000 (Norwalk, CO, USA) gas chromatograph equipped with a

flame ionization detector (FID) was used in this study. A 0.5-μl aliquot of extract was injected into a capillary column (Rtx-2330, 90% biscyanopropyl, 10% phenylcyanopropyl polysiloxane, 30 m×0.53 mm I.D. with a 0.2 μm film thickness) (Restex, Bellefonte, PA, USA). Gas flow was 5.0 ml min^{-1} passing it through a molecular-sieve trap and oxygen trap, and purified nitrogen was used as carrier gas. Data handling from the GC runs was performed on an on-line PE Nelson processor.

Conditions were as follows: injector and detector temperature, 200 and 250 °C, respectively. The column oven temperature was set at 100 °C increased to 180 °C at 3 °C min^{-1}. Quantitative analyses were carried out using calibration curves obtained with standard solutions of the $Cr(acac)_3$ complex.

2.2.3. High-performance liquid chromatography

Liquid chromatography was carried out on a Merck-Hitachi (Chiyoda-ku, Tokyo, Japan) chromatograph consisting of L-6000 and L-6250 pumps, a Rheodyne (Rohnert Park, CA, USA) 7125 injector fitted with a 20-μl sample loop, and a LaChrom (West Chester, PA, USA) model L-7420 UV–Vis detector. Data handling from the HPLC runs was performed on Star Chromatography Workstation Software from Varian (Santa Clara, CA, USA). The column used throughout this study was a Supelcosil LC-18-DB (250×4.6 mm, 5-μm) from Supelco (Bellefonte, PA, USA). The mobile phase was 70% methanol in 0.01 *M* KH_2PO_4, adjusted to pH 7.3 with NaOH, at a flow-rate of 0.4 ml min^{-1}. The detector was fixed at 340 nm. Quantitative analysis was carried out using calibration curves obtained with $Cr(acac)_3$ complex standard solutions.

2.3. Extraction procedures

2.3.1. Extraction parameters with synthetic solutions

In order to determine the optimum extraction parameters, 0.50 ml of 84 μg ml^{-1} solution of Cr^{3+} was spiked onto a filter paper disc and dried in an oven at 60 °C. The filter paper containing the sample was introduced in the extraction vessel, 90 mg of acetylacetone and 5.0 ml of methanol as modifier were added. The system was closed. The CO_2 inlet valve was opened, allowing the mixture of CO_2–methanol to homogenize. Measurements were made between 80 and 140 °C, 1000–3500 p.s.i. of pressure and times between 10 and 60 min of static extraction and 5 min of dynamic extraction. The restrictor was maintained at 70 °C. Under the best conditions of pressure and temperature, extractions with different amounts of ligand (90–900 mg) and methanol (1.0–5.0 ml) were performed. The extracts were received in empty 5.0-ml volumetric flasks. When methanol was completely exhausted from the extractor, it was cooled to 25 °C; then 1–2-ml aliquots of methanol were added to the extraction vessel and this washing solvent was received in the same flask.

2.3.2. Extraction of chromium from spiked urine samples

The method was validated using urine samples that contained chromium undetectable by our method. Five urine samples (5.0 ml) were contaminated with 0.1 μg of chromium and then were spiked onto filter paper and dried in an oven at 60 °C. The filter paper was introduced into the extraction vessel. Subsequently, 450 mg of ligand and 2.0 ml of methanol (optimum amounts determined with synthetic solutions) were added. The best parameters of extractions were applied. The extracts were collected in collection vials. After completion of extraction, the solvent was evaporated to dryness and 0.5 ml of methanol was added to the dry sample.

2.3.3. Extraction of chromium from urine samples

Urine samples from healthy subjects and diabetic patients were collected using 200-ml plastic containers and stored at −18 °C for several months without treatment. Sample age did not have an effect on the results. During that period some analyses were repeated, giving the same results. A 50.0-ml sample was evaporated almost to dryness and brought up to 5.0 ml with water. Of this sample, 1.0–5.0 ml were added to the filter paper and dried in an oven at 60 °C. Next, the filter paper was placed in the extraction vessel, and 450 mg of acetylacetone and 2.0 ml of methanol were added. Extractions were carried out at 3000 p.s.i. and 120 °C for a 30-min static and 5-min dynamic extraction. All the extracts were collected in collection vials. After completion of extraction, the solvent was evaporated to dryness and 0.1–0.5 ml of methanol was added to the dry

sample. Evaporation was very slow to avoid loss of solute.

2.3.4. Limit of detection

The limit of detection (DL) was calculated from $y_{DL} = a + 3s_{x/y}$ and $y_{DL} = a + bx_{DL}$ where a is the intercept, $s_{x/y}$ is the random error in x and y, and b is the slope.

3. Results and discussion

3.1. Supercritical fluid extraction parameters

Fig. 1 shows the recovery of the $Cr(acac)_3$ complex at different pressures and 100 °C during 20 min of static extraction. These results were obtained when 42 μg of chromium, 90 mg of ligand and 5.0 ml of methanol were added to the extraction vessel. These values correspond to the average of three measurements under the same conditions. The highest extraction efficiency was obtained at 3000 p.s.i. Recovery of chromium, however, was only 49.2%. By increasing the temperature from 100 to 120 °C, recovery increased to 64.8% (Fig. 2). The effect of the amount of methanol added to the extraction vessel is shown in Fig. 3. It is seen that the optimum volume of methanol for the extraction of $Cr(acac)_3$ at

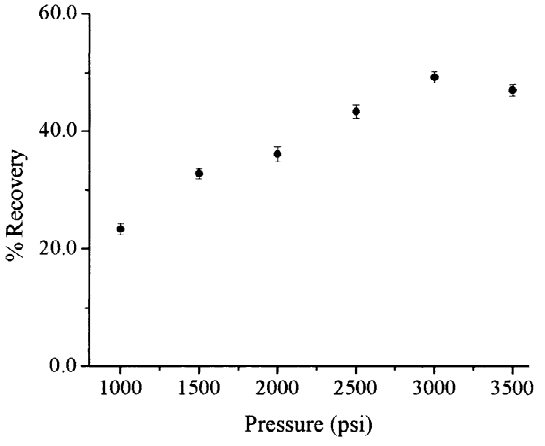

Fig. 1. Percent recovery of $Cr(acac)_3$ at different pressures (1000–3500 p.s.i.). Conditions: 42 μg of chromium; 90 mg of acac; 5.0 ml of methanol (added to a 10-ml extraction vessel); oven temperature, 100 °C; 20 min in the static mode, followed by 5 min in the dynamic mode.

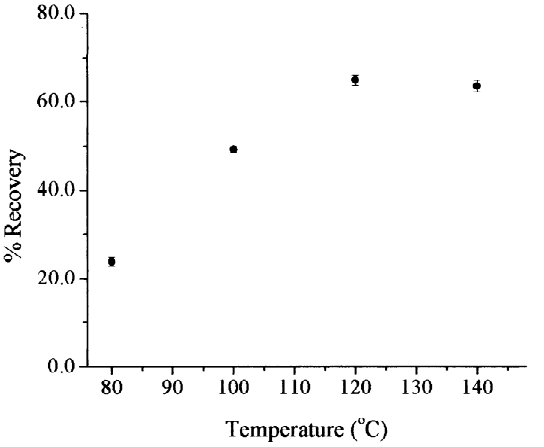

Fig. 2. Percent recovery of $Cr(acac)_3$ at different temperatures (80–140 °C). Conditions: 42 μg of chromium; 90 mg of acac; 5.0 ml of methanol; pressure, 3000 p.s.i.; 20 min in the static mode, followed by 5 min in the dynamic mode.

3000 p.s.i. and 120 °C was 2.0 ml (10-ml extraction vessel). With this volume the extraction efficiency was 82.9%. The previous studies in terms of pressure and temperature were carried out with 5.0 ml of methanol because very bad recoveries were obtained with CO_2 alone. On the other hand, due to the polar character of the metallic complexes, it is believed that quantitative results would be achievable with higher percentages of modifier.

The success of the extraction also depends on the

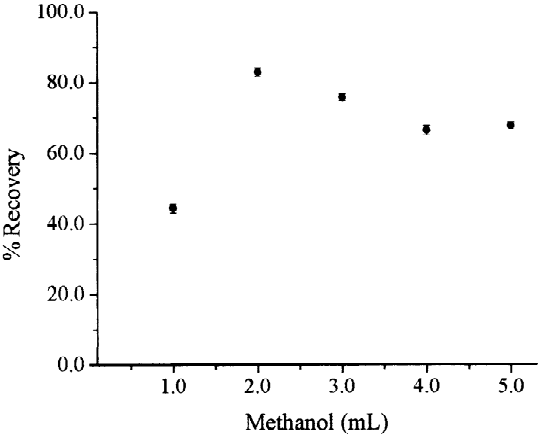

Fig. 3. Percent recovery of $Cr(acac)_3$ with different amounts of methanol (1.0–5.0 ml). Conditions: 42 μg of chromium; 90 mg of acac; pressure, 3000 p.s.i.; oven temperature, 120 °C; 20 min in the static mode, followed by 5 min in the dynamic mode.

concentration and solubility of the ligand. Acetylacetone is an effective chelating agent for chromium and it is a volatile compound that at high temperature could enhance the contact area with the metal ion. With the purpose of observing the effect of all the extraction parameters (pressure, temperature, amount of modifier, etc.) on extraction efficiency, the amount of chromium added to the extraction vessel was always high (42 μg). Next, the amount of acetylacetone added was 90, 270, 450, 630 and 900 mg obtaining 81.26, 98.14, 99.23, 99.07 and 99.38% of recovery, respectively.

Extractions at different static extraction times were performed. For 10, 20, 30, 40 and 60 min of extraction, recoveries of 81.23, 99.23, 100.08, 99.79 and 100.16% were obtained, respectively. The results of this study showed that quantitative extraction was obtained after 20–30 min. The dynamic extraction had to be stopped when methanol was completely exhausted (~5 min).

From these results it can be concluded that the best parameters for the extraction of chromium as $Cr(acac)_3$ were the following: pressure, 3000 p.s.i.; temperature, 120 °C; methanol, 2.0 ml; ligand, 450 mg; 30 min of static extraction and 5 min of dynamic extraction. Under these conditions up to 42 μg of chromium were recovered.

In order to determine the efficiency of the extraction of chromium in the real matrix, this ion was added to urine samples containing chromium at the low detection limit. The same process was repeated five times using 0.1 μg of chromium each time, and recovery was 99.0±0.6%.

3.2. Quantitation by gas chromatography

The urine extracts were injected into the chromatograph. The standard curve was drawn by plotting the peak areas of the $Cr(acac)_3$ complex solutions. The calibration curve of the $Cr(acac)_3$ solutions was linear over a range of 0.50 to 43.0 μg ml^{-1}. The equation of a typical calibration curve was $y = -14023.5 + 489173.3x$. The standard deviation ($s_{x/y}$), the number of standard solutions (N), the standard error in the slope (s_B) and in the intercept (s_A) were: 29,355.4, 10, 690.7, and 12,240.1, respectively. The detection limit (DL) for Cr(III) was 0.18 μg ml^{-1}. The correlation coefficient obtained was $R = 0.9994$, but in this study no internal standard was used. If the chromium concentration is higher than 60.0 μg ml^{-1}, a slight absorption of this ion in the column is observed. After each injection of complex, ligand solution was injected to check the adsorption and clean the stationary phase.

The solid $Cr(acac)_3$ complex in the methanol solution was not stable. The purple color of the solution disappeared after 24 h and the peak area decreased as a function of time. Free ligand was added to these solutions, but it did not stabilize the complex. Surprisingly, extracts obtained with supercritical fluids by in situ complexation and further dilutions were stable for several months. The concentration of chromium in some pure extracts was determined by atomic absorption spectrophotometry. These last solutions were used as standards.

Fig. 4 shows a chromatogram of the urine sample extract of a diabetic person. If iron, aluminum, copper, lead, zinc or cadmium were present in the urine samples, the metal–acetylacetonate complexes could be extracted and then injected in the chromatograph, but all of them had smaller retention times than the chromium complex. These peaks overlap with the high methanol and acetylacetone peaks. However, if a solution in carbon disulfide is prepared

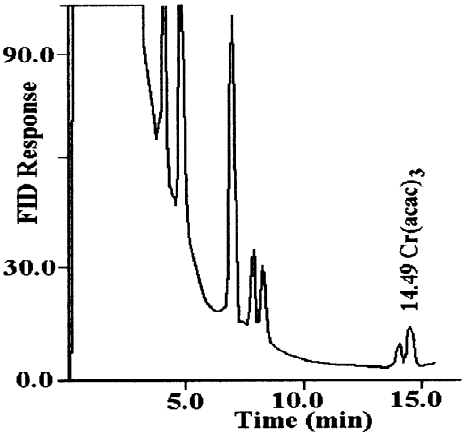

Fig. 4. Chromatogram of urine sample extract of a diabetic patient. Rtx-2330 column; nitrogen flow, 5.0 ml min^{-1}; injector and detector temperature, 200 and 250 °C, respectively; oven temperature, 100 °C increased to 180 °C at 3 °C min^{-1}. FID-detection. The extraction conditions were: 450 mg of ligand, 2.0 ml of methanol, 3000 p.s.i. of pressure, 120 °C of temperature, 30 min of static extraction and 5 min of dynamic extraction.

containing the complexes in the presence of a slight excess of ligand and the following temperature program is carried out as a function of time: $T_1 =$ 35 °C, $T_2 = 70$ °C and $T_3 = 220$ °C, $t_2 = 3$ min; $V_1 =$ 3 °C/min and $V_2 = 20$ °C/min, the separation of all of them is achieved ($CS_2 = 1.120$; acac = 4.187; $Pb(acac)_2 = 5.830$; $Cu(acac)_2 = 6.234$; $Co(acac)_3 =$ 13.493; $Fe(acac)_3 = 15.173$; $Al(acac)_3 = 18.640$ and $Cr(acac)_3 = 21.973$ min).

3.3. Quantitation by high-performance liquid chromatography

Because the aim of this work was to develop a rapid and sensitive method for the determination of chromium in urine, HPLC was chosen to compare the results obtained by GC, in the hope of finding a more sensitive technique. The mobile phase, flow-rate and wavelength detection were selected so as to ensure the highest possible sensitivity and good resolution. The main problem was the chromatographic separation of free ligand and complex. If the concentration of excess free ligand is high, the chromatographic peak shows a ''tailing effect'' and the complex is overlapped. Using a 70:30 methanol–water ratio and adjusting the pH to 7.3 a good separation was obtained. For a concentration of free ligand of 9000 μg ml^{-1}, the retention time (t_R) was 5.54 min, and 6.85 min for the complex (Fig. 5).

The $Cr(acac)_3$ calibration curves were linear over a range of 0.013 to 60.0 μg ml^{-1} when using a UV detector at 340 nm, as shown by an *R*-value of 0.9999. The equation of a typical calibration curve was $y = 1067.4 + 659953.8x$. The standard deviation ($s_{x/y}$), the number of standard solutions (N), the standard error in slope (s_B) and in intercept (s_A), were 4552.8, 10, 76.2, and 1657.5, respectively. The detection limit (DL) was Cr(III) 0.02 μg ml^{-1}. The response for a series of six injections of 1.3 μg ml^{-1} $Cr(acac)_3$ solution resulted in a relative standard deviation of 1.29% for peak area.

The $Cr(acac)_3$ complex also absorbs at 556 nm, but at this wavelength the sensitivity is not good because its molar extinction coefficient is about 100 times lower than at 340 nm. When the interference of other metal ions was studied, it was found that the chromium complex with acetylacetone is the most

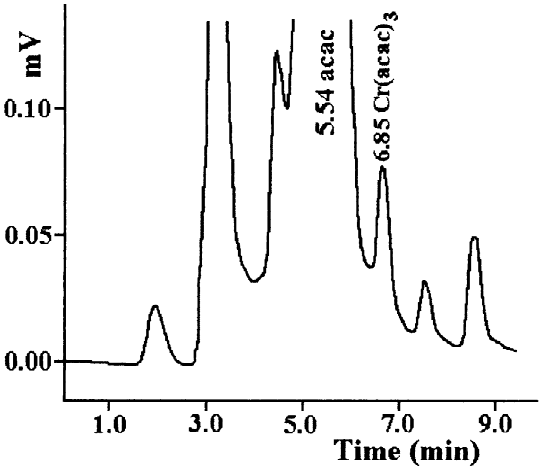

Fig. 5. Chromatogram of urine sample extract from a diabetic patient. Column C_{18}; mobile phase, methanol–water 70:30 (0.01 *M* KH_2PO_4, pH 7.3); flow, 0.4 ml min^{-1}; UV-detection, 340 nm. The extraction conditions were: 450 mg of ligand, 2.0 ml of methanol, 3000 p.s.i. of pressure, 120 °C temperature, 30 min of static extraction and 5 min of dynamic extraction.

apolar one and is the last one to elute. If a solution containing only the complexes in the absence of excess acetylacetone is injected, the signals of the $Pb(acac)_2$, $Cu(acac)_2$ and $Fe(acac)_3$ complexes are detected at almost the same retention time (2.976, 3.078 and 3.245 min, respectively), while $Al(acac)_3$ is not detected at 340 nm.

3.4. Application of the method

The method was applied to the second morning urine samples from 60 diabetic patients and 21 healthy volunteers. The plastic bottles with the diabetic samples were labeled with the following data: date of birth, sex, pH, amount of protein and amount of glucose. Healthy volunteers were young 20- to 30-year-old chemistry students. The diabetic patients were a heterogeneous group that included 20- to 80-year-olds, of which 34 were older than 65 years and 13 were chromium non-detectable. Patients with high glucose levels (1000 mg dl^{-1}) or patients with high levels of total protein (500 mg dl^{-1}) did not show extreme values.

Our general results indicated that the chromium concentration range was 2.5–29.5 μg l^{-1} (average

10.0±6.7 μg l^{-1}) for 47 diabetics and 5.9–12.3 μg l^{-1} (average 8.1±2.3 μg l^{-1}) for the 21 normal subjects. These results demonstrate that diabetic subjects are not associated with a low chromium status but probably impaired chromium utilization. The large standard deviation obtained with the diabetic patients indicates that it is necessary to separate the patients according to age, sex or other characteristics. It could also be important to know how many years the patients have had this deficiency.

Of the two methods used, the HPLC technique was the more sensitive one. The chromatograms of Figs. 4 and 5 correspond to the same patient. When the samples were quantified by GC, it was necessary to perform consecutive evaporations of the initial sample and of the final extract. However, the GC method showed good resolution. Under the experimental temperature and flow program, the separation between ligand and complex was about 10 min. The presence of excess ligand enhances the extraction efficiency and avoids unwanted column interactions between chromium and the stationary phases used in the chromatography. Chromium ion is present in urine samples in extremely low concentration, and the GC–FID method could be applied to other types of samples.

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References

[1] W. Mertz, J. Am. Coll. Nutr. 17 (1998) 544.
[2] R.A. Anderson, Diabetes Metab. 26 (2000) 22.
[3] J.B. Vincent, Nutr. Rev. 58 (2000) 67.
[4] W. Mertz, J. Nutr. 123 (1993) 626.
[5] G.V. Iyengar, Sci. Total Environ. 86 (1989) 69.
[6] A. Alimonti, F. Petrucci, M. Krachler, B. Bocca, S. Caroli, J. Environ. Monit. 2 (2000) 351.
[7] L. Ping, K. Matsumoto, K. Fuwa, Anal. Chim. Acta 147 (1983) 205.
[8] W. Mertz, Physiol. Rev. 49 (1969) 163.
[9] N. Campillo, P. Viñas, I. López-García, M. Hernández-Córdoba, Talanta 48 (1999) 905.
[10] T.W. Lin, D.S. Wang, Anal. Chem. 73 (2001) 4319.
[11] G. Castellari, P. Fiorentini, G. Clin. Med. 54 (1973) 116.
[12] R. Rubio, A. Sahuquillo, G. Rauret, L. Garcia Beltran, Ph. Quevauviller, Anal. Chim. Acta 283 (1993) 207.
[13] R.K. Mugo, K.J. Orians, Anal. Chim. Acta 271 (1993) 1.
[14] L.C. Hansen, W.G. Scribner, T.W. Gilbert, R.E. Sievers, Anal. Chem. 43 (1971) 349.
[15] K. Beyermann Jr., H.J. Rose, R.P. Christian, Anal. Chim. Acta 45 (1969) 51.
[16] S. Bowadt, S.B. Hawthorne, J. Chromatogr. A 703 (1995) 549.
[17] C.M. Wai, S. Wang, J. Chromatogr. A 785 (1997) 369.
[18] M. Ashraf-Khorassani, M.T. Combs, L.T. Taylor, J. Chromatogr. A 774 (1997) 37.
[19] J.H. Shofstahl, J.K. Hardy, J. Chromatogr. Sci. 28 (1990) 225.
[20] K. Robards, E. Patsalides, J. Chromatogr. A 844 (1999) 181.
[21] C. Baiocchi, A. Marchetto, G. Saini, P. Bertolo, Talanta 35 (1988) 685.
[22] J.C. MacDonald, in: P.J. Elving, J.D. Winefordner (Eds.), Inorganic Chromatographic Analysis, Chemical Analysis, Vol. 78, 1985, Chapter 5.
[23] K. Saitoh, M. Satoh, N. Suzuki, J. Chromatogr. 92 (1974) 291.
[24] K. Robards, E. Patsalides, J. Chromatogr. A 844 (1999) 181.
[25] K. Robards, E. Patsalides, S. Dilli, J. Chromatogr. 411 (1987) 1.
[26] P.C. Uden, J.R. Jenkins, Talanta 16 (1969) 893.