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## Biospeciation of Cr(III) Nutritional Supplements in Biological Fluids

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### Cover Page Footnote

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## Biospeciation of Cr(III) Nutritional Supplements in Biological Fluids

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### Abstract

Metal ion speciation in various biological systems has been extensively studied to elucidate its biological role and the toxicity of the element of interest. In the present study, chromium speciation was performed by reacting Cr(III) nutritional supplements  $[\text{Cr}(\text{pic})_3]$ , where  $\text{pic} = 2\text{-pyridinecarboxylato}(-)$ , and a Cr(III) propionate complex,  $[\text{Cr}_3\text{O}(\text{OCOEt})_6(\text{OH}_2)_3]^+$ , in calf serum. Cr(III) complexes in serum were fractionated using size-exclusion chromatography, and the Cr(III) concentrations in each fraction were determined using graphite furnace atomic absorption spectroscopy. The results showed that Cr(III) bound to both high- and low-molecular weight serum fractions. While Cr(III) was mainly bound to albumin or transferrin, unknown low-molecular-weight serum fractions were also important in Cr binding. The Cr(III) distribution in serum fractions was found to be time-dependent.

### Abstrak

**Biospesiasi dari Cr(III) yang Digunakan sebagai Suplemen Kesehatan pada Cairan Biologis.** Studi mengenai spesiasi ion logam dalam sistem biologi dilakukan untuk mengetahui peran biologis dan juga toksisitas dari suatu ion logam. Pada penelitian ini telah dilakukan spesiasi ion Cr(III) yang digunakan sebagai suplemen kesehatan, yaitu  $[\text{Cr}(\text{pic})_3]$ , di mana  $\text{pic} = 2\text{-piridinkarboksilat}(-)$ , dan kompleks dari Cr(III) propionat,  $[\text{Cr}_3\text{O}(\text{OCOEt})_6(\text{OH}_2)_3]^+$ , pada serum. Komplek Cr(III) pada serum difraksinasi terlebih dahulu dengan metode kromatografi, kemudian konsentrasi Cr(III) pada masing-masing fraksi ditentukan dengan metode AAS. Hasil yang didapatkan menunjukkan bahwa Cr(III) terikat pada fraksi dengan berat molekul besar dan kecil. Fraksi serum dengan BM besar adalah albumin dan transferrin, sedangkan fraksi dengan BM kecil tidak diketahui. Distribusi Cr(III) pada serum ditemukan bergantung pada waktu inkubasi (*time-dependent*).

**Keywords:** AAS, chromium, size-exclusion chromatography, speciation

### Introduction

Chromium is probably the most controversial transition element in terms of its nutritional activity and toxicity [1]. While Cr(VI) was one of the first chemicals classified as a human carcinogen [2], Cr(III) has been widely accepted as an essential nutrient that can play a role in carbohydrate and lipid metabolism [3]. As such, many of its complexes have been used as nutritional supplements for animals and humans as a means of enhancing glucose metabolism, inducing weight loss, building muscle, and regulating blood glucose levels in type 2 diabetes for over four decades.

Further, Cr(III) is officially classified by the World Health Organization as an essential trace element for animals

and humans [4]. Chromium deficiency is believed to lead to signs and symptoms similar to those observed in type 2 diabetes and cardiovascular diseases, including elevated blood glucose, serum cholesterol, and triglycerides levels as well as decreased insulin binding, impaired glucose tolerance, hypoglycaemia, and glycosuria [5,6].

Tris(2-pyridinecarboxylate)chromium(III), also known as Cr(III) picolinate  $[\text{Cr}(\text{pic})_3]$ , where  $\text{pic} = 2\text{-pyridinecarboxylato}(-)$ , has become the most popular Cr(III) nutritional supplement and the second largest-selling metal supplement after calcium in the United States [7]. The wide use of  $[\text{Cr}(\text{pic})_3]$  as a nutritional supplement is a result of its high stability in biological media and high bioavailability compared with other Cr(III) complexes [8]. It has been claimed that  $[\text{Cr}(\text{pic})_3]$  not only has positive

effects as an anti-diabetic agent, but also has effects on body tone, including increasing muscle mass and promoting weight reduction [5,9]. However, studies have reported that  $[\text{Cr}(\text{pic})_3]$  has no beneficial effects on metabolism in healthy people, even when taken in combination with an exercise program [10,11].

Stearns et al. were the first to provide evidence for the potential genotoxicity of  $[\text{Cr}(\text{pic})_3]$  in the ovary cells of Chinese hamsters [12]. They also subsequently reported mitochondrial damage [13] and mutagenic effects [14] in these cells. The coordination of Cr(III) with picolinic acid could be the factor that makes  $[\text{Cr}(\text{pic})_3]$  more genotoxic than other Cr(III) complexes.

These findings raised concerns about the safety of using  $[\text{Cr}(\text{pic})_3]$  as a nutritional supplement, particularly for long-term and high-dose use. Several complexes of Cr(III) with other ligands, such as carboxylates ( $[\text{Cr}_3\text{O}(\text{O}_2\text{Cet})_6(\text{OH}_2)_3]^+$ ) [15,16], amino acids ( $[\text{Cr}(\text{L})_3]$ , L = histidine (his) or phenylalanine (pa)) [17], niacin-bound chromium (NBC) [18], and chromium-enriched yeast [19], have been investigated as alternative Cr(III) nutritional supplements.

The trinuclear Cr(III) oxo-carboxylato complex, Cr(III) propionate complex ( $[\text{Cr}_3\text{O}(\text{O}_2\text{Cet})_6(\text{OH}_2)_3]^+$ ), was reported to have similar biological activities and a similar molecular structure to those of chromodulin and was thus proposed as a functional biomimetic of chromodulin [20]. This complex has been patented by Vincent and Davis for the treatment of disorders related to chromium deficiency or as a nutritional supplement [21].

The binding of metal ions to small biomolecules, such as small peptides and amino acids in biological fluids, is considered to have physiological importance [22]. However, these species have not been well characterized, due to isolation and purification problems. Thus, the identification and characterization of low-molecular-weight (LMW) protein-bound chromium, which could have implications in the cellular mechanism of Cr action, are highly important. For these reasons, the current work investigates Cr(III) complexes, focusing on Cr(III) nutritional supplements used for their purported anti-diabetic activity.

## Materials and Methods

**Materials.** All reagents of analytical or higher purity grade were purchased from Merck or Sigma-Aldrich and used as received: HCl (37% aqueous solution),  $\text{HNO}_3$  (trace pure, 65% w/w in  $\text{H}_2\text{O}$ ), NaOH (99.9%),  $\text{NH}_4\text{OAc}$ , NaCl,  $\text{Na}_2\text{Cr}_2\text{O}_7$ , acetonitrile (MeCN), picolinic acid (pyridine-2-carboxylic acid, picH), ethanol, and Cr(VI) standard solution (1000 ppm in 0.10 M HCl). Water was purified using the Milli-Q technique.

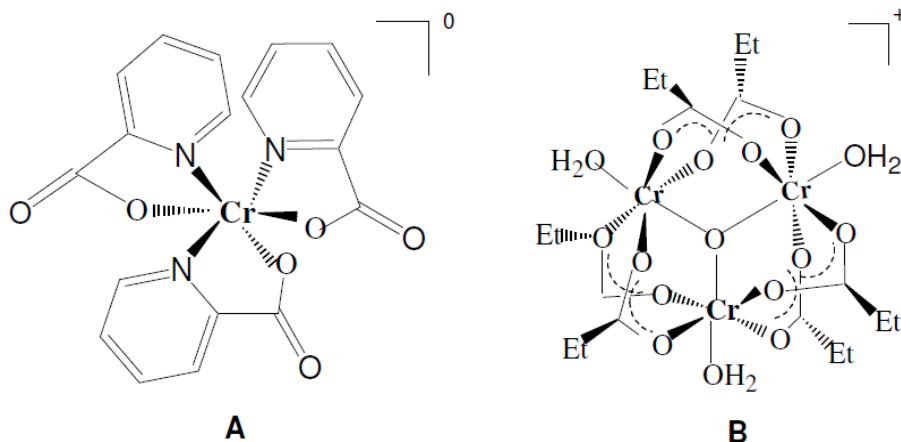
Commercial biochemicals from Sigma-Aldrich were used as received: bovine serum albumin (BSA), bovine apotransferrin, and myoglobin. Commercial calf serum was purchased from Invitrogen. The SeeBlue® Plus2 pre-stained standard (protein markers) and SDS-NuPAGE® Bis-Tris 4-12% electrophoresis systems were supplied by Invitrogen (Cat. No 0323BOX).  $[\text{Cr}(\text{pic})_3] \cdot \text{H}_2\text{O}$  (**A**) and  $[\text{Cr}_3\text{O}(\text{O}_2\text{Cet})_6(\text{OH}_2)_3](\text{NO}_3) \cdot 3\text{H}_2\text{O}$  (**B**) were synthesized according to the method recommended in existing research [23].

**Instrumentation.** Size-exclusion chromatography (SEC) was conducted in the AKTA Purifier 100/10 system at room temperature using a Superdex™ 200 10/200 GL (GE Healthcare Bio-Sciences, Uppsala, Sweden), with an optimum separation range between 10 and 600 kDa (globular proteins) for the gel filtration column and a bed dimension of  $10 \times 300\text{--}310$  mm. Atomic absorption spectroscopy was performed using a Varian SpectrAA20 spectrometer with a GTA 96 graphite tube atomizer to determine the Cr(III) concentration in SEC fractions.

**Sample Preparation.** A stock solution of **B** in  $\text{H}_2\text{O}$  ( $[\text{Cr}] = 100$  mM) was prepared immediately before the experiments. Due to the low solubility of **A**, its solutions in an aqueous buffer or blood serum were prepared by sonication (~10 min at 50 W and 298 K), and undissolved solids were removed by centrifugation (~10 min at 10,000 rpm and room temperature). The total concentration of Cr in the saturated solutions of **A** ( $[\text{Cr}] = 0.30$  to  $1.0$  mM) and **B** ( $[\text{Cr}] = 1.0$  mM) in an aqueous buffer or blood serum were determined by atomic absorption spectroscopy. Figure 1 shows the molecular structure of complexes **A** and **B**.

**Separation of Serum Proteins Spiked with Cr(III) Nutritional Supplements Using Size-Exclusion Chromatography.** The column buffer was prepared by dissolving  $\text{NH}_4\text{OAc}$  in Milli-Q water. It was filtered through a  $0.45\text{-}\mu\text{m}$  filter before use. SEC was performed on an AKTA Purifier 100/10 system (Amersham Biosciences) at room temperature with a Superdex 200 10/300 gel filtration column equilibrated in column buffer (0.10 M  $\text{NH}_4\text{OAc}$ , pH 7.2) at a flow rate of  $0.50$  mL  $\text{min}^{-1}$ . Cr(III) compounds (saturated solutions of **A** or **B** in blood serum, as prepared in section 2.3) were incubated for 0, 1, 24, or 48 h at  $37^\circ\text{C}$ . The resulting sample was dissolved in five volumes of column buffer and filtered through a  $0.20\text{-}\mu\text{m}$  filter before the mixture was injected onto the column. An aliquot of the sample (200  $\mu\text{L}$ ) was injected onto a Superdex 200 10/300 GL column. The chromatographic separation was conducted at a flow rate of  $0.50$  mL  $\text{min}^{-1}$ , followed by UV detection of bands at 260 nm. Isocratic elution with the buffer was applied for 70 min. All operations were carried out at room temperature. The same procedures were applied to a sample of blood serum (unspiked, no

chromium compounds) diluted one in five in buffer solution (0.10 M  $\text{NH}_4\text{OAc}$ , pH 7.2).



**Figure 1. Structures of Cr(III) Complexes used as Nutritional Supplements in this Study. Designations: A =  $[\text{Cr}(\text{pic})_3]$ , B =  $[\text{Cr}_3\text{O}(\text{O}_2\text{CET})_6(\text{OH}_2)_3]^+$**

**Chromium Determination in SEC Fractions Using Graphite Furnace Atomic Absorption Spectroscopy (GFAAS).** The sample solution was prepared by mixing each SEC fraction (0.20 mL) with an aqueous  $\text{HNO}_3$  solution (65%, 0.20 mL) overnight at room temperature to aid digestion of the protein. Aqueous  $\text{HCl}$  (0.10 M, 0.60 mL) was added to each sample prior to the analysis. GFAAS was performed on a Varian SpectrAA20B spectrometer with a GTA 96 graphite tube atomizer. Three replicates for all samples were performed. A standard solution of  $\text{Na}_2\text{Cr}_2\text{O}_7$  in 0.10 M  $\text{HCl}$  was used for calibration (range of 0-25 ppb). Samples that had concentrations out of the calibration range were diluted 10-50 fold until they were within range.

**SDS-PAGE Analysis.** The freeze-dried protein samples and protein standards (BSA and bovine apo-transferrin) were dissolved in a minimal amount of water; the final concentration was  $\sim 1 \text{ mg mL}^{-1}$ , as determined by the Bradford method. An aliquot of 4× NuPAGE® LDS sample buffer (containing 106 mM Tris  $\text{HCl}$ , 141 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol red, pH 8.5) was added to all gel samples. An aliquot of sample (10  $\mu\text{L}$ ) was loaded per well onto a precast Bis-Tris 4-12% SDS NuPAGE® gel. Gels were run at 200 V for 40 min using 20× NuPAGE® MES SDS running buffer (containing 50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.3) in an XCell™ SureLock Mini-Cell apparatus (Invitrogen). Gels were then visualized with an EZBlue™ gel staining reagent (Sigma) and destained with water.

## Results and Discussion

Figures 2 and 3 show typical chromatograms of serum spiked with **A** or **B**, under the specified conditions (0-48 h, 37 °C) as well as the content of Cr(III) within the serum fractions. The addition of **A** to the serum resulted in an additional peak in the chromatogram for a species that eluted at approximately 20 to 22 mL. This absorbance was likely due to the aromatic ligands in complex **A**. With time, a slight shift was observed in the position of this peak. Immediately after the addition of **A** to the serum (0 h), Cr(III) accumulated, predominantly in fractions 19, 20, and 21. This corresponded to 19-21 mL of the eluant, which were predicted to have low molecular weights (LMW). The gradual binding of Cr(III) to high-molecular-weight (HMW) fractions, or fractions 12, 13, and 14, was shown to occur during 1 to 24 h from the beginning of the reaction (310 K). After 48 h of reaction, the amounts of Cr(III) bound to HMW and LMW fractions of serum were roughly equal.

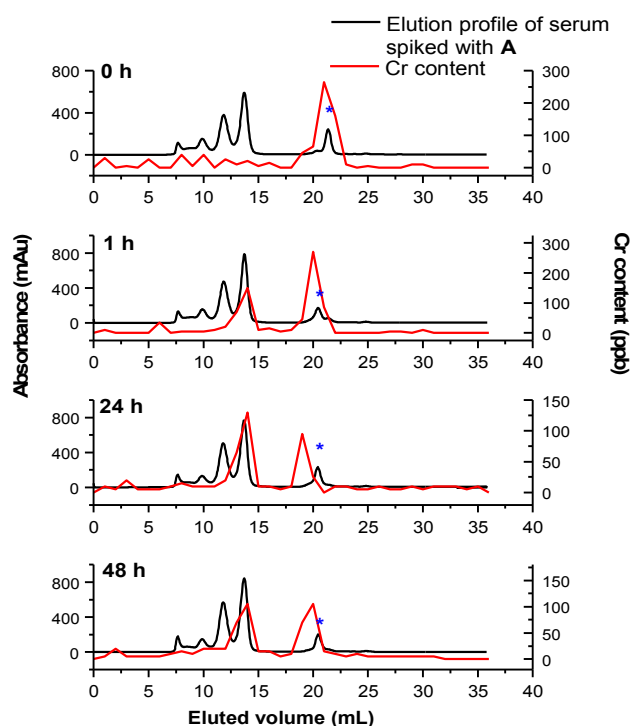
Similar results were observed when **B** was added to the serum. However, a higher total amount of Cr(III) was distributed in serum fractions than when the serum was spiked with **A**. This may be due to **A** having a lower Cr(III) concentration than **B** (Figure 3). No additional peaks were observed after the addition of **B** to the blood serum. At the beginning of **B**'s reaction with the serum (0-1 h, 310 K), Cr(III) eluted primarily in LMW serum fractions. After longer reaction times (24-48 h, 310 K), Cr(III) accumulated mainly in HMW fractions (fractions 12-14).

Numerical data for Cr(III) binding to serum fractions are given in Table 1. The total amount of Cr(III) recoveries were calculated from the Cr(III) concentration dissolved in a saturated solution of **A** or **B** in serum. As shown in Table 1, the total amount of recovered Cr(III)

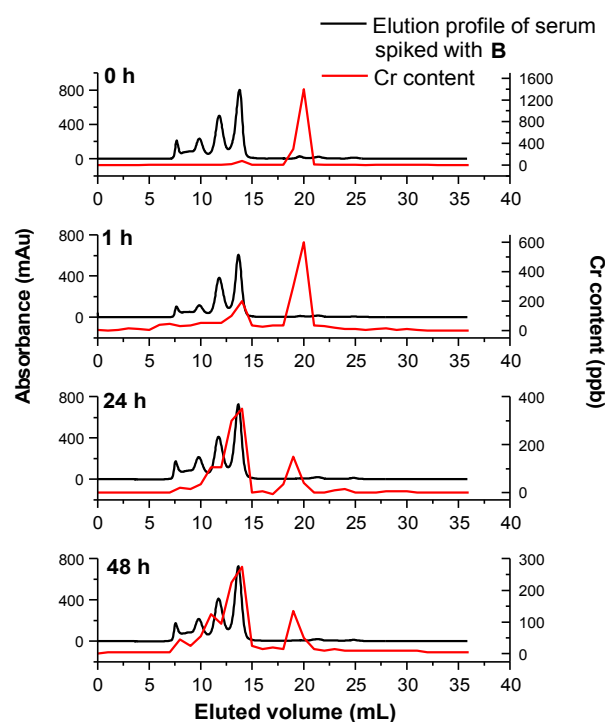
never reached the amount of Cr(III) that was initially introduced into the system, and the amount of unrecovered Cr(III) increased with increasing reaction times.

Interestingly, it was observed that during the 48 h of reaction times, Cr(III) was consistently found to bind to both LMW and HMW fractions. An additional point to be made is that the speciation within serum fractions including Cr(III) was time dependent. For instance, a small amount of Cr(III) was found to accumulate in

HMW fractions (8% and 5% for **A** and **B**, respectively) immediately upon the addition of the compound (0 h, 310 K), even though Cr(III) bound mostly to LMW fractions (68% and 89% for **A** and **B**, respectively). During longer reaction times, as in the case of 24 h at 310 K, 36% and 43% of 44 total Cr(III) in **A** and **B**, respectively, were bound to HMW fractions; 20% and 16% of total Cr(III) in **A** and **B**, respectively, were bound to the LMW fractions. These findings are consistent with the slow kinetics of Cr(III) substitution.



**Figure 2.** Comparison of Size-exclusion Chromatography Profiles and Cr(III) Content in Serum Fractions Spiked with A (0-48 h, 37 °C) on a Superdex 200 Column. A Volume of 1 mL of Each Fraction was Collected

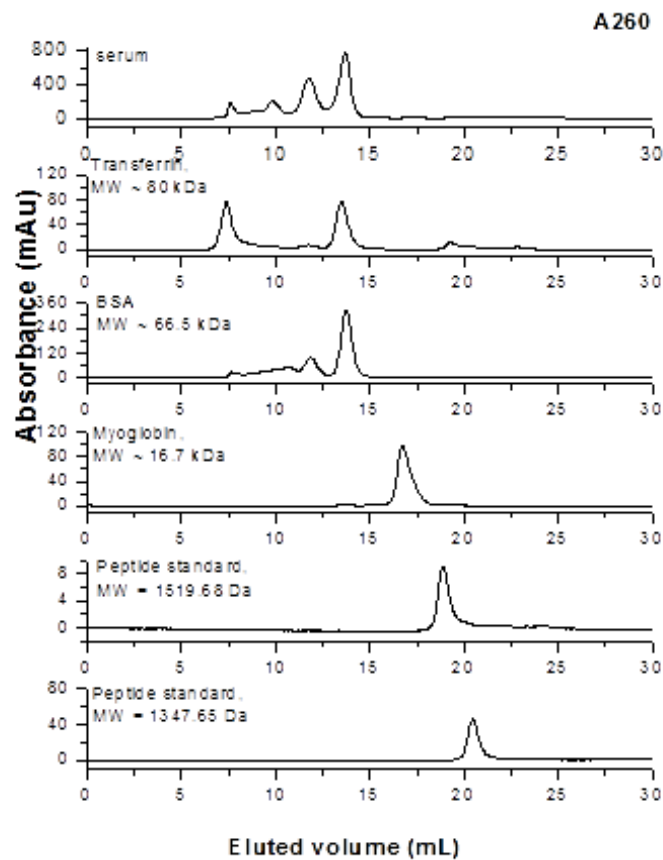


**Figure 3.** Comparison of Size-exclusion Chromatography Profiles and Cr(III) Content in Serum Fractions Spiked with B (0-48 h, 37 °C) on a Superdex 200 Column. A Volume of 1 mL of Each Fraction was Collected

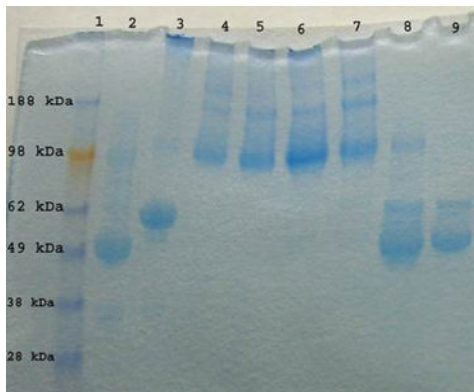
**Table 1.** Summary of GFAAS Analyses of Serum Fractions Spiked with A or B Under the Specified Conditions (0-48 h, 310 K) Collected from SEC

Samples	Quantity of Cr(III) in Serum Fractions (%) <sup>a, b</sup>		Total Cr(III) Recovered (%)
	HMW Fractions (fractions 12-14)	LMW Fractions (fractions 19-21)	
<b>A</b> (0 h)	8 ± 0.5	68 ± 1	84 ± 1.5
<b>A</b> (1 h)	31 ± 2	32 ± 1.1	75 ± 1
<b>A</b> (24 h)	36 ± 2.1	20 ± 1.9	66 ± 2
<b>A</b> (48 h)	22 ± 1.5	23 ± 1.5	57 ± 1.6
<b>B</b> (0 h)	5 ± 2	89 ± 2.1	95 ± 1.5
<b>B</b> (1 h)	10 ± 2	51 ± 1.1	79 ± 1.3

B (24 h)	43 ± 0.5	16 ± 0.6	71 ± 3.1
B (48 h)	36 ± 1.5	14 ± 1.1	62 ± 1.2



**Figure 4.** Chromatograms of Standard Proteins on a Superdex 200 Column (bed dimensions 10 × 300-310 mm). The Fractions were Eluted with 0.10 M NH<sub>4</sub>OAc Buffer (pH 7.2, 298 K, Flow Rate of 0.50 mL min<sup>-1</sup>), with UV Detection at 260 nm. The Concentration of Each Protein was ~1 mg mL<sup>-1</sup>



**Figure 5.** SDS-PAGE Gel Electrophoresis of Serum Fractions in Comparison with Standard Proteins. Lane 1 is a Pre-stained Protein Marker Mixture from Invitrogen: Myosin (188 kDa), Phosphorylase (98 kDa), BSA (62 kDa), Glutamic Dehydrogenase (49 kDa), and Alcohol Dehydrogenase (38 kDa). Lane 2 is a BSA (bovine serum albumin) Standard. Lane 3 is a Bovine APO-transferrin Standard. Lanes 4-9 are Size-

**exclusion Chromatographic Fractions of Serum (unspiked): Fractions 9, 10, 11, 12, 13, and 14, Respectively**

Binding of Cr(III) to HMW and LMW protein fractions has previously been reported [24,25]. These findings suggest that the binding of Cr(III) to HMW proteins LMW proteins was not as specific as previously assumed [26]. In addition, despite five decades of research, no functional Cr-containing enzymes or cofactors have been characterized conclusively, and some hypotheses regarding their possible structure have been refuted [27]. Cr(III)'s mode of action at a molecular level is still an active area of debate. A recent study has suggested that Cr(III) could act as a second messenger or amplify insulin signaling, due to this ion's ability to bind to both HMW and LMW protein masses [28].

Preliminary assignments of the serum fraction natures that bound Cr were performed by comparing the elution times of standard proteins on the same column. Figure 4 shows that several peaks were observed for transferrin

and albumin standards. For these two proteins, the most intense peak resulted from molecular weight calibration, particularly the monomer; the weaker peaks most likely resulted from dimers or other complexes [29]. The HMW serum fractions that bound Cr could be either albumin or transferrin, as the peaks of these proteins were close together. However, separation using size-exclusion chromatography for albumin and transferrin generally resulted in poor resolution of their peaks [30]. In addition, the Cr(III)-binding LMW fractions are likely to correspond to standard peptides with molecular weights around 1.3 to 1.5 kDa. However, since the position of the peak in size-exclusion chromatography is dependent on the nature of the compounds, these results only give a rough indication of molecular weight.

The identification of serum fractions that bound Cr was carried out using protein gel electrophoresis via SDS-PAGE, as presented in Figure 5. A difference in the spot position of BSA (lane 2) with those of the protein markers (lane 1) was observed in this gel. This is because the mobility of protein markers can be affected by the color labels attached to the markers or by components of the sample buffer. The BSA spot of the standard protein was used for molecular weight calibration. Chromatographic fractions 9 to 12 (lanes 4 to 7) were detected as HMW proteins (molecular weights of more than ~80 kDa). Small but distinct differences were observed in the protein position of fractions 13 and 14 (lanes 8 and 9). It is likely that fractions 13 and 14 contained more albumin than transferrin as the main spot position of these fractions corresponds to the spot position of the albumin standard (lane 2). Gel electrophoresis analysis could not be run for the LMW serum fractions (fractions 19 to 21) due to the low protein concentrations within these fractions (less than ~1 mg mL<sup>-1</sup>).

## Conclusion

A combination of size-exclusion chromatographic and atomic-absorption spectroscopic techniques have demonstrated that Cr(III) compounds used as nutritional supplements can bind to both HMW and LMW serum fractions and that the distribution of Cr(III) within this serum was time dependent. At the beginning of reactions, Cr(III) was bound mostly to LMW serum fractions; over ~24 h of reactions, it accumulated to a larger extent in HMW serum fractions. The HMW fractions associated with Cr(III) were albumin and transferrin, the most abundant proteins in the serum. In contrast, the identification of Cr(III)-containing LMW substances has proven to be a demanding task. The study of Cr biospeciation in biological fluids is necessary to understand the biologically active forms of Cr(III) nutritional supplements. Further research is still required to elucidate the role and outcome of Cr that binds to LMW fractions of serum. In summary, the

results of the current work support the hypothesis that common reactive intermediates, including Cr(III)-biomolecules, are likely responsible for both the anti-diabetic activity of Cr(III) compounds and for the genotoxicity and carcinogenicity of Cr(VI).

## Acknowledgments

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