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Journal of Chromatography A, 1054 (2004) 313–319
JOURNAL OF CHROMATOGRAPHY A
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Investigation of the elemental composition and chemical association of several elements in fulvic acids dietary supplements by size-exclusion chromatography UV inductively coupled plasma mass spectrometric

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Abstract

Four fulvic acid dietary supplement samples were obtained for this study with the intention of investigating the elemental composition and association of fulvic acids found in fulvic acid supplements. This was achieved by coupling size-exclusion chromatography (SEC) sequentially with UV–vis and inductively coupled plasma mass spectrometric (ICP-MS) detectors. The combination of UV and ICP-MS offered highly sensitive and selective detection. This technique was used in the present study to initially investigate the chemical association of several different elements including, Cr, Co, Ca, Fe, I, Mg, Zn, Se, Cu, Mn, Mo, As, Hg, Pb, and Ag, by observing the elution profile of the fulvic acids obtained with UV detection and matching their retention times with the peaks measured with ICP-MS. The results found based on this type of analysis suggest that there was some association of the elements to the fulvic acids. It was also of interest to observe the stability of these complexes upon human digestion; therefore a gastric digestion was mimicked. In the fulvic acid dietary supplement samples studied, fulvic acids were present in the samples and there was elemental association based on the retention time overlap in the UV as well as the ICP-MS. The fulvic complexes found in the samples were of a low molecular weight As a result of the digestion the SEC–ICP-MS chromatographic profile in some of the samples changed, which may infer that the elemental association had changed.

Keywords: Fulvic acids; Fractionation; Elemental speciation; Dietary supplements

1. Introduction

The formation of humic substances is a subject that is not fully understood. Research in this area has been extensive and there are numerous studies on the subject that provide theories as to the pathways of formation [1,2]. The simplest being the result of the decomposition of environmental organic matter, mainly including plants and animals. From there, humic substances can be classified into one of three areas; humic acids, humin, and fulvic acids. Humic acids are the fraction of humic substances that is not soluble in water under acidic conditions usually at pH <2. Humin is the fraction that is not soluble in water at any pH. Fulvic acids which are the fraction of interest for this study are the only part of humus soluble in basic, acidic as well as neutral environments [1].

Fulvic acids are useful in part because of their unique solubility properties. This has been beneficial to plants since they absorb nutrients in solution; therefore, fulvic acids effectively transport minerals and nutrients from the soil to the plant [3]. A positive effect on the growth and development of crops has also been observed with the following benefits; an improvement in soil structure, an increase in crop fertility, as well as a bio-stimulant effect [4]. It has also been reported that use of fulvic acids in agriculture has helped increase the metabolism of proteins and activity of enzymes in plants, and detoxify pesticides and herbicides [5,6].

It is suggested that fulvic acids enter the food chain from ingestion of cropland foods that are naturally subjected to the presence of fulvic acids. When elements come in contact with fulvic acids in a water medium, the elements bind with the fulvic acid forming complexes. Once the elements are complexed by the fulvic acid, they are said to become more bioactive and bioavailable. Additionally, the

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doi:10.1016/j.chroma.2004.08.066

elemental complex form, in most cases, is a safer form for ingestion [2]. However, the increase in the use of pesticides, genetically modified crops, and in the overall use of more sterile soil; fulvic acids are often eliminated from the growth process. Practices, such as these result in mineral deficiencies in plants, which is the argument currently being used to market the sale of fulvic acids as dietary supplements.

There have been several approaches to studying fulvic acids; they often involve an initial fractionation/separation technique, usually size exclusion chromatography. The separation is followed by UV–vis or fluorescence detection, both of which are the more common detectors [7]. The lack of retention time standards has caused researchers to try techniques, such as mass spectrometry [8–13]. Nuclear magnetic resonance (NMR) and infrared (IR) have also been used as a non-destructive approach to the problem [7,14]. In addition to the methods described above for humic substances, element specific detectors, such as atomic absorption (AAS), emission (AES), and inductively coupled plasma mass spectrometry (ICP-MS) have all been listed as effective alternatives because of the combination of their selectivity and sensitivity [15,16].

The aim of this study was to investigate the chemical association of several elements of nutritional and toxicological interest as well as their distribution among different molecular weight fractions of the fulvic acid substances present in commercially available dietary supplements. This was achieved by on-line coupling of SEC–UV–vis with successive ICP-MS detection. Different chromatographic variables including mobile phase composition and pH were carefully optimized to obtain accurate information. Changes on the molecular weight distribution of the elements and stability of the fulvic acids complexes were studied and evaluated at pH 6, 8, and 10. The human digestion system was mimicked in order to study the stability of the element–fulvic acid complexes and, therefore, the potential bioavailability of the elements in the body.

2. Experimental

2.1. Instrumentation and reagents

An Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a binary HPLC pump, an autosampler, a vacuum degasser system, a thermostated column compartment, and a diode array detector was used. The ICP-MS was also from Agilent (Agilent Technologies, Tokyo, Japan) and was a model 7500c with an octapole collision reaction cell system, (the instrument was not operated in collision cell mode for this study). The size-exclusion chromatographic column was a Superdex HR 10/30 Peptide (Amersham Biosciences Inc., Piscataway, NJ, USA). The fulvic acid standard was obtained from Sigma–Aldrich (St. Loius, MO, USA).

All reagents used were analytical grade and purchased from Sigma–Aldrich except were noted (Sigma–Aldrich, St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Fisher Scientific Fairlawn, NJ, USA). Solutions were prepared in 18 MΩ cm distilled deionized water (Sybron Barnstead, Boston, MA, USA).

2.2. Sample preparation

Four fulvic acid dietary supplements were purchased from nutritional supplement distributors. The full sample set along with their descriptions is listed in Table 2. The liquid samples were diluted 100-fold, and filtered, using a 0.45 μm nylon filter prior to analysis. For FA4 sample, all of the capsules were emptied and the sample was ground to a homogenous powder with a mortar and pestle. Then, 2 g of the sample was dissolved in 100 mL of deionized water prior to filtering the solution through a filter.

2.3. Intestinal digestion mimic

The intestinal digestion mimic used was a modification of the one developed by Glahn et al. for the determination of iron in different foods [17,18]. First, solutions of pepsin and pancreatin were made as follows: 0.2 g of pepsin (800–2500 U/mg protein) was dissolved in 5 mL of 0.1 M HCl. The solution was combined with 25 mg of chelex resin (Bio-Rad Laboratories, Hercules, CA) and stirred, using a magnetic stirrer for 30 min. Finally, the pepsin solution was filtered. For the pancreatin-bile extract solution, 0.05 g of pancreatin and 0.3 g of bile was dissolved in 25 mL of 0.1 M $NaHCO_3$, the solution was also treated with a chelex resin and stirred for 30 min. Finally, the solution was filtered and set aside.

Ten milliliters of sample was pH adjusted to 2.0 with 5 M HCl, then 0.5 mL of the pepsin solution was added for every 10 mL of sample. Samples were shaken at 37 °C for 60 min. Next, the pH was raised to 6 with the drop wise addition of 6 M $NaHCO_3$ and 2.5 mL of the pancreatin-bile extract was added per 10 mL of sample (based on the original volume). The samples were brought to a final volume of 15 mL with 120 mM NaCl and 5 mM KCl, and shaken for an additional 12 h. Prior to analysis samples were centrifuged, filtered and diluted depending on there concentrations.

2.4. Elemental analysis

The detection of the different fractions was performed using an on-line sequential SEC–UV–ICP-MS detection system. On-line coupling of the SEC–UV system to the ICP-MS detector was performed, using PEEK tubing of 0.5 mm i.d. from the outlet of the UV detector directly to the inlet of the pneumatic nebulizer. The on-line use of the UV–vis detector prior to ICP-MS did not produce dispersion of the ICP-MS chromatographic signals. The ICP-MS was calibrated with a multi-element solution of 10 $\mu g\,l^{-1}$ of each element,

Table 1
Summary of instrumental operating parameters for SEC–UV–ICP-MS technique

Chromatographic separation parameters	
Column superdex peptide	HR 10/30
Mobile phase I	50 mM Tris–HCl, pH 8
Mobile phase II	10 mM CAPS buffer, pH 10
Mobile phase HI	10% Acetonitrile/0.1% TFA, pH 6
Flow rate	0.6 mL min^{-1}
Injection volume	100 μL
Detection parameters	
UV–vis	
λ-Monitored	210, 230, 280
ICP-MS	
Forward power	1300 W
External flow	15.0 L min^{-1}
Internal flow	1.0 L min^{-1}
Carrier gas flow	0.89 L min^{-1}
Isotopes monitored	^{53}Cr, ^{59}Co, ^{44}Ca, ^{56}Fe, ^{127}I, ^{24}Mg, ^{66}Zn, $^{77,78,82}Se$; ^{63}Cu, ^{55}Mn, ^{95}Mo, ^{75}As, ^{202}Hg, ^{208}Pb, ^{107}Ag

including elements of diverse atomic weights, such as Ba, Cd, Ce, Cu, Ge, Mg, Pb, Rh, Sc, Tb, and Tl. Because of the complexity of samples, polyatomic interferences are an issue often encountered; this was avoided by a proper selection of the isotopes for the elements of interest. Additionally, such interferences could be avoided or eliminated with the use of collision cell or dynamic reaction cell technologies or high resolution instruments. For the total analysis an internal standard method was employed. A volume 0.2 mL of each sample was diluted to 20 mL with 2% HNO_3 and 10 μg l^{-1} Ge, In, and Y.

2.5. Size-exclusion chromatography

To assure a proper molecular weight distribution study of the fulvic acid samples, the column was calibrated, using a standard mixture of (Gly) 6: (0.36 kDa), substance P: (1.35 kDa), lysozyme: (14.40 kDa), and myoglobin: (17.00 kDa) at three different pH values (pH 6, 8, and 10). Chromatographic conditions and instrumental operating conditions are all summarized in Table 1.

The mobile phases used for this study initially were two of the more common mobile phases for SEC–ICP-MS; 50 mM Tris–HCl pH 8, and 10 mM CAPS buffer pH 10. However, since the pH of the samples was 6.2, a third mobile phase (10% acetonitrile with 0.1% TFA adjusted to pH 6 with sodium hydroxide solution) was later employed for the SEC–UV–ICP-MS studies of the fulvic acid dietary supplements. The use of the buffer solutions helped to ensure that the elemental association remained unchanged during the study and was and minimized the chances of changing the species originally present in the samples.

3. Results and discussion

The bioavailability of elements is one of the major selling points for these supplements; specifically the fact the elements are claimed to be bound to the fulvic acid structure making them more absorbable, and therefore bioavailable. It is also stated that these complexes stay intact from the point of ingestion up to the point that they enter into the bloodstream. It was, therefore, of interest to investigate the elemental distribution before and after a simulated intestinal digestion by SEC–UV–ICP-MS to study the complexes' stability. The influence of the pH on the elemental distribution in the dietary supplements was also studied at pH 6, 8 and 10. Additionally, the elemental distribution results for the samples were contrasted with those obtained for a fulvic acid standard, which was also studied at the pH 3 values mentioned above.

3.1. ICP-MS elemental analysis of the fulvic acid supplements

The elements labeled on the commercial products were the ones chosen for this study; they are listed in Table 2. It was also of interest to monitor some of the more toxic elements, including As, Hg, Pb, and Ag; since the origin of the fulvic acids was unknown and it is not specified on any of the labels. A total analysis of the samples was performed to confirm the elemental composition/concentration of the samples. The experimental results showed that the elemental concentrations found are in agreement with the labeled values (Table 3). Furthermore, some of the more toxic elements were also monitored and non-quantifiable levels were found for As, Hg, Pb, and Ag.

3.2. Qualitative information obtained from SEC–UV studies

Size exclusion chromatography was the best choice for chromatographic separation because of its ability to separate large biomolecules [19,20]. The absorbance of the chromatographic eluent was initially monitored in the 200–500 nm wavelength range. It was observed that all the fractions

Table 2
List and description of fulvic acid dietary supplement sample set studied

Sample ID	Label description	Physical description	Elements added
FA1	Fulvic acid mineral water	Vitamin enhanced water	Co, Ca, Fe, Mg, Zn, Cu, Cr
FA2	Fulvic acid mineral water	Vitamin enhanced water	Co, Ca, Fe, Mg, Zn, Cu,
FA3	Fulvic mineral complex	Liquid dietary supplement	Co, Ca, Fe, I, Mg, Zn, Se, Cu, Mn, Cr, Mo
FA4	Fulvic acid dietary supplement	Capsule	Co, Ca, Fe, Mg, Zn, Se, Cu, Mn, Cr, Mo

Table 3
Results of total elemental analysis of fulvic acids dietary supplements

Element	Experimental (reported) concentrations (μg/g, n = 3)			
	FA1	*FA*2	FA3	FA4
Co	60 ± 4 (68)	72 ± 4 (85)	83 ± 6 (85)	80 ± 6 (85)
Ca	1985 ± 20 (2380)	2259 ± 32 (2380)	11669 ± 7 (2720)	6994 ± 22 (6800)
Fe	201 ± 10 (255)	296 ± 12 (255)	152 ± 12 (340)	978 ± 17 (1360)
I			38 ± 3 (50)	38 ± 6 (50)
Mg	890 ± 8 (1020)	3043 ± 22 (3400)	7199 ± 23 (8160)	
Zn	156 ± 7 (187)	260 ± 5 (290)	181 ± 16 (204)	2200 ± 15 (3400)
Se			35 ± 4 (39)	48 ± 2 (55)
Cu	22 ± 4 (30)	32 ± 8 (45)	34 ± 7 (45)	27 ± 4 (35)
Mn			88 ± 10 (102)	1548 ± 20 (1700)
Cr	74 ± 6 (85)		93 ± 7 (102)	886 ± 9 (1070)
Mo			37 ± 4 (45)	48 ± 4 (55)

showed good response at 280 nm, therefore this was the wavelength chosen.

The calibration of the SEC column performed with the different molecular weight standards allowed for a curve with a linear response from 0.36 to 17 kDa, plotting the log of molecular weight versus retention time ($y = -0.071x + 5.156$, $r^2 = 0.988$). The calibrated range was appropriate for the samples analyzed, since they show a molecular weight distribution over low molecular weights, which is in agreement with previous reports [15,16]. This was compared with the observation of the fulvic acids standard by SEC–UV at pH 6 (Fig. 1). Since, the fulvic acid present in the dietary supplement samples and the fulvic acid standard could have resulted via a different formation pathway, the comparative experiments were performed just to give a rough idea of how a fulvic acid would behave under such conditions. Upon comparing the fulvic acid sample and standard chromatographic profile at pH 6 (Fig. 1), an overlap in their retention times is observed. The differences in the chromatographic profiles suggest, as previously stated, that the fulvic acids substances present in the sample and the standard are different. However, since the peak obtained with UV detection is in the same molecular weight range of the fulvic acid standard, it is reasonable to assume that the compounds present in the dietary supplements are actually fulvic acids rather than some other unknown compounds. The fulvic acid standard was later run by SEC–ICP-MS and no elements were found in the fulvic standard in the molecular weight range of the column used for this study.

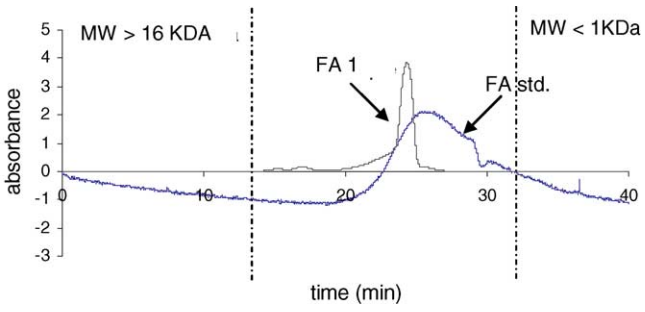

Fig. 1. SEC–UV chromatogram at 280 nm showing elution profile of fulvic acids at pH 6 in a FA standard and a FA sample.

3.3. Effect of the chromatographic variables on the elemental fractionation

Different buffer solutions were used as mobile phases in order to optimize the separation of the different elemental fractions in the SEC peptide column. Thus, 10 mM CAPS (pH 10.0), 50 mM Tris (pH 8.0), and a solution consisting of 10% acetonitrile with 0.1% TFA (pH 6.0) were employed for elemental fractionation of the four different dietary supplements analyzed in this study. The use of different buffers as mobile phases for the chromatography did not yield significant differences in the SEC–UV and SEC–UV–ICP-MS chromatograms (Fig. 2). Therefore, the pH value did not affect the distribution profile of the elements among the different molecular weight fractions and the metal–fulvic acid complexes remained unchanged. As stated above, the pH of the samples was 6.2; therefore, a mobile phase with a composition of 10% acetonitrile with 0.1% TFA at pH 6, was selected for the remainder of the study.

An elemental recovery study of the size exclusion process in the column was estimated by comparison with a flow injection signal from the sample. This was performed to ensure that the compound was not sticking to the column. With the individual estimated elemental mass balances, it was possible to calculate that the chromatographic recovery varied between 82 and 97%.

3.4. Effect of a simulated intestinal digestion on the fractionation profiles

Fig. 3 shows the SEC–UV results for an actual sample (3A) with the enzymes prior to digestion, and 3B shows the results for the same sample after the simulated digestion process. As expected, comparison of the elution profile before and after digestion reveal significant differences because of the enzymatic digestion process. However, it should be noted that there is still a peak eluting between 20 and 30 min in the

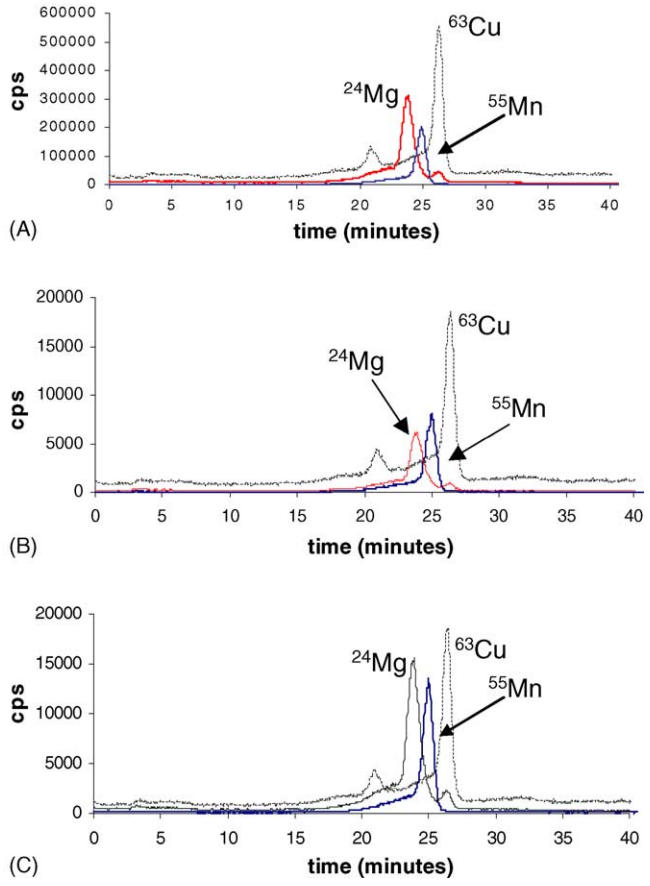

Fig. 2. Chromatographic profile obtained with SEC–ICP-MS showing the elemental distribution of FA3: (A) pH 10, (B) pH 8, and (C) pH 6.

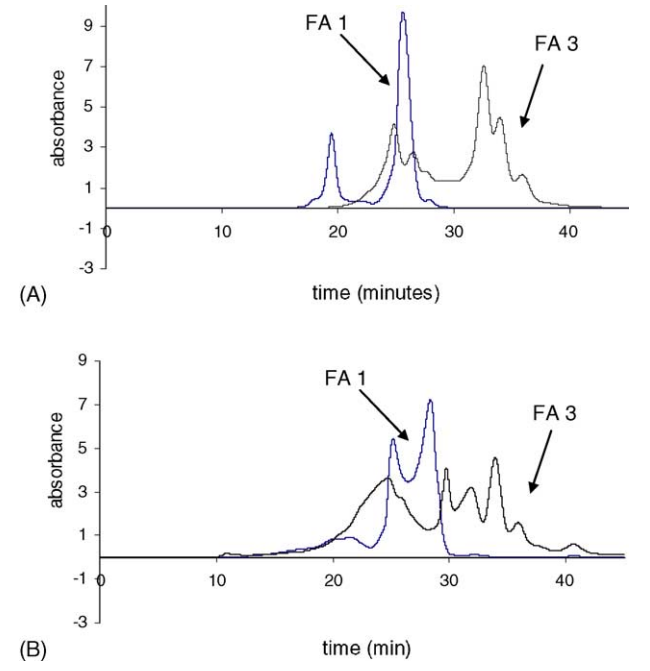

Fig. 3. SEC–UV chromatograms of samples at 280 nm: (A) before (with enzymes) and (B) after simulated intestinal digestion.

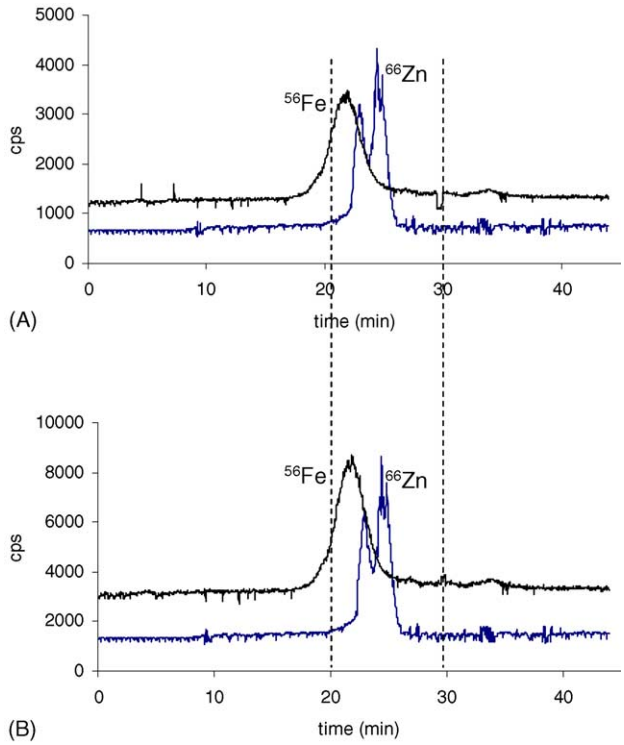

Fig. 4. SEC–ICP-MS chromatograms of sample FA1: (A) before and (B) after intestinal digestion.

UV profile; this was similar for all of the samples. The results obtained by SEC–UV show that the intestinal digestion produced a change in the fulvic acids present in the dietary supplement samples. Since, SEC–UV provided no elemental data, the ICP-MS technique was coupled on-line to the UV detector for subsequent elemental detection and more conclusive information.

Fig. 4 includes the chromatogram obtained with ICP-MS for ^{56}Fe and ^{66}Zn of sample FA1. It shows that this particular sample had no change in retention time or peak shape before and after digestion. This would suggest that the digestion had no affect on the metal associations forming the fulvic acid complex, and it remained intact. This same behavior was observed for both FA1 and FA2. In contrast, upon running the fulvic acid samples FA3 and FA4, the SEC–ICP-MS results were very different. Fig. 5 shows the chromatograms for ^{56}Co and ^{127}I, of FA3 before as well as after the simulated intestinal digestion. As seen in the figure, there was a significant change in the retention time for the chromatographic peak of these elements. The change occurred from 20–30 min to 30–45 min for both, this behavior was common at all of the measured masses and for both FA3 and FA4. The observed shift in retention times implies that the elemental association with the fulvic complex has been altered and, consequently it may affect the bioavailability of the complexes.

These differences in retention times for FA3 and FA4 versus FA1 and FA2 may be because in these particular samples the elements were associated to significantly different types

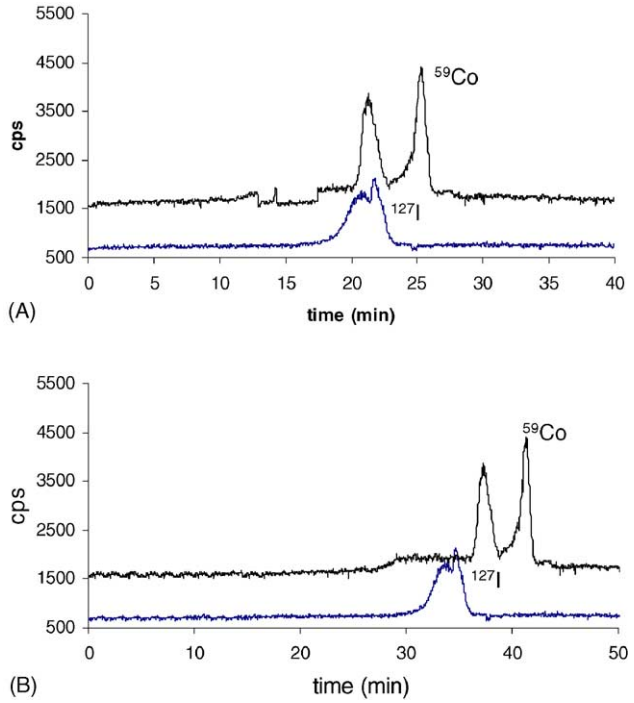

Fig. 5. SEC–ICP-MS chromatograms of sample FA3: (A) before and (B) after intestinal digestion.

of fulvic acids or even to a different ligand type. Possible differences in the fulvic acid structure may have contributed to a particular selectivity with the enzymatic digestion for some samples. Another observation is that, although the chromatographic peaks changed in elution time, the chromatographic profile did not change shape suggesting that the selective enzymatic digestion did not affect the particular metal binding with the appropriate fulvic acid site. In some cases, this may not have physiological significance, for example, in the case of iodine and cobalt (Fig. 5) the ingested form of these elements may not matter because of the way that they are metabolized [21]. However, in the case of chromium, which was also present in some of the supplement samples, a change in the form could be detrimental. Chromium in dietary supplements is often sold as chromium picolinate its trivalent form, however in the hexavalent form the same element is potentially toxic [22,23]. In another case, improper dosages of certain forms of manganese can lower iron levels, and throw Ca, Na, and K levels out of balance [21].

4. Conclusions

Fulvic acids have held a position of relatively high interest because of potential environmental benefits. However, the additional focus on fulvic acids involving nutritional supplements is new and slow moving for valid reasons. Since, there is still much unknown about potential toxic and beneficial effects for these substances, it is likely that care should be taken regarding the ingestion of dietary supplements containing metal–fulvic acid complexes. The results obtained in this work suggest the possibility of employing the SEC–UV–ICP-MS coupling to monitor the association of metals–fulvic acid complexes and develop studies to evaluate their stability before and after a pseudo-digestion. Changes in the molecular weight distribution of different elements were observed for some of the samples after the digestion process, indicating that the bioavailablity of those elements could have been changed. In order to fully access those changes, further studies will need to be carried out to characterize the samples. As it is well-known SEC chromatography is not the most accurate method of determining molecular weight information, and ICP-MS provides no structural information. In the future, two-dimensional chromatography followed by electrospray mass spectrometry will an excellent route to pursue, in order to further characterize these molecules.

Acknowledgements

The authors are grateful to NIEHS Grant No. ES04908 and NSF Grant No. 9904643 for partial financial support.

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