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~~New -~~ ~~arsenosugar~~ Arsenosugar standards extracted from algae: isolation, characterization and use for identification and quantification purposes

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ABSTRACT: Sulfate (SO_4-sug) and sulfonate (SO_3-sug) arsenosugar standard solutions were obtained using preparative liquid chromatography. Several commercial algae samples were characterized (total contents and speciation) to select the most appropriate in relation to their arsenosugar contents. Water extracts from the selected sample (*Fucus vesiculosus*) were fractionated using a Hamilton PRP-X100 preparative column, and the presence of arsenic species in the isolated fractions was ascertained by ~~LC~~IC-ICP-MS. Two of the fractions successfully presented only one arsenic species corresponding to sulfate and sulfonate arsenosugars at suitable concentrations. To unequivocally confirm the presence of both compounds, high-resolution mass spectrometry (ESI-TOF/MS) was used and the exact mass determined with errors lower than 0.5 ppm. The standard solutions obtained were successfully used to identify and quantify SO_4-sug and SO_3-sug in several edible algae samples purchased in local market. Total arsenic content for analysed samples ranged from 34 to 57 mg·kg^{-1}, concentration values found for SO_3-sug ranged from 5 to 36 mg As·kg^{-1} and SO_4-sug was only found in fucus with a concentration of 9,3 mg As·kg^{-1}.

KEYWORDS: Algae, Arsenic speciation, Arsenosugar standards, Preparative chromatography, ~~HPLC~~IC-ICP-MS, ESI-TOF/MS

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1. Introduction

Algae are known to accumulate high arsenic contents [1, 2] and, as primary producers, they accumulate inorganic arsenic and transform it into complex water- or lipid-soluble organic arsenic compounds. When ingested by higher trophic levels, these organoarsenic compounds are further metabolized into other distinct arsenicals or are accumulated unchanged [3 - 5]. It is well known that organic and inorganic arsenic compounds display extremely different degrees of toxicity [6, 7]. In most algae genera, the most abundant species are the arsenic-containing ribofuranosides, commonly called arsenosugars (As-sugars) [8 - 11]. As-sugars play a pivotal role in the transformation and cycling of arsenic in the marine environment, and these mechanisms have been studied [12, 13]. Nuclear magnetic resonance spectroscopy has revealed the structures of arsenosugars shown in Figure 1. The four compounds vary only in the side chain attached to the R position of the sugar.

Although it seems that As-sugars are not acutely toxic, they have the potential to present slight chronic toxicity and, due to high seaweed consumption, assessment of exposure to different As-sugars is needed [14 - 16]; until now, very little reliable information on toxicity exists.

Analytical methods for arsenic speciation are generally based on coupling a separation technique (mainly liquid chromatography) with a suitable detection system. Inductively coupled plasma mass spectrometry (ICP-MS) has been widely used to identify and characterize different arsenic-containing compounds in marine samples, since it offers high element sensitivity, low limits of detection and can be easily coupled to HPLC. Different modalities of liquid chromatography have been used for the initial separation of the arsenic species such as ion exchange (cationic and anionic) and reverse-phase (with ion pairing) [17 - 20]. Additionally, HPLC coupled with electrospray mass spectrometry (ES-MS) is used to provide structural information on the arsenic compounds [21 - 24].

However, analysis of arsenosugars is hampered by the very similar physical and chemical properties of these species and the lack of standards. Although standards are commercially available for some arsenic species, this is not the case for arsenosugars. In the literature some attempts are reported for the synthesis of arsenosugars [25 - 29]. These procedures are time consuming (nine days for one step), involving a high number of steps and the reported overall yield is low (ranging from 5% to 22% and only in one case up to 68%). Moreover, certified reference materials (CRMs) are scarce and

published data can only be found regarding some As-sugar contents (phosphate and sulfonate arsenosugars) in a kelp dietary supplement [30, 31], which could be useful for method validation.

The aim of this study is to obtain algae extracts containing isolated and well characterised arsenosugar species to be used as standards for quantitative and qualitative purposes to support IC-ICP-MS analysis~~The aim of this study is to obtain and characterize solutions containing isolated arsenosugar species to be used as standards for quantitative and qualitative purposes to support HPLC-ICP-MS analysis~~. To this end, a suitable algae matrix was selected from those that are commercial available and the suitability of preparative chromatography was assessed as an isolation method to obtain solutions containing single arsenic species. Once isolated, arsenosugars were unequivocally identified by high-resolution mass spectrometry. Solutions containing sulfate (SO_4-sug) and sulfonate (SO_3-sug) arsenosugars were successfully used as standards for further identification and quantification of these species in commercially available edible algae samples by ~~HPLC~~IC-ICP-MS.

2. Materials and methods

2.1. Reagents and standards

Analytical-grade reagents were used throughout the study: nitric acid (69%) (PanReac, Hiperpur), 98% formic acid (PanReac), ammonium dihydrogen phosphate (PanReac), 25% aqueous ammonia solution (PanReac), pyridine (Scharlau) and 31% hydrogen peroxide (Merck, Selectipur). Doubly deionized water used as the HPLC solvent was purified with a Millipore water system (18.2 MΩ cm^{-1} resistivity and total organic carbon <30 µg L^{-1}).

The stock standards used for inorganic arsenic species were a solution of As (III) with a certified concentration of 1002±4 mg ~~As~~ L^{-1} (Inorganic Ventures) and a solution of As (V) with a certified concentration of 1003 ± 6 mg ~~As~~ L^{-1} (Inorganic Ventures), both traceable to NIST (National Institute of Standards and Technology).

Other stock standard solutions (500 mg As L^{-1}) were aqueous solutions prepared from $(CH_3)AsO(Ona)_2 \cdot 6H_2O$ (Carlo Erba) for methylarsonic acid (MMA), from $(CH_3)_2AsNaO_2 \cdot 3H_2O$ (Fluka) for dimethylarsonic acid (DMA), from $(CH_3)_3As^+(CH_2)CH_2OHBr^-$ (Argus Chemicals SRL) for arsenocholine (AC), from $(CH_3)_3As^+ CH_2COO^-$ (Argus Chemicals SRL) for arsenobetaine (AB), and from $(CH_3)_3AsO$ (Argus Chemicals SRL) for trimethyl arsenic oxide (TMAO). These solutions were standardized against

As(III) certified standard solutions. All stock solutions were kept at 4 °C in polyethylene containers. Further diluted solutions for analysis were prepared daily.

2.2. Instruments and apparatus

A microwave digestor (Milestone Ethos Touch Control) was used for total arsenic determination ~~and for the extraction of species~~. An end-over-end shaker and a centrifuge Rotanda 460RS were used for arsenic species extraction.

An Agilent 7500ce inductively coupled plasma mass spectrometer (ICP-MS) (Agilent Technologies, Germany) with a ~~BURGENER~~ Burgener Ari Mist HP type nebulizer was used for arsenic measurement. For arsenic quantification, ion intensity at m/z 75 (^{75}As) was considered. Additionally, ion intensities at m/z 77 ($^{40}Ar^{37}Cl$) and m/z 35 (^{35}Cl) were monitored to detect possible chloride interference ($^{40}Ar^{35}Cl$) at m/z 75.

The columns and chromatographic systems used in the study are summarized in Table 1, for both analytical and preparative chromatography.

Mass spectrometry measurements were performed in an MSD-time-of-flight (MSD-TOF) series 2006 (Agilent Technologies, Palo Alto, CA, USA) equipped with Agilent Mass Hunter software that was used for MS control, data acquisition, and data analysis. The sample was directly introduced by using a quaternary pump of an 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA) was used.

2.3. Reference material

A seaweed certified reference material for total arsenic content was used throughout this work. Bladderwrack (*Fucus vesiculosus*) (ERM-CD 200) from the Institute for Reference Materials and Measurements (IRMM) has a certified total arsenic content of 55.0 ± 4 mg ~~As~~ kg^{-1}.

Additionally, an aliquot of a freeze-dried extract of *Fucus serratus*, kindly donated by Prof. K.A. Francesconi (Karl-Franzens University, Graz, Austria) [32], was used for arsenosugar identification. The extract is extensively used for arsenosugar identification in algae samples [33].

2.4. Samples and sample pretreatment.

The algae samples used in this study were purchased at local specialized shops in Barcelona (Spain), during January 2018. Samples are sold as alimentary supplements or foodstuff. All samples are *Fucus*

vesiculosus presented in two different forms: pellets (samples A, B, C) and dried portions at low temperature (samples D, E). Additionally, to check the performance of the new isolated standards, several edible algae samples were analyzed: wakame, nori and kombu (dried form) and fucus (pellets). Pellets were finely powdered in a glass mortar. Samples available as dried leaves (D, E) required the use of a ring mill of tungsten carbide for grinding~~The dried portions are hard and need to be hydrated before consuming. Sample pretreatment in these cases required the use of a ring mill of tungsten carbide~~. The resulting fine powders were manually homogenized and stored in polyethylene containers at room temperature until analysis.

2.5 Procedures

2.5.1 Moisture determination

Aliquots of 0.2 g of sample were dried in triplicate, at 100 ± 5 °C to constant weight in an oven with natural convection. The moisture values obtained ranged from 10 to 12% and all results refer to the dried mass.

2.5.2 Total arsenic ~~analysis~~determination

The total arsenic contents in samples were determined in triplicate by ICP-MS after microwave acid digestion as described elsewhere [9]. In summary, 0.5 g was weighed into the digestion vessels. After addition of 8.0 mL of 69% nitric acid and 2.0 mL of 31% hydrogen peroxide, the vessels were closed and digested following a temperature program up to 190 ^{o}C for a total digestion time of 80 min. Digestion blanks were also prepared in each sample digestion series. After cooling to room temperature, the digests were transferred to vials and diluted with double deionized water up to a final weight of 25 g. Digested samples were kept at 4 °C until analysis and filtered through 0.45 µm nylon filters prior to analysis.

The extracts were analyzed by ICP-MS after further dilution by a factor of 50. Helium gas was used in the collision cell to remove interferences. Arsenic was quantified using internal standard calibration ~~and~~ (^{103}Rh was used as the internal standard). ~~The sample was quantified by means of an external calibration curve from a~~Arsenate standards in the working range of 0–50 ~~µg~~ µg·~~As m~~L^{-1} were used. For quality control purposes, standards were run before and after each sample series.

2.5.3 Arsenic species analysis

For extracting As species, 0.1000 g of the samples were weighed into 15 mL polypropylene tubes and 10 mL of doubly deionized water were added. Samples were extracted using an end-over-end shaker at 30 rpm for 16 h at room temperature. The suspensions were centrifuged at ~~3000~~ 1855 ~~rpm~~ *g* for 20 min and supernatant extracts were filtered through 0.45 µm nylon filters and kept at 4 °C until analysis. In order to guarantee stability, arsenic species in the extracts were analyzed by ~~HPLC~~IC-ICP-MS no later than 24 h after extraction. Instrumental conditions for anion exchange chromatography and cation exchange chromatography are detailed in Table 1 together with the species determined in each case. These analytical methods are based on previously established conditions [9]. Quantification was performed by external calibration curves to the nearest eluted standard compound. Sulfate and sulfonate arsenosugars were quantified with As (V) standards, phosphate arsenosugar was quantified with MMA standards, and glycerol arsenosugar was quantified with AC standards.

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Extraction blanks were also performed in each session and, as an internal quality control check, the total arsenic content in the extract was compared with the sum of quantified species.

2.5.4 Fractionation of algae extracts by preparative column

An aqueous extract from 0.1000 g of sample was prepared as described in Section 2.5.3. After filtrating the extract, 2 mL were injected in the preparative column and were eluted ~~on, the supernatant was eluted isocratically with the 40 mM NH_4HCO_3 (pH = 8.0) in 1% MeOH solution~~ at the working conditions shown in Table 1. Sixty fractions of 5 mL were manually collected during one hour of elution. The total arsenic content in each fraction was ~~analyzed~~ determined as described in Section 2.5.2 and arsenic species were determined as in Section 2.5.3.

2.5.5 Mass spectrometry analysis of algae extracts

Mass spectrometry analysis of the algae extracts was performed with a time-of-flight mass spectrometer (Agilent Technologies) equipped with an ESI source. Each sample was introduced by flow injection using a HPLC Agilent 1100 pump working at a flow of 200 µL min^{-1} of H_2O:CH_3CN (1:1 v/v). Full-scan data acquisition was performed from 80 to 840 m/z using electrospray ionization in positive and negative ion mode. Working conditions for ESI are the following: N_2 was used as nebulizing gas at 15 psi pressure; source was heated to 325 ^{o}C; capillary voltage was 4.00 kV (positive mode) and 3.5 kV (negative mode).

2.5.6 *Use and performance of standards*

The isolated arsenosugar standard solutions were used for identification purposes (through fortification studies) and for quantification of four commercial available algae: kombu, wakame, nori and fucus. In all extracts, arsenic species were determined by anion-exchange chromatography as described in Section 2.5.3.

3. Results and discussion

3.1. Selection of raw algae material

The total arsenic and arsenic species content in algae differ tremendously between samples, depending on genera or origin. Therefore, the criteria that should be taken into consideration before selecting the appropriate algae for the identification, separation and isolation of arsenosugars include the following: contents of total arsenic and the presence of arsenic species, especially the proportion of arsenosugars. Accordingly, five algae samples, A, B, C, D and E (see the experimental section), were tested with the aim of selecting the best material for the purpose of this study.

The total arsenic content in the samples was determined by ICP-MS after microwave digestion as stated in the experimental section. For quality control purposes, the certified reference material ERM-CD 200 was measured with each sample batch and no significant differences were observed when comparing obtained values with certified values using a t-test at 95% confidence level~~good agreement was obtained with respect to the arsenic content in the CRM~~. As seen in Table 2, the total arsenic content ranged from 37.57 ± 0.73 ~~μγ/g~~ As kg^{-1} in algae E to 85.26 ± 0.99 mg As kg^{-1} in algae C. ~~These relatively high values in relation to fresh algae samples are in agreement with the fact that the samples studied were processed, in this case having been dried to moisture contents below 12%.~~

Arsenic species analysis was performed by ~~LC~~IC-ICP-MS. The arsenic compounds (i.e. arsenosugars) analyzed are polar and extremely soluble in water. Water is considered a suitable extractant since it is capable of penetrating the sample matrix [34]. Hence, water was chosen for arsenic species extraction in this study.

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The extraction efficiency was evaluated by calculating the ratio of total arsenic present in the extracts to the total arsenic in the samples, resulting from acid digestion. The extraction efficiencies are also presented in Table 2 for samples and the CRM, with values ranging from 78.8% to 101.7%, and 93.6%, respectively. Thus, it can be concluded that water proved to be an effective solvent in the extraction of arsenic species in the samples studied. Additionally, the table summarizes the limits of detection (LOD) and quantification (LOQ) for each arsenic species in the samples, the determination of which has been described in detail previously [35]. Furthermore, the results show that arsenosugars are the main arsenic compounds in the samples studied, showing percentages of the arsenic extracted ranging from 39.2% (algae E) to 66.7% (algae C). Appreciable amounts of other arsenic species were also found (Table 2) showing considerable variation in species distribution for the different samples. Column recovery was 51.7% to 70.6% and 104.6% for samples and the CRM respectively, which can be considered acceptable in this kind of study. According to our results, it can be seen that sample C, with the highest total arsenic content, also presents the highest proportion of arsenosugar species (66.7% of the extracted arsenic).

The chromatograms obtained for sample C are shown in Figure 2. Arsenic species were identified by comparison with the retention times of a *Fucus serratus* reference sample. As seen in the assignment of peaks, PO_4-sug, SO_3-sug, and SO_4-sug can be identified in the anion-exchange chromatogram, whereas Gly-sug was identified in the cation-exchange chromatogram. Two peaks (see Figure 2a) were not identified via anion-exchange chromatography due to the difference of retention times in relation to those observed in the *Fucus serratus* chromatogram. Therefore, further identification was undertaken by adding standard solutions of 10 µg L^{-1} of DMA and MMA to the sample C extract. As seen in the insert of Figure 2a, the addition of the standards yields an increase of the supposed DMA and MMA peaks (dotted line in the insert), confirming their occurrence. In addition, similar spiking experiments using AB and TMAO species standards were carried out using cation-exchange chromatography, confirming the absence of these species in the extracts.

Sulfonate arsenosugar is the predominant species in the selected sample, accounting for 50.8% of the extracted arsenic. Lower concentrations of sulfate arsenosugar and phosphate arsenosugar were obtained with percentages of extracted arsenic around 11.7% and 2.2%, respectively. Gly-sug was also found in small proportions (below 3% of the extracted arsenic). DMA and MMA were also identified in small proportions and below 1% of the extracted arsenic. Additionally, the results clearly show that

227 inorganic arsenic was found in small proportions in the algae: 0.23 ± 0.07 mg As kg^{-1} of As(V),
228 accounting for 0.3% of the extracted arsenic; and 1.99 ± 0.08 mg As kg^{-1} of As(III), which is below 3%
229 of the extracted arsenic. The As(III) concentration was estimated from the difference between the
230 concentration of the integrated front peak containing As(III) plus cations, which co-eluted in the
231 anionic column, and the sum of the cations eluted in the cationic column (AB, AC, TMAO and Gly-
232 arsenosugar). These results are in agreement with Llorente-Mirandes *et al*. [35], who reported the
233 identification of arsenosugars in water extracts of marine algae. According to our results, sample C
234 proved to be the most suitable matrix for further experiments.
235
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237 ***3.2. Fractionation of algae water extract***

238 Further experiments focused on testing the capability of preparative chromatography to isolate
239 arsenosugars by fractionation. Preliminary studies by the research group showed that good
240 arsenosugar separation could also be achieved using ammonium hydrogen carbonate as the mobile
241 phase even if, in this case, the total analysis time would be longer. Whereas for analytical
242 chromatography ammonium dihydrogen phosphate was used as the mobile phase, for preparative
243 chromatography ammonium hydrogen carbonate was preferred in order to make the isolated
244 fractions suitably compatible with the molecular mass spectrometry detector for further
245 characterization purposes.
246 Fractionation of sample C water extracts and a standard solution containing the available arsenic
247 species was carried out as described in Section 2.5.4. Sixty fractions were collected during one hour of
248 elution (one fraction of 5 mL each minute) and they were analyzed for their total arsenic content. The
249 results of two replicate experiments are shown in Figure 3. It can be seen that arsenic in the algae
250 extract eluted in five main peaks: Peak I corresponding to the fraction collected at 12 min (0.34 mg kg^{-1}),
251 Peak II corresponding to fractions collected from minute 15 to minute 17 (3.79 mg kg^{-1}), Peak III
252 corresponding to fractions from 20 to 23 min (2.72 mg kg^{-1}), Peak IV corresponding to fractions
253 collected from 28 to 33 min (43.0 mg kg^{-1}), and Peak V corresponding to fractions from 45 to 50 min
254 (13.6 mg kg^{-1}). Both experiments provided highly reproducible results and, in all cases, preparative
255 column recovery was higher than 85% with respect to the total arsenic content in the water extract.

Each peak was analyzed by ~~HPLC~~IC-ICP-MS to determine the possible arsenic species present. Figure 4 shows the speciation results obtained, compared with the chromatogram of a mixture of anionic species standard solution, and with the chromatogram of a *Fucus serratus* extract which is used for arsenosugar identification purposes. It can be seen that Peak I contained cationic species and inorganic arsenic, Peak II contained As(III), cations and DMA, and no arsenosugars were present in the peaks corresponding to higher elution times. Peak III mainly contained the phosphate arsenosugar (PO_4-sug) with small amounts of MMA and thus isolation of PO_4-sug was not achieved under the working conditions adopted. In contrast, Peaks IV and V successfully presented only one arsenic species corresponding to SO_3-sug and SO_4-sug, respectively.

The separation achieved by the preparative column was also checked by eluting a standard solution containing all available arsenic species in the same way. The speciation analysis of the eluted peaks of the standard solution by both cationic and anionic exchange chromatography provided peaks at the theoretically expected retention times, and none of the available species used as standards were observed in Peaks IV and V, whereas MMA was obtained in Peak III.

In order to increase the concentrations obtained in the fractionation procedure, a more concentrated extract (1.0 g sample to 10 mL water) was fractionated and analyzed as described before. The results show that elution times of arsenic compounds are consistent with the more dilute extract but the observed peaks show a pronounced front that indicates a possible overloading of the separation system. Although certain loss of separation efficiency is observed when injecting more concentrated extracts, both Peak IV and Peak V show only one arsenocompound. However, concentrations increased by a factor of 10 in both cases. Thus, preparative chromatography has proved to be a powerful tool to isolate arsenosugar species from a certain volume of algae extract, as is the case of the fractions corresponding to Peak IV and Peak V.

3.3. Characterization of isolated arsenosugars by ESI-TOF/MS

As mentioned in the introduction, ESI-MS allows the characterization of arsenosugars and is likely to be applicable to most organoarsenic compounds found in environmental samples [21, 22]. Moreover, the high mass resolution of TOF-MS allows the calculation of the molecular formulas with the data obtained for the molecular ion. Besides exact mass measurements, the isotope patterns of the protonated molecular ions can also be used for further confirmation of the structural identification.

285 The abundances of the molecular ion isotope peaks ($[M+H]^+$) and ($[M-H]^-$) could be monitored to
286 assign the compounds. Figure 5a shows the LC-full scan TOF/MS spectra and isotope pattern of
287 $[M+H]^+$ and $[M-H]^-$ ions obtained by direct infusion of Peak IV. Ions corresponding to m/z 393.0197 in
288 positive mode and m/z 391.0079 in negative are proposed to be protonated and deprotonated SO_3-
289 sug ions, respectively. The experimental mass data for these ion peaks are consistent with their
290 theoretical values, since the chemical formula of the compound $C_{10}H_{22}AsO_9S$ obtained matches the
291 SO_3-sug. The experimental isotope pattern data of the protonated molecular ion exactly matched with
292 the theoretical isotope pattern presenting a mass error of -0.51 ppm. Moreover, several structurally
293 informative product ions were observed. Two product ions observed under ESI operating conditions,
294 m/z 130 and 119, can be assigned to structural features of SO_3-sug in positive-ion and negative-ion
295 modes, respectively. These two ions occur at significantly lower relative intensity. For example, the
296 ion occurring at m/z 130 is believed to have resulted from cross-ring cleavage of the ribose moiety
297 yielding an ion at m/z 148, and subsequent loss of H_2O.
298 Mass spectra were also recorded by direct infusion of Peak V into ESI-TOF/MS. As shown in Figure 5b,
299 a total of four major $[M+H]^+$ and $[M-H]^-$ product ions can be assigned to structural features of SO_4-sug.
300 The mass spectrum clearly shows m/z 409.0145 for positive and 407.0013 for negative corresponding
301 to the protonated and deprotonated molecular ions of SO_4-sug, respectively. Moreover, the
302 comparison of the obtained mass with the theoretical exact mass yields an error of -0.17 ppm
303 considering that the elemental composition is $C_{10}H_{22}AsO_{10}S$, and the experimental isotope pattern
304 data of the protonated molecular ion exactly matches the theoretical one. Other product ions were
305 also observed at m/z 329 and 407 (positive mode), and m/z 255, 310 and 407 (negative mode) that
306 are consistent with the fragmentation of the proposed structure. Our results are in agreement with
307 data reported in the literature [23, 36, 37], and therefore SO_3-sug and SO_4-sug were identified as the
308 only species containing arsenic in the collected fractions corresponding to Peak IV and Peak V,
309 respectively.

310 ***3.4. Use and performance of standard solutions***

311 The fractions obtained containing SO_3-sug and SO_4-sug species were standardized against As(V)
312 certified solutions to establish the concentration as arsenic which were 493 $\mu g \cdot L^{-1}$ and 101 $\mu g \cdot L^{-1}$,
313 respectively. Once standardized, calibration graphs containing all available anionic species (As(III),

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As(V), DMA, MMA, SO_3-sug and SO_4-sug) were prepared in the concentration range from 1 to 50 μg L^{-1} (as arsenic). As expected, the slopes obtained were similar in all cases.

Four commercially available samples were analyzed. Example chromatograms of algae extracts and fortified extracts with arsenic species are shown overlapped in Figure 6. It can be seen that with the standards obtained, the identification of SO_3-sug and SO_4-sug in unknown samples is straightforward. Although neither of these two As-sugars were present in the nori sample, SO_3-sug was present in wakame and kombu, and both were found in fucus sample. The concentrations determined using calibration graphs prepared with the new standards are shown in Table 3 for quantification runs corresponding to two different days, confirming the suitability of their use as standard solutions.

4. Conclusions

Quantification of arsenosugars is difficult due to the lack of commercially available standard solutions. At present the only available tool for laboratories in the field of As-sugar speciation is a kelp powder aimed at method validation.

After a screening study on different available algae samples regarding arsenic content and speciation a Fucus vesiculosus sample was considered appropriate for the study. The use of preparative ionic chromatography has proved to be a good approach for isolating SO_4-arsenosugar and SO_3-arsenosugar as the unique arsenic species present in solution. These solutions were standardised against arsenic (V) traceable standard and the presence of both arsenosugars was confirmed by means of high-resolution mass spectrometry (ESI-TOF/MS). Finally, these solutions were used for the successfully identification and quantification of these As-sugars in commercially available edible samples as nori, kombu, wakame by IC-ICP-MS.

~~In this work, the use of preparative chromatography has proved to be a good approach for isolating SO_4-sug and SO_3-sug standard solutions from *Fucus vesiculosus* samples. After the confirmation of isolated standards by means of high-resolution mass spectrometry (ESI-TOF/MS), identification and quantification of these As-sugars in several commercially available edible algae was successfully achieved by HPLC-ICP-MS.~~

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Figure captions

Fig. 1. Chemical structure of the arsenosugars analyzed.

Fig. 2. Chromatograms of reference material (*Fucus serratus*) and sample (*Fucus vesiculosus*) extracts from anion exchange (a) and cation exchange (b) by ~~LC~~IC-ICP-MS.

Fig. 3. Arsenic contents in fractions obtained by preparative chromatography from algae extracts.

Fig. 4. Anion exchange chromatograms of standards and selected fractions containing arsenic species.

Fig. 5. HPLC-ESI-TOF/MS spectra, isotope pattern of $[M+H]^+$ and $[M-H]^-$ ions of SO_3-sug (a) and SO_4-sug (b). Ion peaks labeled with an asterisk (*) are believed to contain arsenic.

Fig. 6. Anion exchange ~~LC~~IC-ICP-MS chromatograms of algae extracts (direct and fortified samples): (a) kombu, (b) wakame, (c) fucus and (d) nori.

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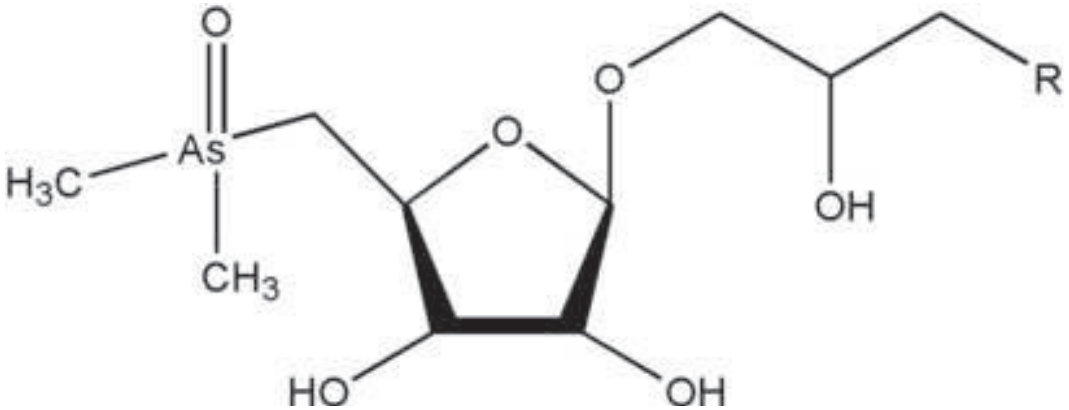

1 R=OH
2 R=$OPO_3CH_2CH(OH)CH_2OH$
3 R=SO_3H
4 R=OSO_3H

Figure 2a (tiff file)
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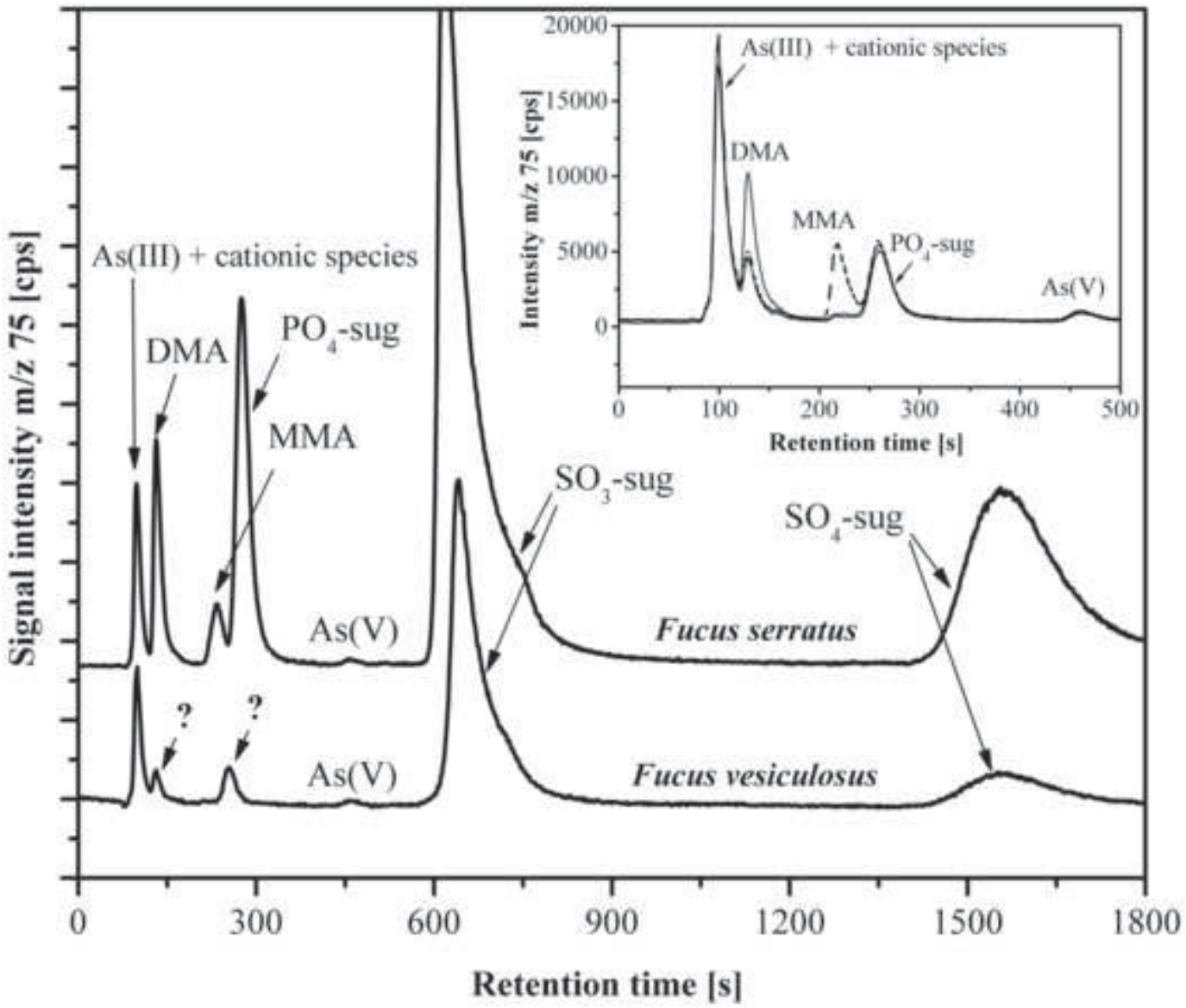

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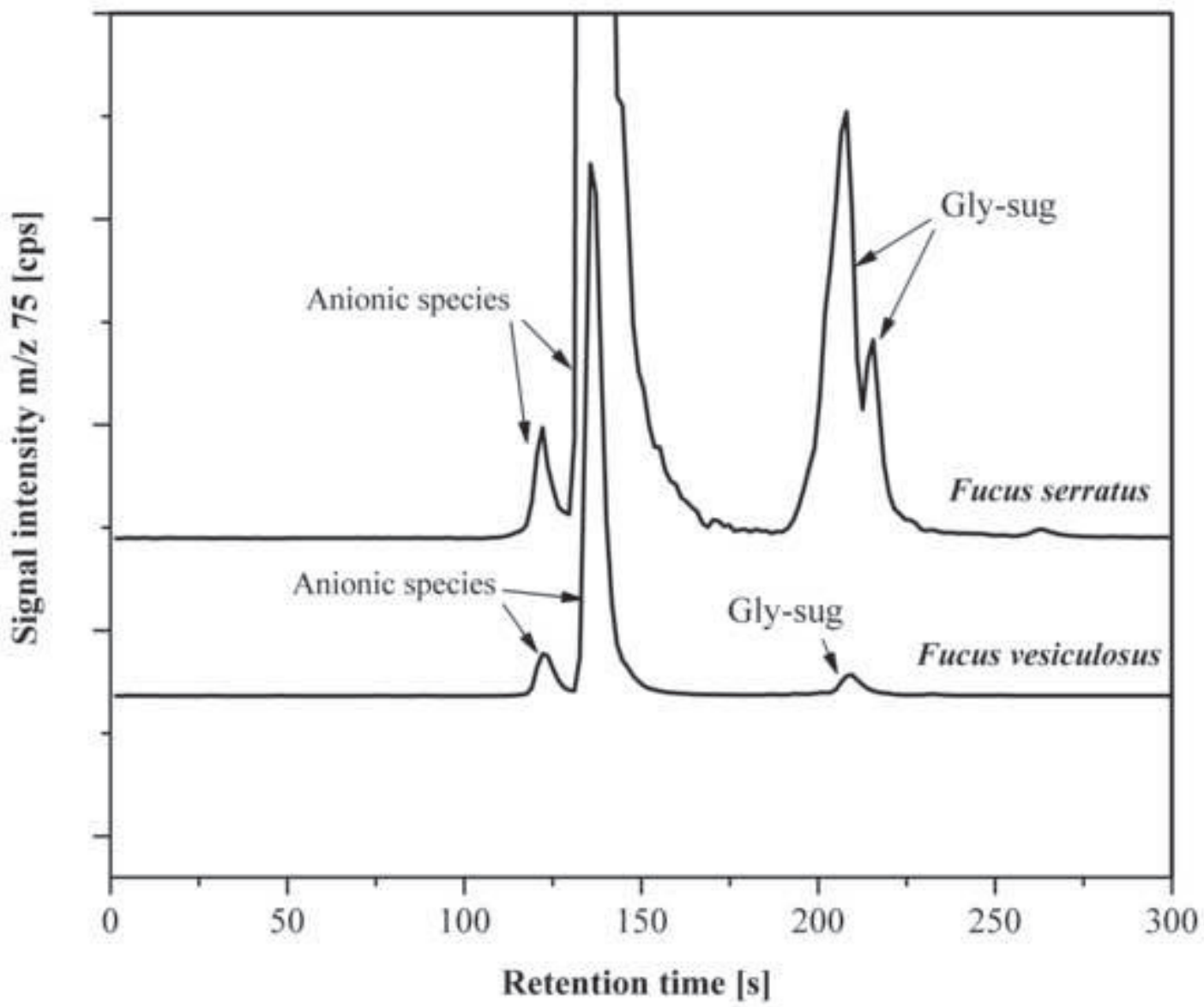

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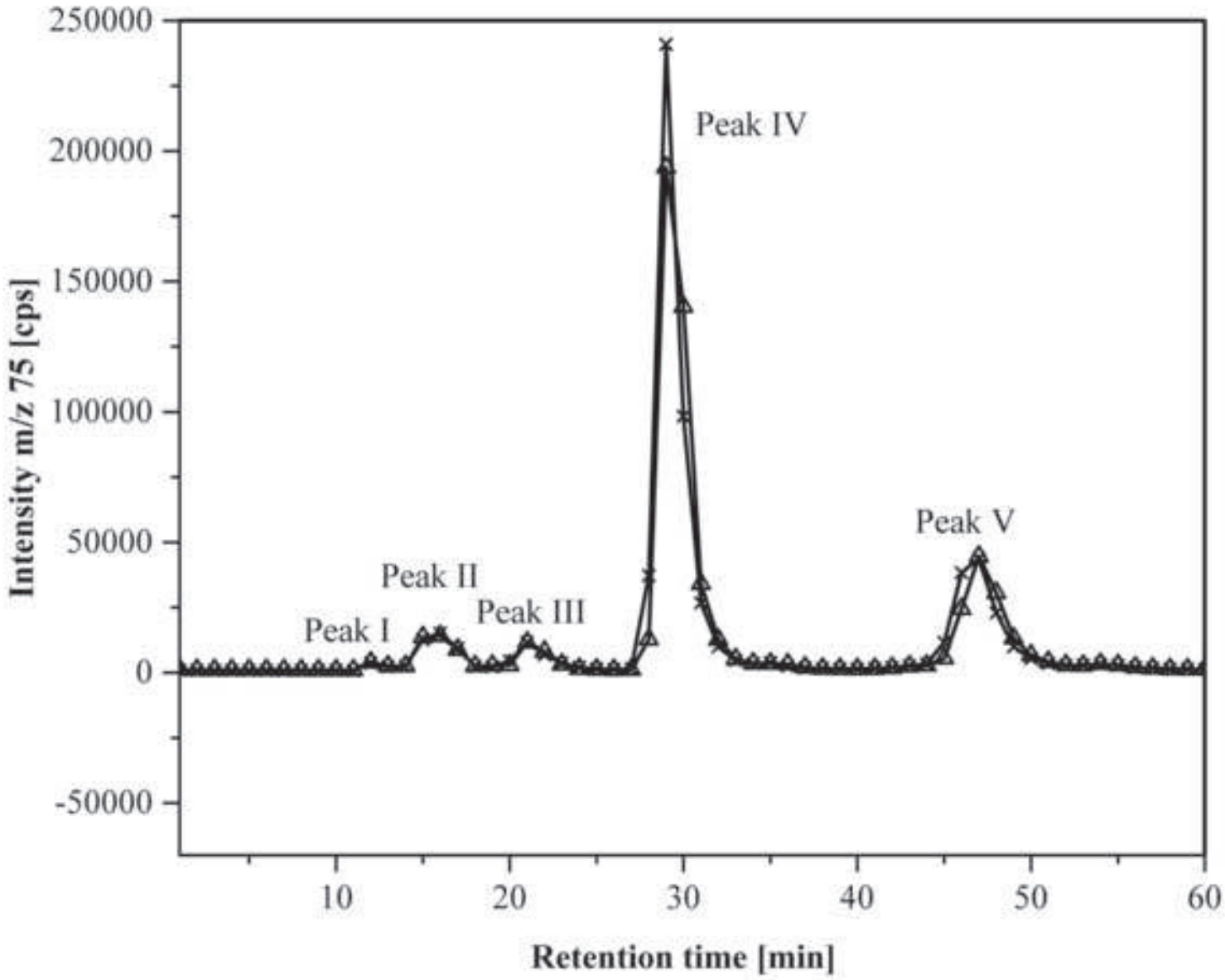

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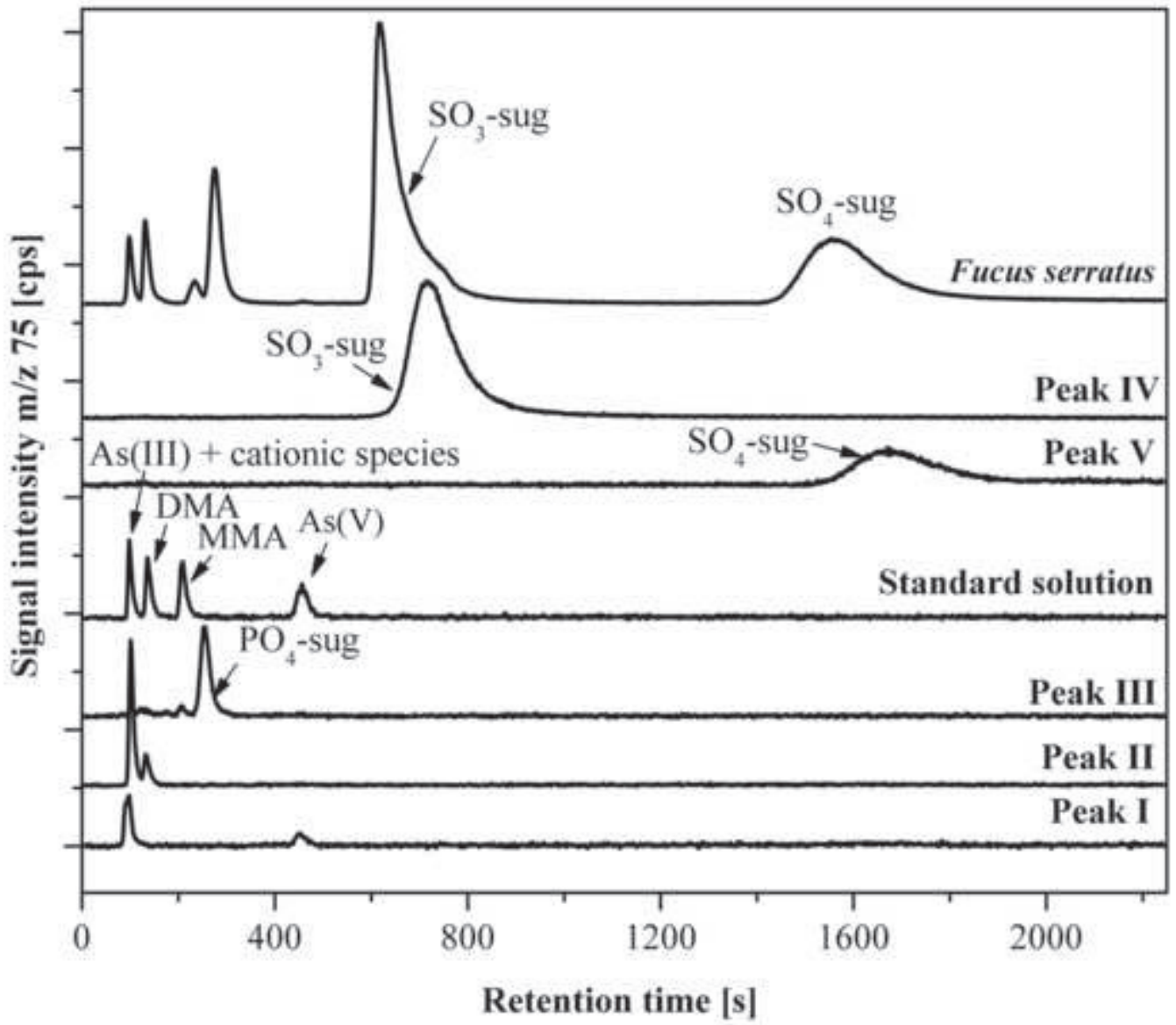

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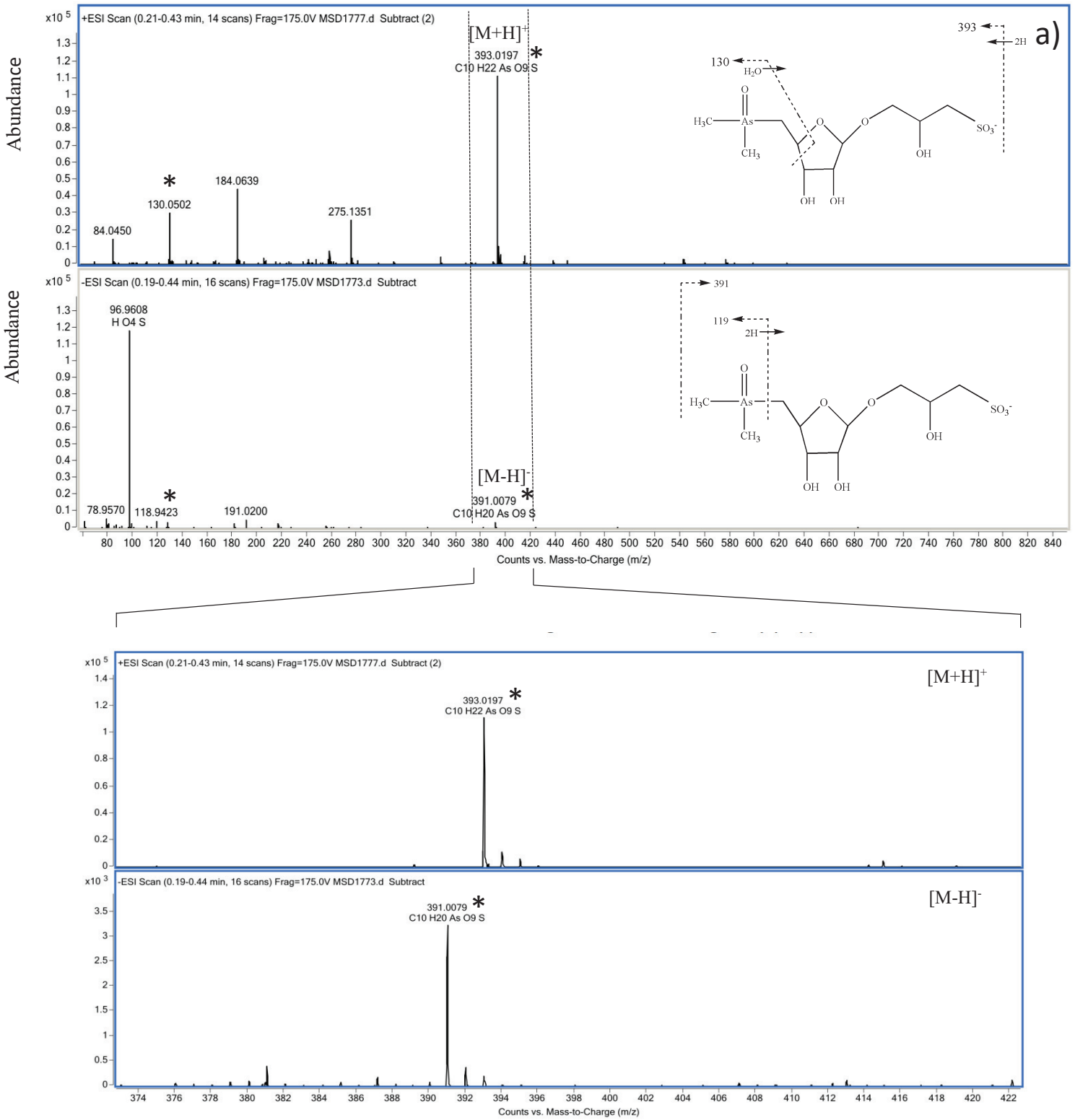
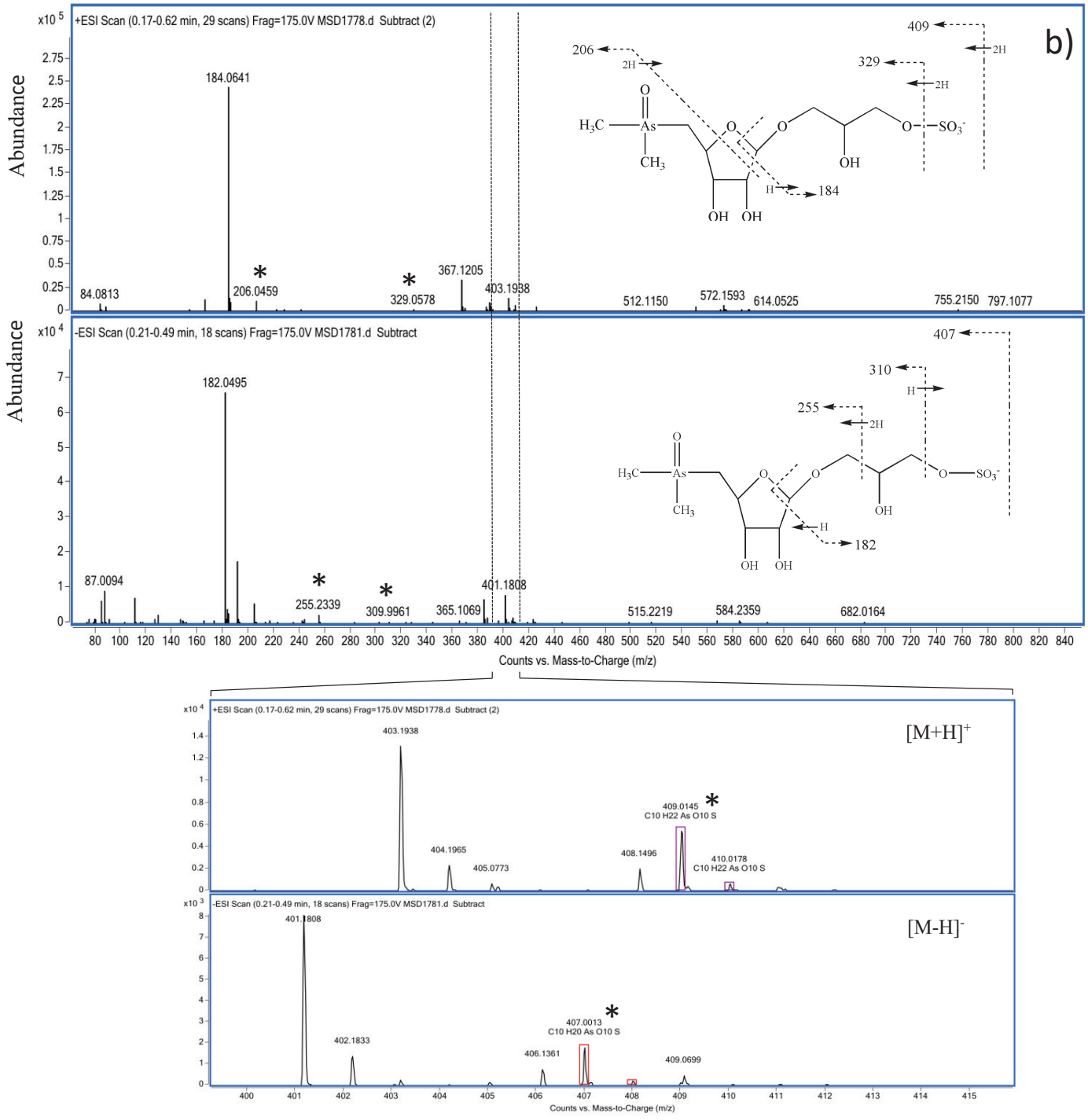


Figure 5b (pdf file)



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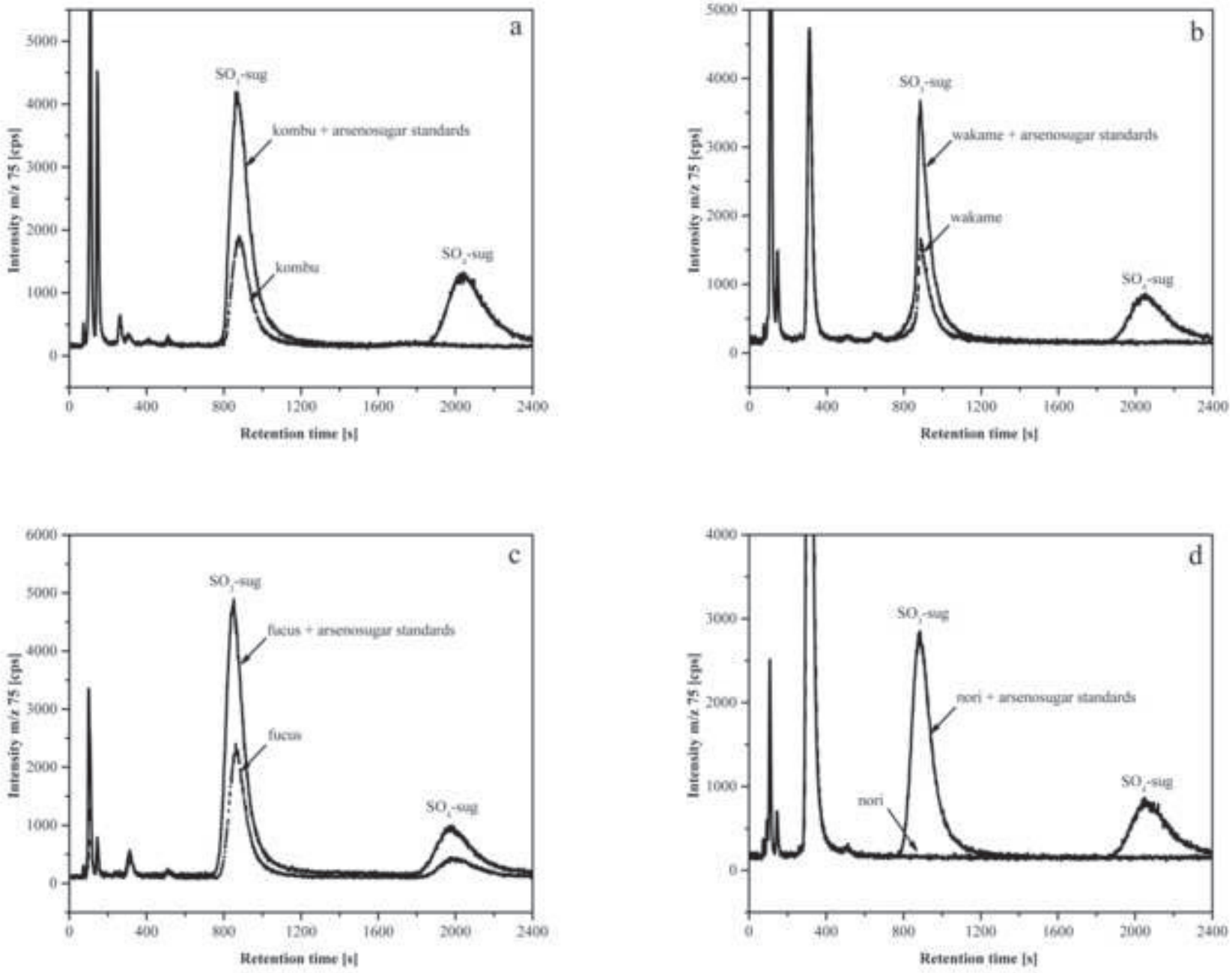

Table 1 (Word file)

Table 1 Chromatographic conditions used for the separation and preparation of arsenic species

	Analytical Chromatography Anionic exchange	Analytical Chromatography Cationic exchange	Preparative Chromatography Anionic exchange
LC	Quaternary pump, Agilent 1200 equipped with an autosampler	Quaternary pump, Agilent 1200 equipped with an autosampler	1260 Infinity II with manual injection
Column	Hamilton PRP-X100 (250 mm × 4.1 mm i.d, 10 µm)	Zorbax 300-SCX (250 mm × 4.6 mm i.d, 5 µm)	Hamilton PRP-X100 (250 mm × 21.5 mm i.d, 12-20 µm)
Precolumn	Hamilton PRP-X100 (20 × 2.0 mm i.d., 10 µm)	Zorbax 300-SCX. (12.5 mm × 4.6 i.d., 5 µm)	No precolumn
Mobile phase	20 mM $NH_4H_2PO_4$ pH = 5.8 (adjusted with aqueous ammonia)	20 mM pyridine pH = 2.6 (adjusted with formic acid)	40 mM NH_4HCO_3 pH = 8.0 in 1% MeOH (adjusted with aqueous ammonia)
Flow rate (mL min^{-1})	1.5	1.5	5.0
Injection volume (µL)	100	100	2000
Pressure (bar)	140	137	100
Arsenic species	As(III), As(V), MMA, DMA, PO_4-sug, SO_3-sug, SO_4-sug	AB, AC, TMAO, Gly-sug	As(III), As(V), MMA, DMA, PO_4-sug, SO_3-sug, SO_4-sug

Table 2 (Word file)

Table 2 Concentrations expressed as mg As kg^{-1} on dry mass (mean ± SD, n = 3) of total arsenic and arsenic species in algae. Detection and quantification limits for the As species in algae (mg As kg^{-1}).

Algae species	Total As	Total extracted As	Asenic species											Sum of As species	Column Recovery (%)	Extraction efficiency (%)
			As(III)	DMA	MMA	As(V)	AB	AC	PO_4-sug	SO_3-sug	SO_4-sug	Gly-sug	TMAO	Unknown cation[b]		
A	51.77 ±1.68	47.48 ±4.97	1.50 ±0.04	0.57 ±0.02	＜LOD	0.49 ±0.03	< LOD	< LOD	0.57 ±0.03	16.90 ±0.22	4.22 ±0.33	0.31 ±0.04	< LOD	–	24.56 ±0.71	51.7	91.7
B*	54.33 ±0.63	55.23 ±0.62	1.51	0.68	0.08	0.44	< LOD	< LOD	0.80	18.93	8.91	0.006 ±0.002	< LOD	–	31.36	56.8	101.7
C	85.26 ±0.99	74.53 ±1.31	1.99 ±0.08	0.66 ±0.09	0.04 ±0.004	0.23 ±0.07	< LOD	< LOD	1.62 ±0.08	37.86 ±1.64	8.71 ±0.86	1.52 ±0.18	< LOD	–	52.63 ±3.00	70.6	87.4
D	45.99 ±0.81	36.25 ±0.89	0.74 ±0.15	0.53 ±0.06	< LOD	< LOD	< LOD	< LOD	0.63 ±0.03	4.78 ±0.20	9.15 ±0.70	4.64 ±0.34	< LOD	0.22 ±0.01	20.69 ±3.19	57.1	78.8
E*	37.57 ±0.73	32.37 ±1.17	4.48	0.97	0.08	0.77	< LOD	< LOD	0.51	4.84	7.32	0.032 ±0.003	< LOD	–	19.00	58.7	86.2
ERM-CD 200 (*Fucus Vesiculosus*)[a]	56.80 ±0.24	53.17 ±0.99	3.60 ±0.06	11.99 ±0.75	0.07 ±0.009	0.09 ±0.009	< LOD	< LOD	1.56 ±0.06	29.16 ±1.47	7.47 ±0.42	1.68 ±0.16	< LOD	–	55.62 ±2.94	104.6	93.6
Detection limits (LOD)	0.033		0.005	0.007	0.009	0.017	0.002	0.005	0.015	0.061	0.089	0.008	0.009				
Quantification limits (LOQ)	0.109		0.016	0.025	0.031	0.058	0.007	0.018	0.05	0.205	0.297	0.028	0.030				

* n=1

[a] Certified value: mean ± uncertainty, 55.0 ± 4 mg As kg^{-1}

[b] Unknown cation arsenic species with a retention time of 192 s.

Table 3

Table 3 Concentrations expressed as mg As kg^{-1} on dry mass (n = 2) of arsenic species in algae.

Sample	Total As content (%RSD)	Arsenic species*	Mean	SD	RSD (%)
Fucus	55,8 ± 2,9 (5,2 %)	SO_3-arsenosugar	36,52	0.63	1.72
		SO_4-arsenosugar	9,27	0.11	1.22
Kombu	56,7 ± 3,5 (6,1 %)	SO_3-arsenosugar	23,66	0.25	1.08
Wakame	34,4 ± 1,4 (4,1 %)	SO_3-arsenosugar	5,65	0.06	1.13

*Calibration graphs for SO_3-arsenosugar and SO_4-arsenosugar are y = 115040c+31030 (R^2= 0,9998) and y = 115043*c+47649 (R^2 = 0,9999), respectively.