Development of anion-exchange/reversed-phase high performance liquid chromatography–inductively coupled plasma-mass spectrometry methods for the speciation of bio-available iodine and bromine from edible seaweed

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A B S T R A C T

Anion exchange high performance liquid chromatography hyphenated with inductively coupled plasma-mass spectrometry has been novelly applied to assess inorganic (iodide and iodate) and organic (3-iodotyrosine – MIT, and 3,5-diiodotyrosine – DIT) iodine species in a single chromatographic run. The optimized operating conditions (Dionex IonPac AS7, gradient elution with 175 mM ammonium nitrate plus 15% (v/v) methanol, pH 3.8, as a mobile phase and flow rates within the 0.5–1.5 mL min^{-1} range) have also been used to perform inorganic bromine speciation analysis (bromide and bromate). The developed method has been applied for determining the bio-available contents of iodine and bromine species in dialyzates from edible seaweed. Reverse phase high performance liquid chromatography (Zorbax Eclipse XDB-C8, gradient elution with 0.2% (m/m) acetic acid, and 0.2% (m/m) acetic acid in methanol, as mobile phases, and a constant flow rate of 0.75 mL min^{-1}) also hyphenated with inductively coupled plasma-mass spectrometry was used to confirm the presence of organic iodine species (MIT and DIT) in the dialyzates. The verification of the presence of iodinated amino acids (MIT and DIT) in the extracts was also performed by reverse phase high performance liquid chromatography–electrospray ionization-mass spectrometry (LTQ Orbitrap). The developed methods have provided good repeatability (RSD values lower than 10% for both anion exchange and reverse phase separations) and analytical recoveries within the 90–105% range for all cases. The in vitro bio-availability method consisted of a simulated gastric and an intestinal digestion/dialysis (10 kDa molecular weight cut-off – MWCO) two-stage procedure. Iodide and MIT were the main bio-available species quantified, whereas bromide was the major bromine species found in the extracts.

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1. Introduction

Iodine and bromine are two elements typically found in the marine environment. Seafood, mainly seaweed [1,2], has been recognized as an important source of these elements for the human diet. However, the action of these elements in humans varies greatly. Bromine, and especially bromate species, is believed to be a carcinogenic compound [3–5], while iodine, mainly as MIT and DIT forms, is an essential micronutrient and participates in the biosynthesis of thyroid hormones (thyroxin, T4, and triiodothyronine, T3) [6] by incorporation at the moieties of the protein thyroglobulin [7]. These hormones play important roles in the growth and development of living organisms as well as in the immune defense system [8,9].

In addition to the total contents of an element, the different chemical species in which an element occurs can affect the toxicity, mobility and availability of that element in the different biological and environmental systems. Moreover, the bio-availability of elements from foodstuff is also dependent on the specific chemical forms [10], and elemental speciation studies are needed for knowing the significance of the essential and non-essential elements in food. Inorganic iodine (iodide, I$^-$, and iodate, IO$_3^-$) and bromine (bromide, Br$^-$, and bromate, BrO$_3^-$) are commonly present in environmental and biological systems, and hence in seaweed. These aquatic plants have also been reported to contain numerous volatile iodoalkanes, mainly methyl-iodide [11,12], as well as iodinated and brominated ketones, alcohols and carboxylic acids [11–13]. Iodinated species of more complex structure such as iodinated amino

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acids (3-iodotyrosine-MIT- and 3,5-diiodotyrosine-DIT-) have also been found in seaweed such as *Laminaria japonica* (Kombu) and *Undaria pinnatifida* (Wakame) [14], and in microalgae such as *Chlorella vulgaris* [15].

Although gas chromatography (GC) is the chosen analytical technique when assessing volatile halogenated compounds, and although some developments based on capillary electrophoresis (CE) have been developed for iodinated compounds determination, most of the applications for iodine speciation studies use high performance liquid chromatography (HPLC), mainly hyphenated with selective inductively coupled plasma-mass spectrometry (ICP-MS). HPLC can function in size exclusion mode (size exclusion chromatography, SEC) for assessing iodine bound to bio-molecules, reverse phase mode (reverse phase chromatography, RPC) for determining organic iodinated forms, or anion exchange mode (anion exchange chromatography, AEC) which is mainly focused on separating inorganic iodine species. Concerning seaweed, AEC (Ion Pac AS11 [14] and Agilent G3154A/101 [15] columns) has been used to assess inorganic iodine species (iodide and iodate) under isocratic elution with 5 mM sodium hydroxide [14] or 20.0 mM ammonium nitrate (pH 5.6) [15] as mobile phases. Organic iodinated species in seaweed (MIT and DIT) have been resolved by RPC (Altima C18 column) [14] using a gradient elution program involving two different mobile phases (10 mM/10 mM Tris/hydrochloric acid pH 7.3 and 10 mM/10 mM Tris/hydrochloric acid pH 7.3 plus 50% methanol). In this application, inorganic iodine forms co-elute [14]. Other developments based on RPC have allowed the separation of MIT and DIT, and also iodothyronines (tetra-iodothyronine – T4, tri-iodothyronine – T3, reversed tri-iodothyronine – rT3, and di-iodothyronine – T2) in human blood and urine samples [16–18] and also in certain tissues from animals [19]. Applications by SEC have also been developed to assess iodine-binding bio-molecules in seaweed [14] and microalgae [15]. Superdex 75 column (isocratic separation with 0.03 M/0.03 M Tris/hydrochloric acid buffer at pH 8.0 as a mobile phase) was used to detect iodine bound to high and low molecular weight compounds, as well as to polyphenols after subjecting seaweed samples (Wakame and Kombu) to different extractive procedures [20]. This SEC column has also been used as preparative chromatography to separated iodine-containing bio-molecules from microalgae *C. vulgaris* before AEC determination of inorganic iodine forms in aqueous extracts [15].

As commented above, some liquid chromatography methods have been developed for assessing iodine species in seaweed. However, these methods did not report quantitative data on iodide, iodate, MIT and DIT [14,15]. In addition, chromatographic methods based on AEC have been mainly focused on assessing inorganic iodine, chromatographic mode which did not allow organic iodine (MIT and DIT) resolution. The novelty of the current work was the development of an AEC method for resolving simultaneously inorganic and organic iodine species, as well as inorganic bromine species. ICP-MS was used as a selective detector. An alternative RPC, also hyphenated with ICP-MS, was also optimized for confirming the presence of organic iodine in the samples. The presence of MIT and DIT species was further performed by MS with the LTQ-Orbitrap Discovery mass spectrometer. The developed methods were applied to assess the bio-available iodine and bromine species from different edible Atlantic seaweeds after an *in vitro* bio-availability approach based on simulated gastric and intestinal digestion/dialysis conditions. In this sense, only two papers have reported total contents of bio-available iodine in seaweed after *in vivo* [21] and *in vitro* [22] approaches (the latter also gave total contents for bio-available bromine). The presence/determination of bio-available iodine and bromine species has not been yet reported.

2. Materials and methods

2.1. Instrumentation

An 820-MS inductively coupled plasma mass spectrometer (Varian, Mulgrave, Australia), equipped with an SPS3 autosampler (Varian) and a MicroMist nebulizer (Varian), was used for total bromine and iodine determinations, and for bio-available bromine and iodine contents in the dialyzates. A Dionex HPLC UltiMateO 3000 LC (Dionex, Sunnyvale, CA, USA), equipped with a GP50 gradient pump (Dionex), an AS50 thermal compartment (Dionex) and an AS50 autosampler (Dionex), was used for HPLC separations. Iodine and bromine speciation was performed with an IonPac AS7 (250 mm × 4 mm i.d.) anion-exchange column (Dionex) coupled to an IonPac AG7 (Dionex) guard column; and with a Zorbax Eclipse XDB-C8 (4.6 mm × 150 mm i.d.) from Agilent (Palo Alto, CA, USA) coupled to a 4 mm × 3.0 mm i.d. Phenomenex C8 guard column (Torrance, CA, USA). The chromatographic system was coupled to an ICP-MS Thermo Finnigan X Series (Thermo Fisher Scientific Inc., Waltham, MA, USA). An LTQ-Orbitrap Discovery mass spectrometer (Thermo) coupled to an Accela pump (Thermo) and to an Accela autosampler (Thermo) was used for confirming the presence of organic iodine species. An Ethos Plus microwave laboratory station (Milestone, Sorisole, Italy) with 100-mL closed Teflon vessels, Teflon covers and an HTC adapter plate and HTC safety springs (Milestone) was used for alkaline digestion of seaweed samples (determination of total iodine and bromine contents). An RM100 mortar grinder mill (Retsch, Haan, Germany) equipped with an agate pestle and an agate mortar was used to pulverize dried seaweed samples. An ORION 720A plus pH-meter with a glass-calomel electrode (ORION, Cambridge, UK) was used for pH measurements. A Boxcult incubator situated on a Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain) was used to control the temperature during the *in vitro* procedure. Cellu Sep® H1 high grade regenerated cellulose tubular membranes (molecular weight cut-off 10 kDa, 50 cm length, diameter dry 25.5 mm and 5.10 mL cm^{-1}) were from Membrane Filtration Products Inc (TX, USA) and were used for dialysis. Albet®LabScience 0.20 μm cellulose acetate syringe filters (25 mm diameter) were from Albet-Hahnemuehle (Dassel, Germany). A 77530 LYPH-LOCK 6-L freeze–dry system from Labconco Corp. (Kansas City, MO, USA) was used to freeze–dry the canned seaweed in brine.

2.2. Reagents

Ultrapure water, resistivity 18 MΩ cm, obtained from a Milli-Q water-purification system (Millipore, Bedford, MA, USA) was used throughout this work. Tetramethylammonium hydroxide (TMAH) 25% (m/m) in water and methanol (gradient grade) was from Merck (Darmstadt, Germany). HPLC-PAI grade 99.8% (m/m) acetic acid was from Panreac (Barcelona, Spain). Acetonitrile (supragradient HPLC) was from Scharlau (Barcelona, Spain). Digestive enzymes (porcine pepsin P-7000, porcine pancreatin P-1750), bile salts (approx. 50% sodium cholate and 50% sodium deoxycholate) and piperazine-N,N-bis(2-ethane-sulfonic acid) disodium salt (PIPES), were obtained from Sigma Chemicals (St Louis, MO, USA). Hydrochloric acid 37% was from Panreac and sodium hydrogen carbonate and ammonium nitrate salts were from Merck. Stock standard solutions, 1000 mg L^{-1}, were prepared from 99.5% potassium iodide (Merck), 99.95% potassium bromide (Sigma Chemicals), 99.7–100.4% potassium iodate (Merck), 99.8% potassium bromate (Merck), 3-iodo-L-tyrosine (MIT) (Sigma Chemicals) and 98% (NT) 3,5-diiodo-L-tyrosine dihydrate (DIT) (Sigma Chemicals). Tellurium chloride and yttrium nitrate standard solutions, 1000 mg L^{-1}, were from SCP Science (Montreal, Canada). A NIES 09 (Sargasso, *Sargassum fulvellum*) from the National Institute for

Environmental Studies (Ibaraki, Japan) was used to assess accuracy for total iodine and bromine determinations.

To avoid contamination, all glassware and plastic ware were washed and kept for 48 h in 10% (v/v) nitric acid, and then rinsed several times with ultra-pure water before use.

2.3. Seaweed samples

Nine different types of edible seaweed harvested on the Galician coast (Northwestern Spain) were obtained from a local manufacturer. One of these samples is commercialized as cooked and canned in brine (25 g), and it consists of a mixture of two brown seaweeds, Sea Spaghetti (*Himanthalia elongata*) and Furbelows (*Saccorhiza polyschides*). This sample was freeze-dried. The remaining samples are commercialized as dehydrated products (100 g). Red seaweeds: Dulse (*Palmaria palmata*) and Nori (*Porphyra umbilicalis*); brown seaweeds: Kombu (*Laminaria ochroleuca*), Wakame (*U. pinnatifida*) and Sea Spaghetti (*H. elongata*); and green seaweed: Sea Lettuce (*Ulva rigida*). Dehydrated samples were kept in an oven at 40 °C to eliminate water traces before pulverization in an agate mortar, and were preserved in pre-cleaned polyethylene bottles. Other samples studied were the microalgae *Spirulina platensis*, and agar-agar (a hydrocolloid from the red seaweed *Gelidiumm sesquipedale*).

2.4. Microwave assisted alkaline digestion

A microwave assisted alkaline digestion was performed to assess total iodine and bromine contents in seaweed [23]. The procedure consisted of weighing approximately 0.1 g of powdered seaweed into microwave Teflon vessels, and adding 5 mL of ultra-pure water and 5 mL of TMAH. Vessels were then capped and subjected to microwave irradiation from room temperature to 200 °C (temperature ramp of 18 °C/min), followed by a second irradiation stage at 200 °C for 5 min. After cooling down, alkaline extracts were centrifuged at 3000 rpm for 10 min, and the supernatant was transferred to 50 mL volumetric flasks. The solid residue was rinsed with a small volume of water. After centrifugation, the water rinsing was combined with the above-mentioned supernatant before dilution to 50 mL. Extracts were filtered through 0.45 μm cellulose acetate syringe filters (Millipore) before ICP-MS measurements. Each seaweed sample was treated in triplicate and two reagent blanks were prepared for each microwave irradiation set.

2.5. In vitro digestion procedure

An *in vitro* digestion procedure based on a dialyzability approach was performed to assess the bio-available iodine and bromine fraction [22]. The procedure consisted of weighing powdered seaweed (0.5 g) into 100 mL Erlemeyer flasks, adding 20 mL of ultrapure water (seaweed hydration for 15 min), and adjusting pH to 2.0 with 6 M hydrochloric acid. The gastric solution (0.15 g) consisting of 6.0% (m/v) pepsin in 6 M hydrochloric acid was then added. Flasks were covered and incubated at 37 °C with orbital-horizontal shaking at 150 rpm for 120 min. Simulated gastric digestion was stopped by placing the flasks in an ice–water bath. The intestinal solution (5.0 mL) consisting of 4% (m/v) pancreatin and 2.5% (m/v) bile salts dissolved in 0.1 M sodium hydrogen carbonate was then added, dialysis membranes (filled with 20 mL of 0.15 N PIPES at pH 7.5 adjusted with hydrochloric acid) were placed inside the flasks. Simulated intestinal digestion was performed at 37 °C with orbital-horizontal shaking at 150 rpm for 120 min. The enzymatic reaction was then stopped by immersing the flasks in an ice–water bath. The membranes were removed, and their outer surface was rinsed with ultrapure water. The membrane containing solution (dialyzate) and

Table 1
Operating ICP-MS conditions for total iodine and bromine determination.

General	Radiofrequency power (W)	1380
	Peristaltic pump speed (mL min^{-1})	0.45
	Stabilization delay (s)	35
	Number of replicates	3
	Nebulizer type	MicroMist
Gas flows (L min^{-1})	Nebulizer	0.98
	Plasma	17.0
	Auxiliary	1.65
	Sheath	0.27
	Skimmer cone	Nickel
	Sampler cone	Nickel
Torch alignment (mm)	Sampling death	7.0
Ion optics (V)	First extraction lens	−32
	Second extraction lens	−164
	Third extraction lens	−231
	Corner lens	−206
	Mirror lens right	25
	Mirror lens left	24
	Mirror lens bottom	27
	Entrance lens	3
	Fringe bias	−4.9
	Entrance plate	−3.4
	Pole bias	0
CRI (mL min^{-1})	Skimmer gas source	H$_2$
	Sampler gas source	OFF
	Skimmer flow	80
	Sampler flow	0
Mass-to-charge ratio	Br	79
	I	127

the residual or non-dialyzable fraction (remaining slurries in the flasks) were transferred to polyethylene vials and weighed. Dialyzates were kept at −20 °C before measurements. Reagent blanks were obtained to control possible contamination.

2.6. Bromine and iodine determination by ICP-MS

Total bromine and iodine in TMAH extracts and total bromine and iodine in the dialyzates were determined by ICP-MS (Table 1). Tellurium (^{125}Te) at 2 mg L^{-1}, and yttrium (^{89}Y) at 2 μg L^{-1}, were used as internal standards for iodine (m/z 127) and bromine (m/z 79) determinations, respectively. A flow of 80 mL min^{-1} H$_2$ in the collision cell was used to minimize ^{38}Ar^{40}Ar^{1}H^{+} (m/z 79) bromine polyatomic interference [24]. This gave the best sensitivity and linear ranges for bromine and also for iodine determinations [22,23]. Calibrations covered iodine and bromine concentrations between 0 and 1000 μg L^{-1}. Accuracy for total iodine and bromine determination was assessed by analyzing the CRM NIES 09. Values of 525 ± 59.5 μg g^{-1} (n = 3) for iodine and 274 ± 17.1 μg g^{-1} (n = 3) for bromine were found, which agree with the indicative values of 520 and 270 μg g^{-1} for iodine and bromine, respectively.

2.7. HPLC–ICP-MS measurements

Anion exchange HPLC conditions were optimized to separate four iodine species (iodide, iodate, MIT and DIT) in a single chromatographic run (Fig. 1(a)). The optimized elution program works in a gradient elution mode. The mobile phase consisted of a 175 mM ammonium nitrate plus 15% (v/v) methanol solution at a pH 3.8;
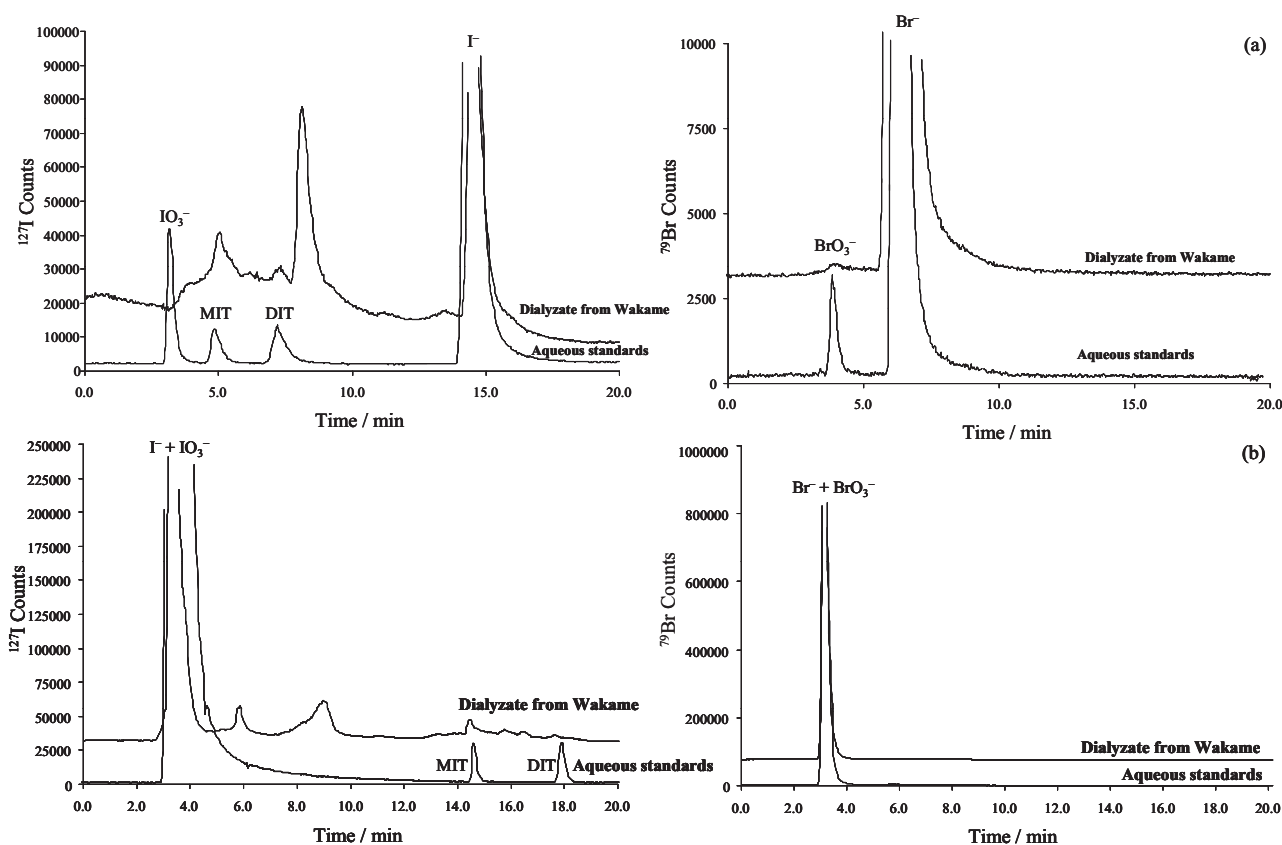
Fig. 1. Anion exchange (a) and reverse phase (b) chromatograms for iodine and bromine species: iodide and bromide at 1.0 mg L^{-1}; iodate, bromate, MIT and DIT at 50 μg L^{-1}.

whereas, the flow rate was set at 0.5 mL min^{-1} for 8 min, then increased to 1.5 mL min^{-1} within 1 min, and finally maintained at 1.5 mL min^{-1} for 8 min (Table 2). For reverse phase HPLC, optimized conditions led to a separation of organic iodinated forms (MIT and DIT) and total inorganic iodine (iodide plus iodate) as shown in Fig. 1(b) when working in a gradient elution mode (0.2% (m/m) acetic acid, and 0.2% (m/m) acetic acid in methanol, as mobile phases). The flow rate was set at 0.75 mL min^{-1} and separation was performed at a controlled temperature of 25 °C (Table 2). For both cases, the outlet of the separation column was directly connected to the Meinhard nebulizer using a PEEK tubing (50 cm length). Under the ICP-MS conditions (Table 2), problems derived from the use of methanol as a component of the mobile phases were not observed. Collision cell technology (H$_2$/Heat 4.5 mL min^{-1}) was used to reduce ^{38}Ar^{40}Ar^{1}H^{+} (m/z 79) bromine polyatomic interference [24,25]. Tellurium (^{125}Te) at a concentration of 2 mg L^{-1}, and yttrium (^{89}Y) at 2 μg L^{-1}, were used as internal standards by continuously mixing the chromatographic eluate with an external flow of the internal standard mixture (0.4 mL min^{-1}) using a T-junction after the column and just before the nebulizer.

2.8. HPLC–Orbitrap MS measurements

Target separation (injection volume of 25 μL) was performed with the Zorbax Eclipse XDB-C8 (4.6 mm × 150 mm i.d.) reverse phase column coupled to a 4 mm × 3.0 mm i.d. Phenomenex C8 guard column and an Orbitrap mass spectrometer for detection. Column temperature was set at 22 °C, and separation was performed in a gradient mode at a flow rate of 0.500 mL min^{-1}. Mobile phase A was 0.2% (m/m) acetic acid in water; and mobile phase B was 0.2% (m/m) acetic acid in methanol. Solvent gradient conditions were as shown in Table 2. Under these conditions, MIT and

DIT elute at 13.7 and 18.3 min, respectively. Detection was performed in negative ion atmospheric pressure chemical ionization (PI-APCI) mode. Ultrahigh-purity nitrogen (99.999%) was used as the sheath (50 arbitrary units) and auxiliary gas (5 arbitrary units). The analyzer was FTMS with a scan range of m/z 125.00–440.00 (resolution 30,000). Capillary temperature was set at 270 °C, tube lens were set at −100 V, and capillary voltage −20 V. MIT and DIT are uncharged compounds and the identification was based on [M−H]$^{-}$ of 305.9634 and 431.8618, respectively. Aqueous standards and enzymatic digests were matched with mobile phase A (0.2% (m/m) acetic acid in water).

3. Results

3.1. Optimization of operating AEC conditions

An isocratic elution of inorganic iodine (iodide and iodate) and bromine (bromide and bromate) forms, and also organic iodinated amino acids (MIT and DIT) with the Dionex AS7 anion exchange column, was tested by using different mobile phases reported in literature such as sodium hydroxide [14,26,27], nitric acid [28–30], and ammonium nitrate [15,31]. Iodide, MIT and DIT co-elution was observed when using sodium hydroxide ranging from 15 mM to 50 mM as a mobile phase, whereas iodide and iodate were separated when using nitric acid (50 mM and 100 mM), although organic iodine species (MIT and DIT) were not eluted from the column. The use of ammonium nitrate at low concentrations (from 15 to 50 mM) as a mobile phase led to the co-elution of iodate and MIT, and good resolution of DIT and iodide. Elution with ammonium nitrate at concentrations up to 75 mM allows iodate and MIT resolution and also a fast elution of iodide. Therefore, ammonium nitrate at higher concentrations (from 75 to 200 mM) was tested as a mobile phase,

Table 2
Operating ICP-MS conditions and anion exchange/reverse phase HPLC–ICP-MS settings for iodine and bromine speciation.

ICP-MS	Radiofrequency power (W)	1400
	Peristaltic pump speed (rpm)	2.5
	Nebulizer type	Beat impact (cooled spray chamber)
Gas flows (L min^{-1})	Plasma	14.0
	Auxiliary	0.8
	Nebulizer	0.85
Torch alignment (mm)	Horizontal	117
	Vertical	317
	Sampling death	210
Ion optics (V)	Extraction	−102
	Lens 1	−1150
	Lens 2	−62
	Focus	−7.8
	D1	−55.7
	D2	−140
	Pole bias	−15
	Hexapole bias	−18
CCT-KED (mL min^{-1})	H$_2$/He	4.5
Mass-to-charge ratio	I	127
	Br	79
	^{125}Te (2.0 mg L^{-1}) and ^{89}Y (5.0 μg L^{-1}) as internal standards (post-column added at 0.4 mL min^{-1})	
AEC–HPLC	Dionex IonPac AS7 (250 mm × 4 mm i.d.) anion-exchange column coupled to a Dionex IonPac AG7 guard column	
	Injection volume	50 μL
	Column temperature (°C)	25
Elution program	Gradient	
Mobile phase composition	175 mM ammonium nitrate, 15.0% (v/v) methanol, pH 3.8	
Mobile phase flow gradient	0.5 mL min^{-1}, 0.0–8.0 min	
	0.5–1.5 mL min^{-1}, 8.0–9.0 min	
	1.5 mL min^{-1}, 9.0–17 min	
	1.5–0.5 mL min^{-1}, 17–18 min	
	0.5 mL min^{-1}, 18–20 min	
RPC–HPLC	Agilent Zorbax XDB-C8 (4.6 mm × 150 mm) reverse phase column coupled to a Phenomenex C8 (4.0 mm × 3.0 mm) guard column	
	Injection volume	50 μL
	Column temperature (°C)	22
Elution program	Gradient	
Flow rate (mL min^{-1})	0.75	
Mobile phase composition	0.2% (m/m) acetic acid in water, pH 2.7 (A); 0.2% (m/m) in methanol, pH 2.3 (B)	
Mobile phase composition gradient	0.0–3.0 min: 100% A	
	3.0–3.5 min: 100 → 90% A, 0 → 10% B	
	3.5–6.0 min: 90% A, 10% B	
	6.0–6.5 min: 90 → 80% A, 10 → 20% B	
	6.5–8.0 min: 80% A, 20% B	
	8.0–8.5 min: 80 → 60% A, 20 → 40% B	
	8.5–14 min: 60% A, 40% B	
	14–15 min: 60 → 100% A, 40 → 0% B	
	15–23 min: 100% A	

and a composition of 175 mM ammonium nitrate was finally selected.

As previously shown in [32], resolution provided by the Dionex AS7 for arsenic speciation is improved when combining aqueous mobile phases with a small proportion of methanol. Therefore, different experiments involving increased methanol concentrations (up to 20%) were performed. As a result, retention times were not affected but the chromatographic peak schemes improved, especially the DIT chromatographic signal. Therefore, the composition of the mobile phase was finally set at 175 mM ammonium nitrate and 15% (v/v) methanol.

The effect of the temperature on the separation of the four-iodinated species was also tested. Resolution of chromatographic peaks, especially iodate, MIT and DIT which are eluted within the first 3 min, worsened when working at high temperatures (30, 35 and 40 °C), whereas the resolution of these iodine species was not changed when working at temperatures close to the room temperature (20 and 25 °C). Experiments at temperatures lower than 20 °C were not performed, and separations were therefore established at room temperature (column oven heated at 25 °C).

Under the chromatographic conditions shown above, good resolution between inorganic bromine species (bromate and bromide) was achieved.

Finally, a flow rate gradient consisting of 0.5 mL min^{-1} for 8 min, a ramp from 0.5 to 1.5 mL min^{-1} within 1 min, and elution at 1.5 mL min^{-1} for another 8 min, was finally established to achieve the best resolution of iodate, MIT and DIT (retention times of 3.2, 4.9, and 7.2 min, respectively), and the fast elution of iodide (retention time of 14.4 min) as shown in Fig. 1(a). Under these gradient flow conditions, a better iodate and MIT resolution was achieved when analyzing dialyzates from seaweeds. In addition, bromate and bromide (Fig. 1(a)) could also be resolved in less than 10 min.

3.2. Optimization of operating RP conditions

Because RPC has been commonly used to assess organic iodine species (MIT and DIT), a RPC method hyphenated with ICP-MS and ESI-MS (LTQ-Orbitrap) was optimized for confirming the presence of MIT and DIT in the dialyzates.

Different mobile phases, aqueous solutions with a moderate amount of organic solvent (mainly acetonitrile and methanol) [16–18,33,34] modified with a low amount of acetic acid, were tested to elute iodine organic forms (MIT and DIT), while elution of inorganic iodine was tested with aqueous solution as mobile phases. Preliminary studies led to a better performance when first eluting inorganic iodine, followed by organic iodine species elution. Under these conditions, iodide (major iodine species) is conveniently eluted within the first few minutes, and organic species can be separated from the iodide peak tail. In addition, the column equilibration was achieved in a shorter time when using a gradient starting with an aqueous solution, and progressively increasing the proportion of organic solvent. The use of acetonitrile led to poor results mainly because MIT and DIT were not stable, and unknown chromatographic signals when using MIT and DIT standards were observed when increasing the amount of acetonitrile in the mobile phases. Methanol was therefore selected and different mobile phase composition gradients were tested to separate inorganic iodine (iodide plus iodate) and organic iodine (MIT and DIT). Iodide plus iodate elute from the column when using a low proportion of methanol in the mobile phase. However, methanol proportion in the mobile phase must to be increased to elute the organic iodine forms (MIT and DIT). The presence of higher amounts of methanol in the mobile phase affects the response of targets because of a major amount of carbon reaching the plasma [35]. Nevertheless, calibrations with each target (MIT and DIT are used as calibrating standards) subjected under the same chromatographic

Table 3
Mean slopes for calibration, and limits of detection and quantification.

	Calibration			
	Mean slope $\pm$ SD[a] (L μg^{-1})	RSD (%)	LOD[b] (ng g^{-1})	LOQ[b] (ng g^{-1})
AE–HPLC–ICP-MS				
I$^-$	$9.3 \times 10^6 \pm 5.7 \times 10^{5}$[c]	6	3.2	11
IO$_3$$^-$	$8.9 \times 10^3 \pm 5.5 \times 10^2$	6	3.5	12
MIT	$4.1 \times 10^3 \pm 1.4 \times 10^2$	3	2.4	8
DIT	$5.2 \times 10^3 \pm 2.5 \times 10^2$	5	3.4	11
Br$^-$	$1.5 \times 10^5 \pm 1.1 \times 10^{4}$[c]	7	112	373
BrO$_3$$^-$	93 ± 13	14	123	410
RP–HPLC–ICP-MS				
Inorganic iodine	$1.2 \times 10^8 \pm 1.1 \times 10^{7}$[c]	9	2.3	7.8
MIT	$6.7 \times 10^3 \pm 2.0 \times 10^2$	3	1.4	4.7
DIT	$8.7 \times 10^3 \pm 3.0 \times 10^2$	3	1.0	3.5
Inorganic bromine	$2.5 \times 10^6 \pm 2.1 \times 10^{5}$[c]	8	83	275

[a] $n = 4$.
[b] $n = 11$.
[c] Units in L mg^{-1}.

conditions ensure that the response of each iodide species is always the same (highly affected or not by the amount of carbon present in the plasma) for all the different runs.

As shown in Table 2, a three-stage program for increasing the amount of methanol (from 0 to 10%, from 10 to 20%, and finally from 20 to 40%) was needed to obtain a stable baseline. In addition, this low rate for methanol increase allows the separation of some unknown iodine compounds in the dialyzates. The amount of acetic acid in the mobile phase was also studied, and acidity in both mobile phases was finally set at 0.2% (m/m). As shown in Fig. 1(b), inorganic iodine elutes within the first few minutes, while MIT and DIT elute at approximately 14.9 and 17.5 min, respectively. Under these conditions, inorganic bromine (bromide plus bromate) co-elute (Fig. 1(b)).

Finally, the retention times for inorganic iodine, MIT and DIT were not affected when performing the separation within the 20–40 °C range, and the oven temperature was finally fixed at 22 °C.

3.3. Analytical performance of the methods

Table 3 lists the mean and the standard deviation of the slopes of calibration graphs for each analyte (bromate, bromide, iodate, iodide, MIT and DIT for anion exchange HPLC–ICP-MS; and also for total inorganic bromine, total inorganic iodine, MIT and DIT for reverse phase HPLC–ICP-MS). Good repeatability of the calibration curves can be seen over four different days for all cases. Calibration curves for both chromatographic methods cover iodide and bromide concentrations of 0, 1, 2, 4 and 5 mg L^{-1}; 0, 37.5, 75, 150 and

Table 4
Concentrations of total iodine and total bromine in seaweeds, total iodine and total bromine in dialyzates from seaweeds, and total iodine and bromine (as a sum of concentrations of iodide/bromine species) in dialyzates from seaweed.

Sample	Total iodine concentrations (μg g^{-1})				
	Iodine[a]	Iodine[b]	Iodine[c]	Percentage (%)[d]	Percentage (%)[e]
Dulse (*Palmaria palmata*)	77 ± 9	5.7 ± 1.7	5.0 ± 0.4	7 ± 2	66 ± 7
Nori (*Porphyra umbilicalis*)	43 ± 4	2.1 ± 0.4	2.2 ± 0.4	5 ± 1	100 ± 19
Sea lettuce (*Ulva rigida*)	66 ± 2	1.4 ± 0.03	1.2 ± 0.1	2 ± 0.1	84 ± 2
Wakame (*Undaria pinnatifida*)	306 ± 42	8.7 ± 3.1	6.8 ± 0.7	3 ± 1	98 ± 15
Sea spaghetti (*Himanthalia elongata*)	117 ± 23	4.4 ± 0.3	3.0 ± 0.4	4 ± 1	69 ± 5
Kombu (*Laminaria ochroleuca*)	6138 ± 314	1075 ± 109	1060 ± 158	18 ± 2	99 ± 12
Canned seaweed (cooked *Himanthalia elongata* and *Saccorhiza polyschides*)	37 ± 1	<0.082	0.69 ± 0.1	–[f]	–[f]
Spirulina (*Spirulina platensis*)	<0.082	<0.082	–[f]	–[f]	–[f]
NIES 09 Sargasso	525 ± 59	23 ± 4.2	26 ± 0.4	4 ± 1	116 ± 1
Agar-agar (from *Gelidiumm sesquipedale*)	27 ± 0.5	4.5 ± 0.5	4.6 ± 0.2	17 ± 0.4	102 ± 12

Sample	Total bromine concentrations (μg g^{-1})				
	Bromine[a]	Bromine[b]	Bromine[c]	Percentage (%)[d]	Percentage (%)[e]
Dulse (*Palmaria palmata*)	451 ± 54	131 ± 9	115 ± 4	29 ± 4	88 ± 6
Nori (*Porphyra umbilicalis*)	42 ± 2	20 ± 0.7	17 ± 0.8	48 ± 3	85 ± 3
Sea lettuce (*Ulva rigida*)	529 ± 21	95 ± 28	90 ± 6	18 ± 5	94 ± 28
Wakame (*Undaria pinnatifida*)	709 ± 75	149 ± 6	143 ± 1	21 ± 2	97 ± 4
Sea spaghetti (*Himanthalia elongata*)	377 ± 42	128 ± 2	108 ± 6	34 ± 4	84 ± 2
Kombu (*Laminaria ochroleuca*)	972 ± 31	194 ± 27	174 ± 1	20 ± 3	90 ± 13
Canned seaweed (cooked *Himanthalia elongata* and *Saccorhiza polyschides*)	105 ± 11	22 ± 3	26 ± 0.8	21 ± 3	115 ± 14
Espirulina (*Spirulina platensis*)	12 ± 1	4.1 ± 0.5	4.3 ± 0.5	34 ± 5	105 ± 14
NIES 09 Sargasso	274 ± 17	29 ± 2	26 ± 0.5	11 ± 1	91 ± 8
Agar-agar (from *Gelidiumm sesquipedale*)	77 ± 0.5	14 ± 1	13 ± 1	18 ± 0.1	95 ± 9

[a] Total iodine/total bromine in seaweed (TMAH alkaline digestion and ICP-MS measurement).
[b] Total iodine/total bromine in dialyzates from seaweed (dialyzability approach and ICP-MS measurement).
[c] Total iodine/total bromine in dialyzates from seaweed as a sum of iodine/bromine species concentrations (*in vitro* bio-availability approach and AEC–ICP-MS measurement).
[d] Percentage of total iodide/bromine in the dialyzates respect to the total iodine/bromine in seaweed samples (dialyzability ratio).
[e] Percentage of total iodide/bromine as a sum of iodine/bromine concentrations respect to the total iodine/bromine concentrations in the dialyzates.
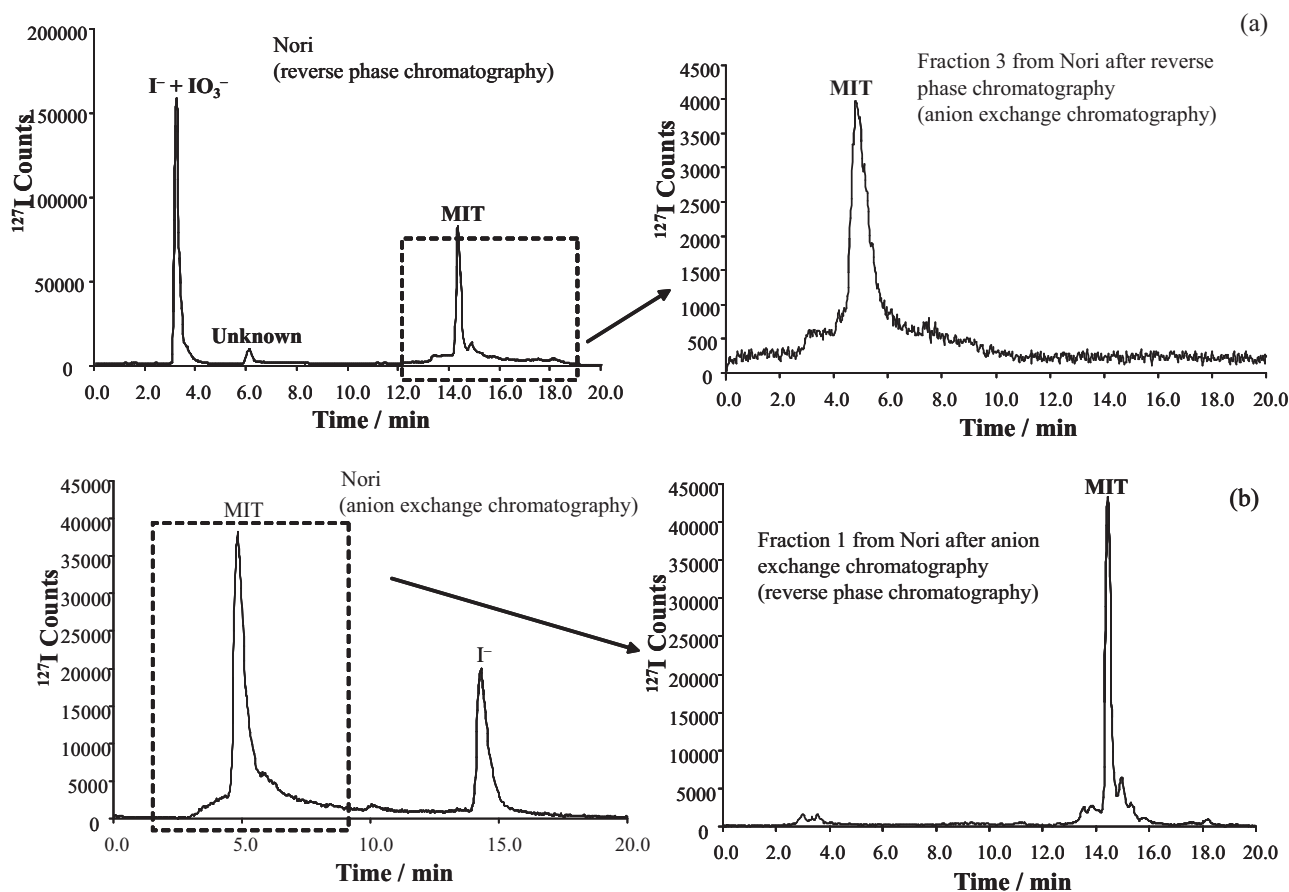[f] Not calculated.

Fig. 2. Anion exchange chromatogram from fraction 3 isolated from a Nori extract by phase reverse chromatography (a), and reverse phase chromatogram from fraction 1 isolated from a Nori extract by anion exchange chromatography (b) for verifying the presence of MIT.

300 $\mu g \, L^{-1}$ for iodate; 0, 27.5, 55, 110 and 220 $\mu g \, L^{-1}$ for bromate; and 0, 25, 50, 100 and 200 $\mu g \, L^{-1}$ for MIT and DIT.

The LODs and LOQs were based on the $3\sigma/10\sigma$ criterion (σ is the standard deviation of 11 measurements of a blank). LODs/LOQs were therefore calculated after dividing the $3\sigma/10\sigma$ values by the mean slopes of the calibration from each target, and taking into account the extract volume (20 mL) and the sample mass (0.50 g). Table 3 lists LOD and LOQ values expressed as $ng \, g^{-1}$. It can be seen that the values are low enough to perform iodine and bromine speciation in dialyzates from seaweed. Repeatability of the over-all procedure ($n = 7$) has provided RSD values lower than 10% (6% for iodide, bromide and bromate; 7% for iodate; and 9% for MIT and DIT) when performing AEC–HPLC–ICP-MS measurements. RSD values ($n = 7$) of 8% were assessed for MIT and DIT, while inorganic iodine and inorganic bromine showed RSD values of 13% and 10%, respectively, for determination by RPC–HPLC–ICP-MS.

The percentage of each iodine/bromine species in the dialyzates taking into account the total amount of iodine and bromine found in the dialyzates was calculated as follows:

$$\% = \frac{[\ ]_{\text{species in the dialyzate extract}}}{[\ ]_{\text{dialyzate extract}}} \times 100$$

where % is the percentage of each bio-available iodine/bromine species, and $[\ ]_{\text{species in the dialyzate}}$ and $[\ ]_{\text{dialyzate extract}}$ are the iodine/bromine concentration in the dialyzate as a sum of iodine/bromine species concentrations, and the total iodine/bromine concentrations in the dialyzate, respectively.

These percentages (Table 4) were from 66 ± 7% (Dulse) to 102 ± 12% (agar-agar) for iodine, and from 84 ± 2% (Sea spaghetti) to 97 ± 4% (Wakame) for bromine. It can be observed that the sum of iodine/bromine species concentrations lead to percentages higher than 80% for most of the seaweed samples. This means that the targets resolved by the AEC (iodide, MIT, DIT, bromide and bromate) are the major species present in the dialyzates. However, unresolved/undetermined iodine compounds must be present in dialyzates from Dulse and Sea spaghetti because low percentages (66 and 84%) are obtained for these samples.

The extraction yields for the over-all procedure were assessed. As the *in vitro* bio-availability procedure is not an exhaustive extraction method the percentage of released targets from the matrix sample is low. In addition, these percentages tend to be lower when simulating a dialysis step during the intestinal digestion stage (dialyzability approaches). These percentages are commonly referred as bio-availability ratios (dialyzability) [22] and they are calculated using the equation:

$$B_{\text{av}} \ (\%) = \frac{[\ ]_{\text{dialyzate extract}}}{[\ ]_{\text{alkaline digest}}} \times 100$$

where B_{av} (%) is the bio-availability percentage, and $[\ ]_{\text{dialyzate extract}}$ and $[\ ]_{\text{alkaline digest}}$ are the total iodine or the total bromine concentrations after *in vitro* digestion and after the microwave assisted alkaline digestion procedures, respectively. Table 4 lists dialyzability ratios which reflect the mass balance with respect to the total iodine/bromine contents (total iodine/bromine concentrations in

Table 5
Concentrations of total bromine, total iodine, iodine species and bromine species in dialyzates from edible seaweed.

Sample	RP–HPLC–ICP-MS					
	Total concentrations (μg g^{-1})			Bromine/iodide species concentrations (ng g^{-1})		
	Bromine	Iodine	Br$^-$ + BrO$_3^{-a}$	I$^-$ + IO$_3^{-a}$	MIT	DIT
Dulse (*Palmaria palmata*)	131 ± 9	7.5 ± 0.7	106 ± 9	2.7 ± 0.08	484 ± 19	<LOD
Nori (*Porphyra umbilicalis*)	20 ± 1	2.1 ± 0.4	15 ± 0.05	0.68 ± 0.05	1.6 ± 0.3[a]	<LOD
Sea lettuce (*Ulva rigida*)	95 ± 29	1.4 ± 0.03	70 ± 3	1.1 ± 0.1	255 ± 10	<LOD
Wakame (*Undaria pinnatifida*)	148 ± 6	7.0 ± 1	140 ± 9	5.4 ± 0.06	572 ± 19	126 ± 20.5
Sea spaghetti (*Himanthalia elongata*)	128 ± 2	4.4 ± 0.3	128 ± 9	2.7 ± 0.2	<LOD	<LOD
Kombu (*Laminaria ochroleuca*)	194 ± 27	1075 ± 109	181 ± 7	1117 ± 124	1.1 ± 0.06[a]	487 ± 17
Canned seaweed (cooked *Himanthalia elongata* and *Saccorhiza polyschides*)	22 ± 3	<LOD	18 ± 0.8	0.43 ± 0.05	103 ± 13	<LOD
Spirulina (*Spirulina platensis*)	4.1 ± 0.5	<LOD	5.1 ± 0.8	< LOD	< LOD	< LOD
NIES 09 Sargasso	29 ± 2	22 ± 0.2	26 ± 0.6	24 ± 0.7	1.2 ± 0.5[a]	2.1 ± 0.4[a]
Agar-agar (from *Gelidiumm sesquipedale*)	14 ± 1	4.5 ± 0.5	14 ± 1	4.1 ± 0.2	136 ± 13	<LOD

Sample	AE–HPLC–ICP-MS					
	Br^{-a}	BrO$_3^-$	I^{-a}	IO$_3^-$	MIT	DIT
Dulse (*Palmaria palmata*)	114 ± 4	495 ± 30	2.4 ± 0.4	<LOD	394 ± 16	<LOD
Nori (*Porphyra umbilicalis*)	16 ± 0.7	576 ± 64	0.70 ± 0.09	<LOD	1.5 ± 0.07[a]	<LOD
Sea lettuce (*Ulva rigida*)	90 ± 6	256 ± 15	0.94 ± 0.1	<LOD	242 ± 21	<LOD
Wakame (*Undaria pinnatifida*)	143 ± 0.3	399 ± 35	6.1 ± 0.7	<LOD	633 ± 48	117 ± 18
Sea spaghetti (*Himanthalia elongata*)	107 ± 6	481 ± 42	3.0 ± 0.4	<LOD	<LOD	<LOD
Kombu (*Laminaria ochroleuca*)	174 ± 1	429 ± 29	1059 ± 158	<LOD	900 ± 109	440 ± 21
Canned seaweed (cooked *Himanthalia elongata* and *Saccorhiza polyschides*)	25 ± 0.8	279 ± 24	0.55 ± 0.1	<LOD	141 ± 20	<LOD
Spirulina (*Spirulina platensis*)	4.3 ± 0.5	<LOD	<LOD	<LOD	<LOD	<LOD
NIES 09 Sargasso	26 ± 0.5	<LOD	23 ± 0.2	<LOD	1.1 ± 0.06[a]	2.0 ± 0.4[a]
Agar-agar (from *Gelidiumm sesquipedale*)	13 ± 1	<LOD	4.5 ± 0.3	<LOD	109 ± 9	<LOD

[a] μg g^{-1}.
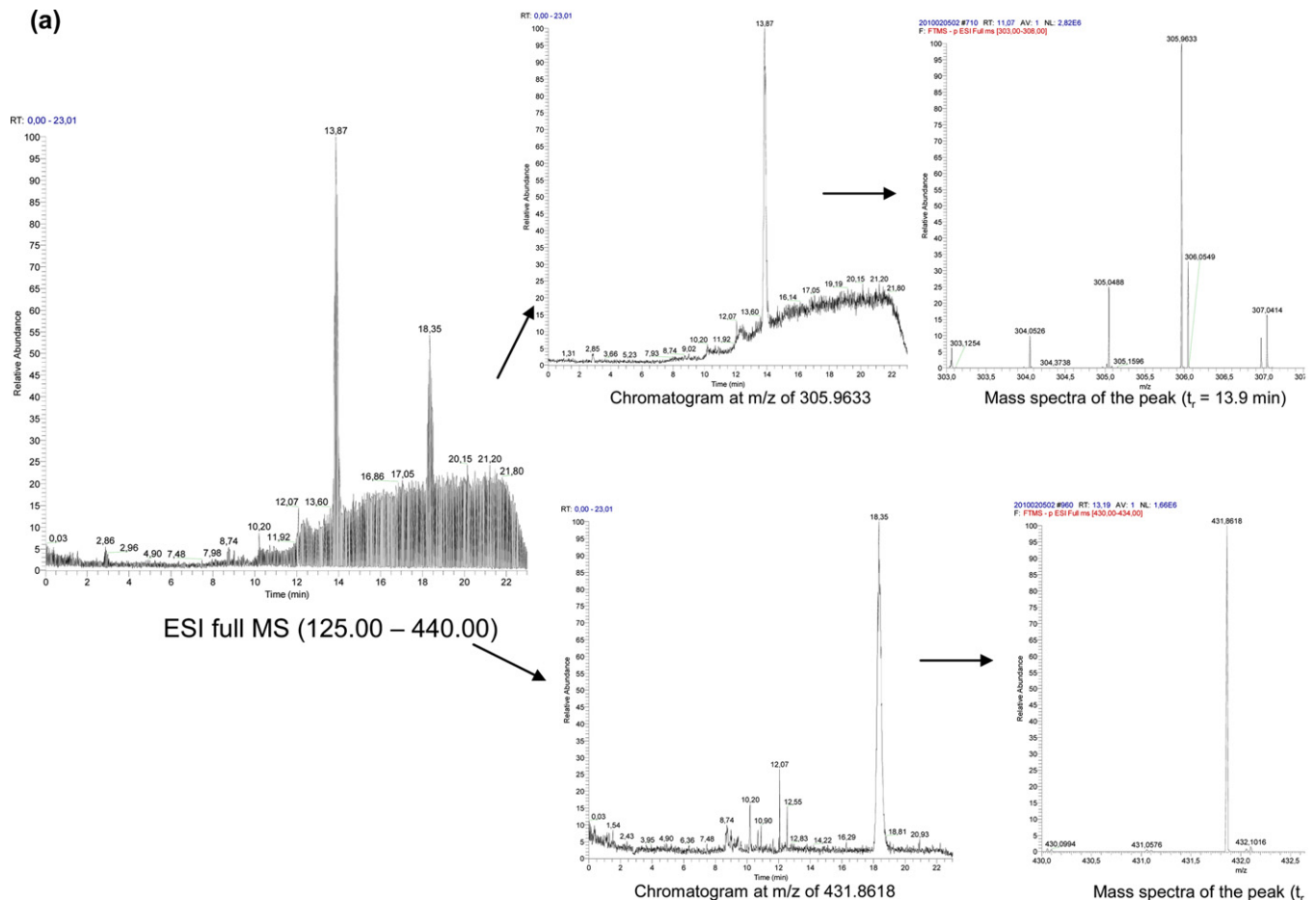
Fig. 3. ESI full MS (125.00–440.00) chromatograms, extracted chromatograms at *m/z* ratios of 305.9633 (MIT) and 431.8618 (DIT), and mass spectra of the peak of the extracted chromatograms, for 100 μg L^{-1} MIT and DIT aqueous standards (a), and for a dialyzate from a Wakame sample (b).

(b)

ESI full MS (125.00 – 440.00)

Chromatogram at m/z of 305.9634

Mass spectra of the peak (t$_r$ = 13.7 m

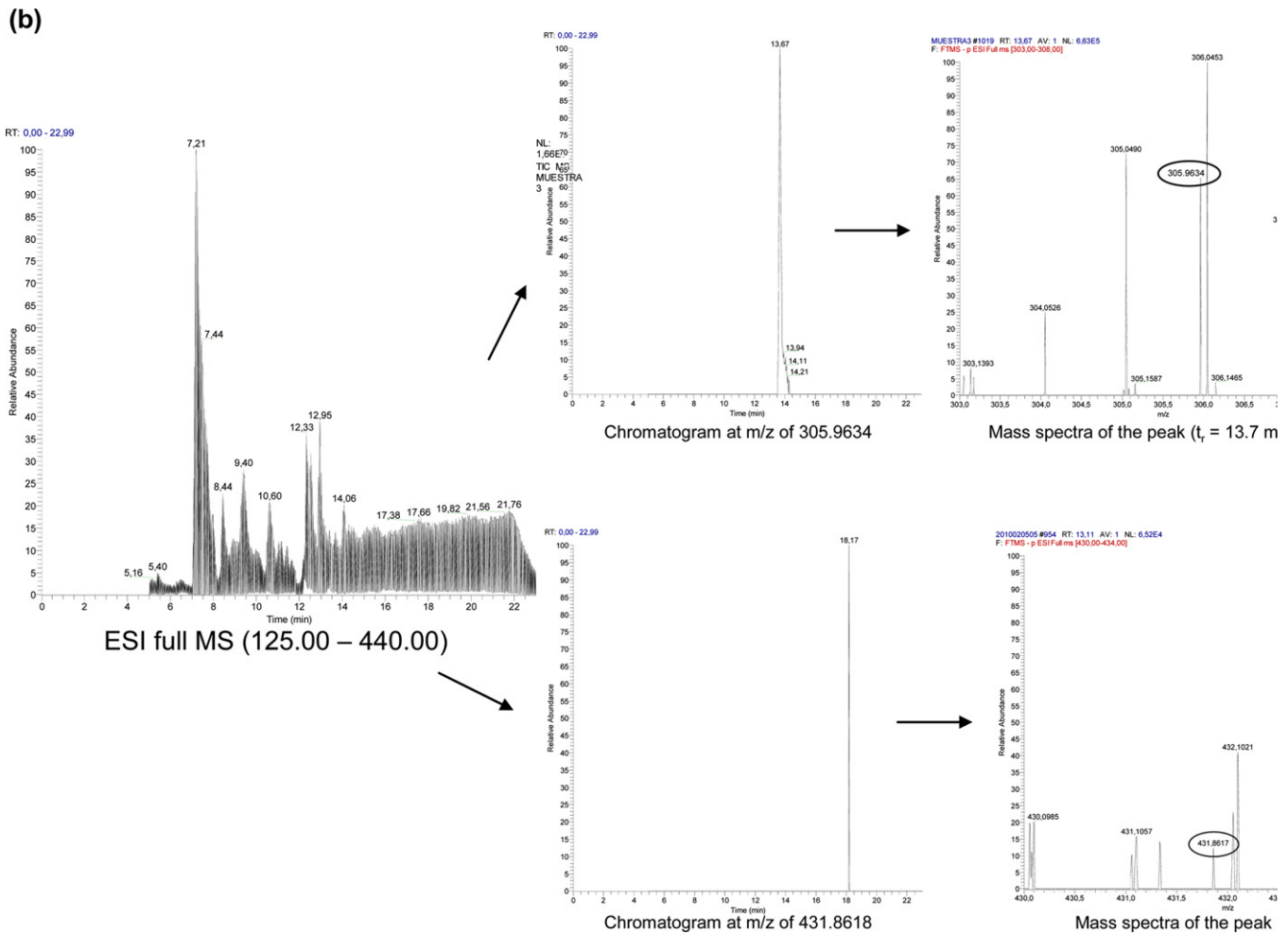Chromatogram at m/z of 431.8618

Mass spectra of the peak

Fig. 3. (*Continued*).

the dialyzate *versus* total iodine/bromine concentrations in the seaweed samples). Ratios within the 10–47% range were found for bromine; whereas, percentages between 2% and 18% were obtained for iodine.

Column analytical recovery studies were performed by spiking different dialyzates with low target concentrations (1 mg L^{-1} for iodide and bromide, 27.5 μg L^{-1} for iodate and bromate, and 25 μg L^{-1} for MIT and DIT) and high target concentrations (5 mg L^{-1} for iodide and bromide, and 200 μg L^{-1} for iodate, bromate, MIT and DIT) in quintuplicate. Column analytical recoveries within the 93–105% and 91–102% ranges for AE and RP columns were obtained.

To verify the presence of MIT and DIT, different chromatographic fractions from a dialyzate from Wakame (both chromatographic signals for MIT and DIT after AEC and RP as shown in Figs. 4 and 6) were collected after RPC and AEC; RPC fractions were then chromatographed by AEC and *vice versa*. For collection, the AE or RP column outlet was disconnected to the ICP-MS detector, the time was monitored with the chronometer of the HPLC, and the eluted volumes within the specified times were directly collected in amber-glass vials. Eluted fractions from the RP column (times within the 13.5–16.0 min range for the MIT elution, and times between 16.0 and 18.5 min for the DIT elution) have allowed the identification of MIT (Fig. 2(a)) after AEC of the first RPC fraction. However, no signal was observed after AEC of the second RP fraction (DIT) because of the low DIT concentration (and also because

of the dilution inherent to this experiment). Similarly, eluted fractions from the AEC column (times between 3.5 and 6.5 min for MIT, and between 6.5 and 10 min for DIT) were also analyzed by RPC. The chromatogram shown in Fig. 2(b) confirms the presence of MIT (DIT concentration in the isolated AEC fraction was not high enough to be detected after RPC separation).

Finally, Orbitrap mass spectrometry (conditions given in Section 2.8) was also used to confirm the presence of MIT and DIT species in the dialyzates from seaweed. The identification was based on [M−H]$^-$ at m/z ratios of 305.9634 and 431.8618, respectively, which were obtained by injecting MIT and DIT standards. Fig. 3 shows the ESI full MS (125.00–440.00), as well as the extracted chromatograms at m/z ratios of 305.9634 (MIT) and 431.8618 (DIT) for MIT/DIT aqueous standards (a) and for a dialyzate from Wakame (b). The mass spectra from each chromatographic peak show the presence of MIT and DIT in the dialyzate from this seaweed, which is in accordance with the detection/quantification of these species (in addition to iodide) in this sample by HPLC–ICP-MS (see next section).

3.4. Applications: bromine and iodine species in dialyzates from edible seaweed.

Optimized AEC and RPC conditions were applied to different dialyzates from six edible dried seaweeds (Nori, Dulse, Wakame, Kombu, Sea spaghetti and Sea lettuce), one edible canned
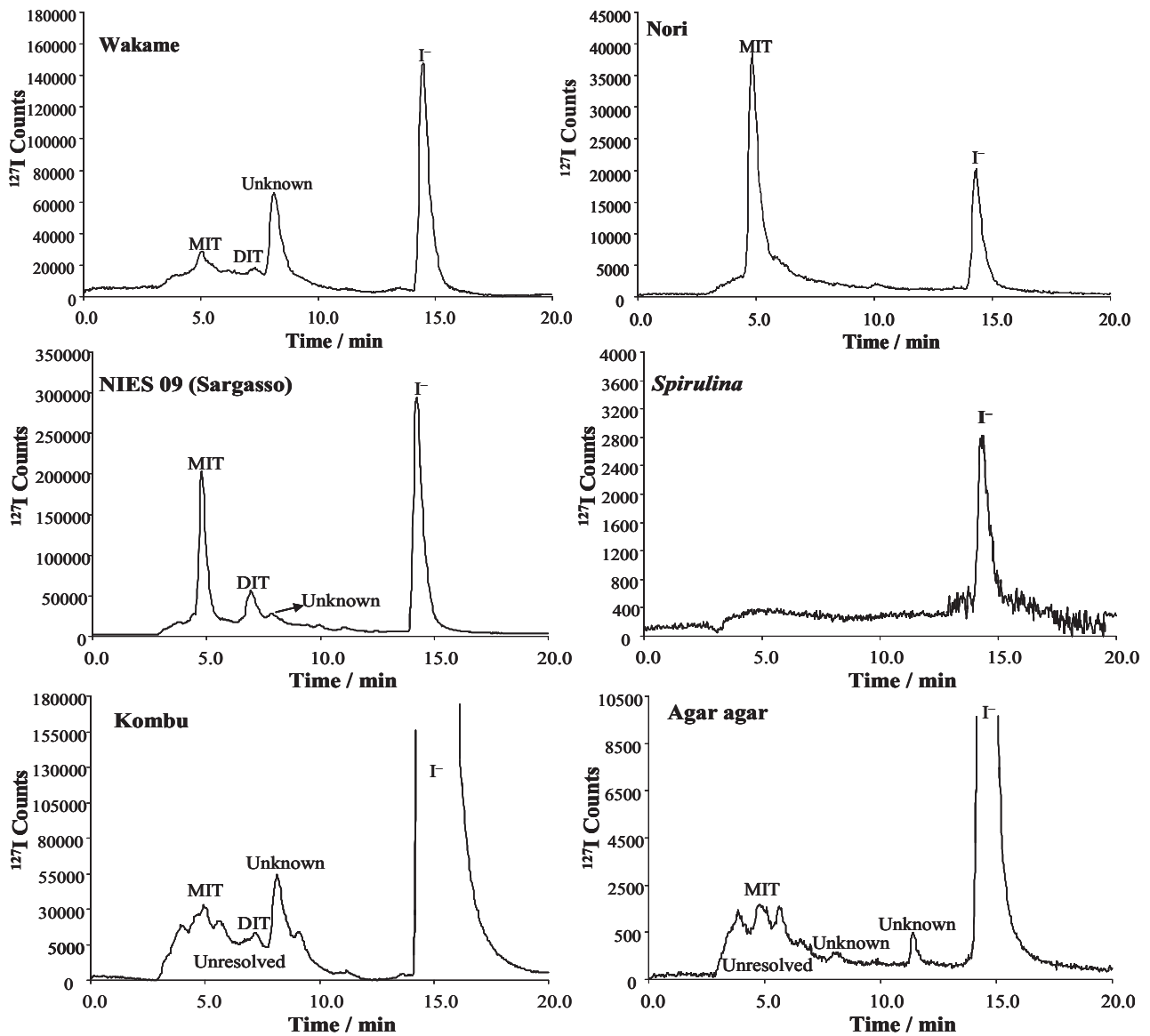
Fig. 4. Anion exchange chromatograms for iodine species for dialyzates from edible seaweed.

seaweed in brine (Sea Spaghetti with Furbelows), and one microalgae (*Spirulina*). Moreover, dialyzates from NIES 09 (Sargasso) and from a seaweed derived product (agar-agar), extracted from *G. sesquipedale*, were also analyzed. Fig. 4 shows AE chromatograms obtained from some dialyzates. Iodide and MIT were observed in Nori and Dulse (red seaweed), and also in Sea lettuce (green seaweed) and in canned seaweed (a mixture of brown seaweeds). Iodide, MIT and DIT (small signal) were observed in the brown seaweed Wakame, and also iodide and DIT (small signal) were obtained in dialyzates from Sea spaghetti (brown seaweed). The chromatogram from *Spirulina* only shows a small signal for iodide, while the dialyzate from NIES 09 Sargasso shows iodide, MIT and DIT, and also an unknown iodinated compound (retention time of 7.0 min). This unknown iodinated compound was also observed in the dialyzates from Wakame and Kombu (Fig. 4). However, unresolved signals in the first part of the chromatograms for Kombu and agar-agar led to a poor resolution of MIT. Finally, iodate was not observed in any dialyzate, while both inorganic bromine species,
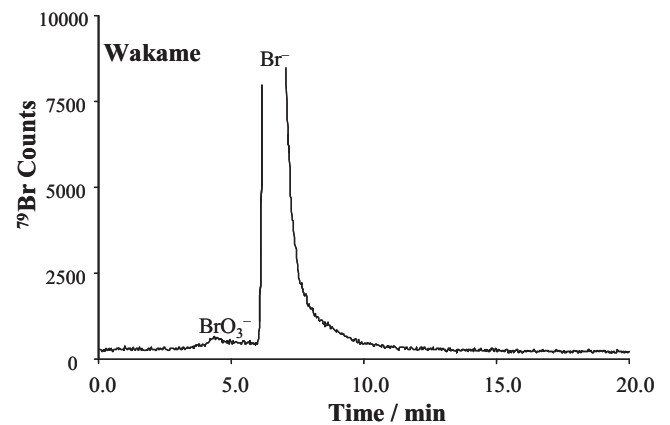
Fig. 5. Anion exchange chromatogram for bromine species for a dialyzate from Wakame.
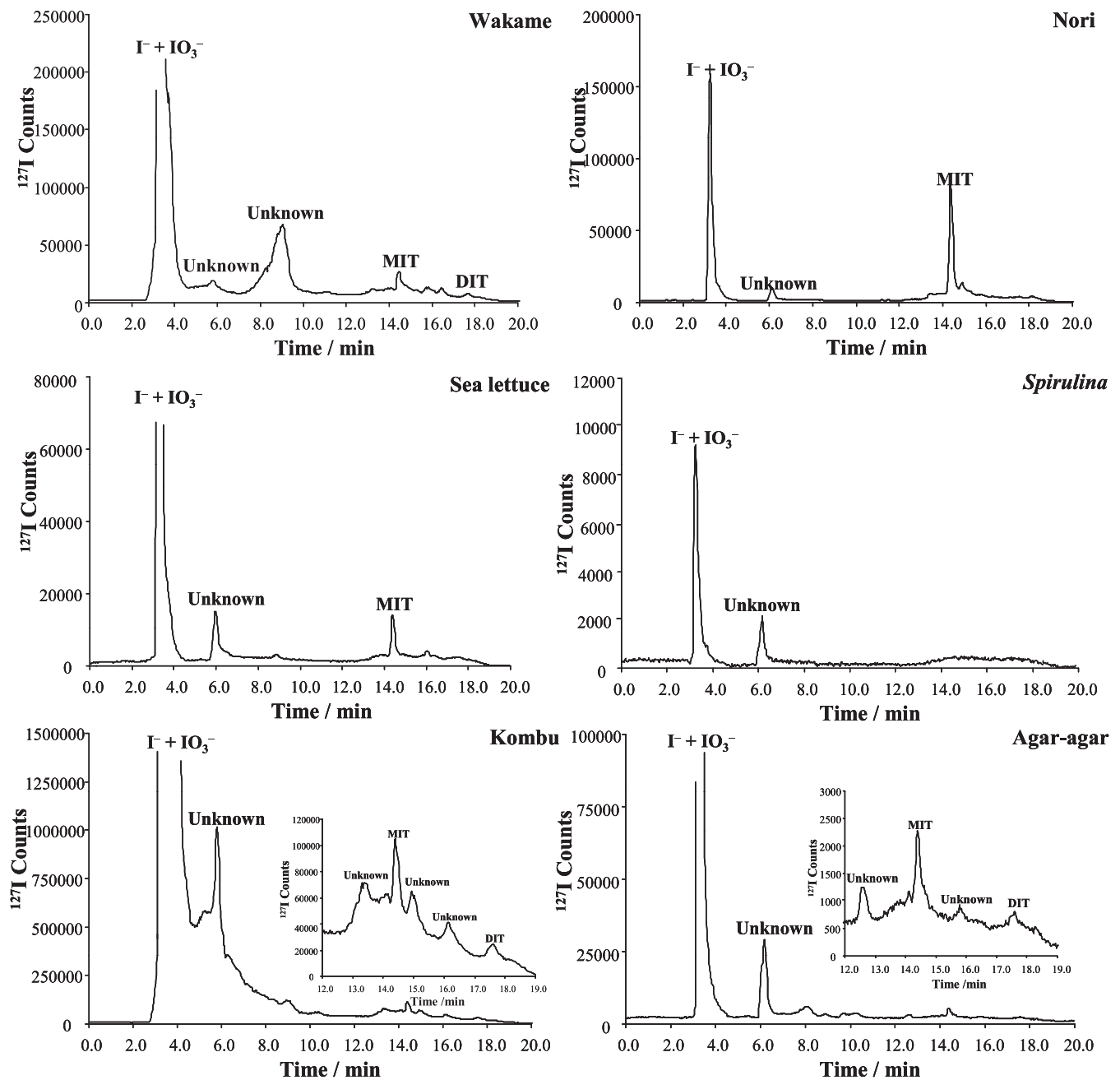
Fig. 6. Reverse phase chromatograms for iodine species for dialyzates from edible seaweed.

bromide as a major species and bromate as a minor species, were observed in all cases (Fig. 5), except for *Spirulina* where only the bromide species was detected.

Similar results have been obtained when performing RPC, although several unknown signals were observed. Inorganic iodine (mainly iodide) was present in all samples, while MIT could be quantified in all dialyzates except for Sea Spaghetti and *Spirulina* (Fig. 6). DIT was present only in the dialyzates from Wakame and Kombu (Fig. 6). This result agrees with those of Shah et al. [14] which reported the presence of DIT in these seaweed types (Wakame and Kombu). An unknown signal (retention time of 5.9 min) was observed in all cases, including in the dialyzates from

Spirulina. Moreover, the dialyzates from Wakame, Kombu and agar-agar have also shown other unknown signals (Fig. 6).

Concerning bromine species, since inorganic bromine species (bromate and bromide) co-elute, only inorganic bromine could be assessed. However, an unknown signal (retention time of 6.8 min) was observed in the dialyzates from agar-agar (Fig. 7), and also, unknown signals (retention times of 13.4 and 15.3 min) were observed in the dialyzates from Sea lettuce (Fig. 7).

Finally, Table 5 lists the concentrations for dialyzable bromine and iodine species in edible seaweed after both chromatographic (AEC and RPC) methods.
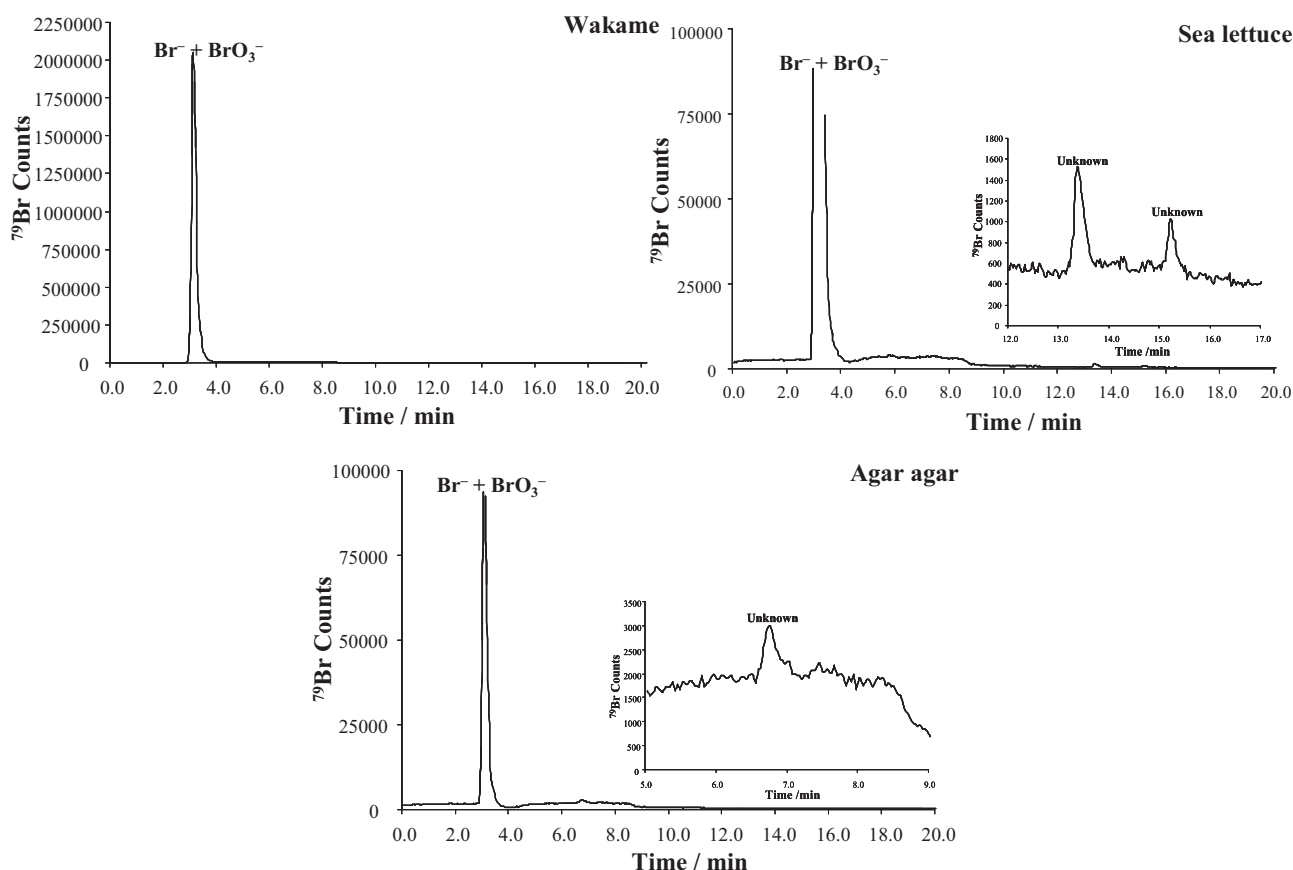
Fig. 7. Reverse phase chromatogram for bromine species for dialyzate from edible seaweed.

4. Conclusions

The use of AEC and RPC hyphenated to ICP-MS has allowed the separation of different iodine species (and also inorganic bromine species) in dialyzates from edible seaweed. Good results have been obtained when using AEC, and four iodine species (iodide, iodate, MIT and DIT) have been resolved in a single chromatographic run working in an isocratic mode. Iodide and bromide were the major species present in all dialyzates, whereas bromate was found in all cases and iodate was always at undetectable levels. MIT was also present in most of the dialyzates from edible seaweed, while DIT was only quantified in Wakame and Kombu (brown seaweed), and in the NIES 09 CRM (Sargasso, green seaweed). Several unknown iodine, and also bromine species have been found in the dialyzates from some seaweed, such as Kombu, Wakame, Nori and Sea lettuce (unknown iodine species) and Sea lettuce (unknown bromine species). Dialyzates from agar-agar have also shown unknown iodine and bromine species. It must be pointed out that Kombu and agar-agar analysis have posed problems to separate most of these unknown species, and both AE and RP chromatograms for these samples have shown some unresolved patterns. Further work is needed to improve iodine species separation in Kombu and agar-agar extracts, and also to elucidate the structure of unknown iodine and bromine species present in the dialyzates, compounds which are bio-available for humans.

Acknowledgments

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