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RESEARCH PAPER

Fast determination of harmala alkaloids in edible algae by capillary electrophoresis mass spectrometry

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Abstract The use of algae as a foodstuff is rapidly expanding worldwide from the East Asian countries, where they are also used for medical care. Harmala alkaloids (HAlk) are a family of bioactive compounds found in the extracts of some plants, including wakame (*Undaria pinnatifida*), an edible marine invasive algae. HAlks are based on a characteristic β-carboline structure with at least one amino ionizable group. In this work, we report the successful separation of a mixture of six HAlks (harmine, harmaline, harmol, harmalol, harmane, and norharmane) by capillary electrophoresis ion-trap mass spectrometry (CE-IT-MS) in less than 8 min. Optimum separation in fused-silica capillaries and detection sensitivity in positive-ion mode were achieved using a background electrolyte (BGE) with 25 mmol L^{-1} ammonium acetate (pH 7.8) and 10 % (*v*/*v*) methanol, and a sheath liquid with 60:40 (*v*/*v*) isopropanol–water and 0.05 % (*v*/*v*) formic acid. The separation method was validated in terms of linearity, limits of detection and quantification, repeatability, and reproducibility. Later, a sample pretreatment was carefully optimized to determine HAlks in commercial wakame samples with excellent recovery and repeatability. For the complex wakame extracts, the MS–MS fragmentation patterns of the different HAlks were useful to ensure a reliable identification. The complete procedure was validated using the standard-addition calibration method, determining matrix effects on the studied compounds. Harmalol, harmine, and harmaline were naturally present in the samples and were quantified at very low concentrations, ranging from 7 to 24 μg kg^{-1} dry algae.

Keywords Capillary electrophoresis · Mass spectrometry · MS–MS · *Undaria pinnatifida* · Harmala alkaloids · Validation

Introduction

Harmala alkaloids (HAlks) are a family of compounds with pharmacological and psychopharmacological effects on humans [1–4]. They are especially renowned for their antidepressant properties, because they are strong inhibitors of monoamine oxidase type A (MAO-A) enzyme, which catalyzes the oxidative deamination of biogenic amines and neurotransmitters [2–4]. HAlks are naturally present at high concentrations in some plants, including *Peganum harmala* (Syrian rue) [4–6], *Banisteriopsis caapi* [7–9], and *Passiflora incarnata* [10]. Harmine and harmaline are the main HAlks in *B. caapi* which is one of the constituents of Ayahuasca, a psychotropic beverage originally used in shamanic rituals of the Amazon-basin tribes [7–9]. Some HAlks, including harmane and norharmane, have been widely reported to occur at lower concentrations in coffee, chocolate, alcoholic beverages, raw or "well done" meat or fish, and cigarette smoke [10–13]. Harmane can also be found as a normal endogenous constituent of some human tissues and body fluids or as a

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biomarker of some diseases, including Parkinson disease [10, 14]. However, compared with exposure to exogenous HAlks, endogenous formation is almost negligible. Exposure levels from dietary sources are estimated to be a maximum of 4 and 1 μg kg^{-1} body weight per day for norharmane and harmane, respectively [10]. There is no regulation for HAlks, nor for the religious or the expanding recreational use of plants, especially *P. Harmala* and *B. Caapi,* that contain high levels of HAlks [6, 8, 13, 15].

The development of efficient and sensitive analytical procedures for HAlks is of interest in pharmacology, toxicology, food safety, and medical and forensic science. HAlks are sparingly water-soluble compounds based on a β-carboline structure (9H-pyrido[3,4-b]indole) (differing only in a substituent or in saturation) (Fig. 1) [16–18]. High-performance liquid chromatography (HPLC) with fluorescence or mass-spectrometry detection has usually been the technique of choice for determination of some HAlks in a variety of samples, including plant extracts, food, beverages, and biological fluids [5–7, 9, 11, 19–22]. Capillary electrophoresis (CE) has also been used [23–27], because HAlks present at least one amino ionizable group [16–18], but to a lesser extent despite the well-known advantages of CE [28]. Few works have revealed the potential of CE, and only for the qualitative analysis of standard mixtures or plant extracts with well-known high levels of HAlks. Only a few authors have optimized methods for the separation of standard mixtures of HAlks using micellar electrokinetic chromatography (MEKC) with ultraviolet or laser-induced-fluorescence detection and capillary electrophoresis mass spectrometry (CE-MS), with limited success [23–25]. More recently, C. Huhn et al. detected HAlks of Ayahuasca and different plant extracts (*B. caapi* and *P. harmala*) by capillary electrophoresis laser-induced-fluorescence mass spectrometry (CE-LIF-MS) and CE-MS with aqueous and nonaqueous background electrolytes (BGEs), without achieving full separation of some extracts [26, 27].

In this work, we developed and validated a method for separation of six HAlks, harmine, harmaline, harmol, harmalol, harmane, and norharmane, by capillary electrophoresis ion-trap mass spectrometry (CE-IT-MS) in less than 8 min. The method was applied to the analysis of wakame (*Undaria pinnatifida*), an edible marine invasive brown algae originally from the East Asian countries, which is commonly used worldwide as a foodstuff, and by traditional oriental medicine practitioners. Although the occurrence of bioactive compounds including alkaloids in algae is known [29–32], there are no previous analytical reports on determination of HAlks and the contents are not yet established [30–32]. The analysis of wakame is a challenging task because of the complexity of the sample and the low concentration of the HAlks. We optimized a high-recovery extraction method for HAlks from wakame before CE-IT-MS. For the complex wakame extracts, the MS–MS fragmentation patterns of the different HAlks were useful to ensure reliable identification. The complete procedure was validated using the standard-addition calibration method, before quantitation of the detected HAlks.

Experimental

Instrumentation

All experiments were performed on an Agilent Technologies $HP^{3D}CE$ system (Waldbronn, Germany) coupled to an MSD Ion Trap mass spectrometer (Agilent Technologies). An electrospray G1603A Agilent Technologies sprayer was used as a sheath-flow–CE–ESI-MS interface. The sheath liquid

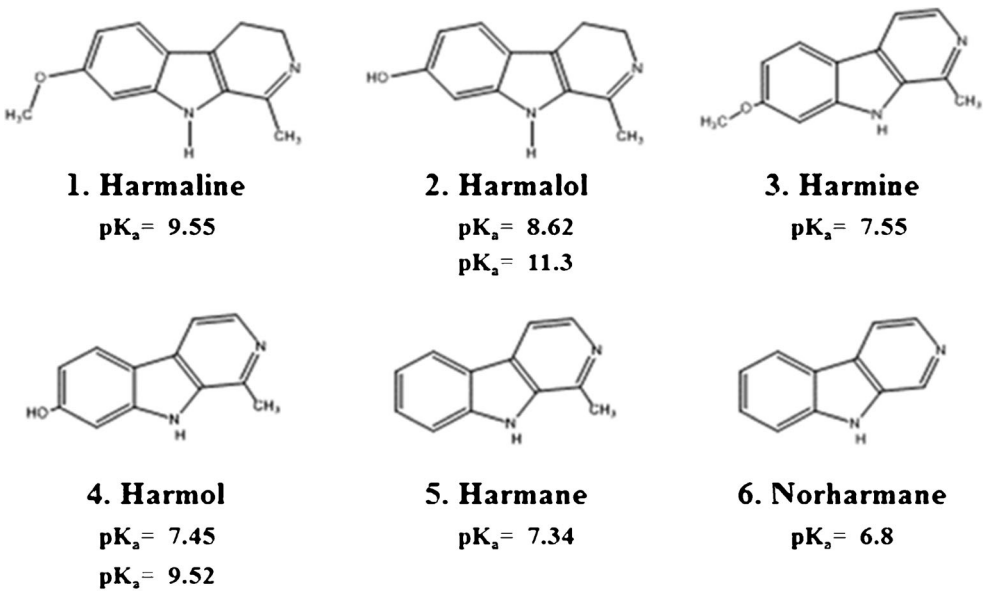

Fig. 1 Chemical structure of the studied harmala alkaloids (HAlks). Monoisotopic molecular mass (*M*): (**a**) harmaline 214.27, (**b**) harmalol 200.24, (**c**) harmine 212.25 (**d**) harmol 198.22, (**e**) harmane 182.22, (**f**) norharmane 168.19

was delivered by an infusion pump KD Scientific 100 Series (Holliston, MA, USA). The detailed conditions affecting CE-MS have been discussed elsewhere, as has the tuning of the IT mass spectrometer [33]. pH measurements were performed with a Crison 2002 potentiometer and a Crison electrode 52–03 (Crison Instruments, Barcelona, Spain). Sample incubation was performed with a Thermo-Shaker TS-100 (Biosan, Warren, USA). Centrifugation was performed in a thermostated Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany).

Chemicals

All solutions were prepared with water provided by a MilliQ® water-purification system (Millipore, Bedford, MA, USA). Methanol, ethanol, formic acid (HFor) (98–100 %), acetic acid (HAc) (glacial), ammonia (25 %), hydrochloric acid (25 %), sodium dihydrogenphosphate, and sodium hydroxide (NaOH) (Merck, Darmstadt, Germany) and isopropanol (Sigma, Saint Louis, USA) were HPLC grade or superior.

Harmane, norharmane, harmalol hydrochloride dihydrate, harmine, and harmaline were purchased from Aldrich (Steinheim, Germany), and harmol hydrochloride dihydrate was donated by Dr F. Cabrerizo (IIB-INTECH, Universidad Nacional de San Martín, Chascomús, Buenos Aires, Argentina). An individual stock standard solution of each alkaloid was prepared by dissolving the solid in methanol to a concentration of 100 μg mL^{-1}. All stock solutions were stored at 4 °C. The working solutions were diluted in water and filtered through a 0.22 μm membrane before use.

Procedures

CE–IT-MS

The electrophoretic runs were performed in a 60 cm total length (L_T), 75 μm internal diameter (id), and 360 μm outer diameter (od) bare-fused-silica capillary supplied by Polymicro Technologies (Phoenix, AZ, USA). Under optimum separation conditions the BGE was 25 mmol L^{-1} ammonium acetate (pH 7.8) with 10 % (*v*/*v*) methanol. All capillary rinses were performed at 930 mbar. New capillaries were activated by flushing with 1 mol L^{-1} NaOH (20 min), water (15 min), and BGE (30 min). This activation was performed off-line to avoid the unnecessary entry of NaOH into the MS system. The capillary was finally equilibrated by applying the separation voltage for 15 min. The capillary was rinsed between runs with methanol (1 min), water (1 min), and BGE (2 min). Samples were hydrodynamically injected at 35 mbar for 3 s. Analyses were performed at 25 °C, applying a voltage of 20 kV under normal polarity. Between workdays, the capillary was conditioned by rinsing successively with methanol (5 min), water (5 min), and BGE (15 min). The capillary was stored overnight filled with working BGE; after longer storage periods it was rinsed with water (10 min).

Under optimum detection conditions in positive-ion mode the sheath liquid was a mixture of 60:40 (*v*/*v*) isopropanol–water with 0.05 % (*v*/*v*) HFor and was delivered at 3.3 μL min^{-1}. The drying gas (N_2) flow and temperature were 5 mL min^{-1} and 250 °C, respectively. The ESI potential was set at 4000 V. Voltages on the capillary exit and skimmer were 145 and 47 V, respectively. Octopole voltages were set at 7.5 and 1.25 V. The trap drive, octopole RF, lens 1, and lens 2 were set at 94.5, 93.5, −14, and −100 V, respectively. Full-scan mass spectra were acquired as an average of seven scans from 100 to 500 *m*/*z*.

The fragmentation of the molecular ion in MS^n detection was optimized independently for each compound by using direct infusion into the IT-MS spectrometer. The MS–MS (MS^2) fragments were useful to confirm the compound identity in real samples. Higher-order fragmentations were also studied with standards, but sensitivity was not sufficient to monitor those transitions in real samples. Because of the structural similarities of HAlks (Fig. 1), the MS–MS fragmentation conditions are also quite similar; thus, a characteristic fragmentation pattern of the six compounds was obtained in a single run. Collision-induced-fragmentation experiments were performed using helium as the collision gas. Fragmentation energies were investigated in the range 0.50–2.00 V, and the useful range for these analytes was 0.75–1.0 V. Therefore, the isolation bandwidth was set at 4 *m*/*z* and collision energy at 1 V. MS^2 mass spectra were acquired as an average of seven scans from 50 to 500 *m*/*z*.

Sample preparation

The wakame (*Undaria pinnatifida*) samples were from three different commercial brands in the original sealed plastic bags. Two were from the Atlantic coast of Galicia (Spain) and the other from the Atlantic coast of Brittany (France). All samples were dried at 40 °C for 24 h and then milled in a disc mill. Finally, the powder was sieved with a sieve of 200 μm and stored at 4 °C.

The optimized sample pretreatment consisted of the extraction of 0.1 g sieved powder with 500 μL methanol and 500 μL HCl 3.5 mol L^{-1}. The mixture was incubated for 6 h at 80 °C in a shaker. After this step, the sample was vortexed for 30 s and then centrifuged at 10,000 *g* and 25 °C for 15 min. The supernatant was ultrafiltrated with a 3 kDa molecular-weight cut-off (MWCO) Amicon® Ultra-0.5 centrifugal device (Millipore) for 15 min at 16,000 *g* and 25 °C. Then, an aliquot of 50 μL MilliQ water was added to the filter and centrifuged at 12,000 *g* for 10 min, to lose the minimum amount of low-molecular-mass HAlks in the retentate. This washing step was repeated twice and all the filtrates were combined. Finally, sufficient water was added to obtain a final volume of 250 μL.

The 3 kDa filters were passivated before the first use with a solution of PEG 5 % to ensure optimum recovery of HAlks [34]. The recovery of the ultrafiltration step with the passivated filters was estimated from a comparison of the peak areas obtained by CE–MS with and without ultrafiltration for a standard mixture of the six HAlks at a concentration of 1 μg mL^{-1}. Recoveries were estimated in a similar way, using spiked samples before and after the incubation step to find the best extraction-mixture composition, incubation time, and temperature.

Method validation

Quality data were calculated from data obtained by measuring peak area and migration time from an extracted ion electropherogram (XIE) of each HAlk. Studies of repeatability (intra-day, n=10 with one capillary) and reproducibility (inter-day, n=30 over three alternate days and with a new capillary each day) were performed by analyzing a mixture of the six HAlks at concentrations of 1 μg mL^{-1} each. These values were calculated as a percentage relative standard deviation (%RSD) of peak areas and migration times. The limit of detection (LOD) and limit of quantification (LOQ) for each HAlk were calculated as three times and ten times the signal-to-noise ratio (S/N), respectively, where the noise values were obtained from each corresponding blank-sample analysis.

External and standard-addition calibration methods were used for quantification of the six HAlks. Calibration was performed at five levels of concentration, in duplicate or triplicate at each level. The studied concentrations were in the range 0.01–10 μg mL^{-1} and 0.016–16 μg mL^{-1} for the external and the standard-addition calibration method, respectively. The slopes of both calibration curves were compared to determine the matrix effects according to Massart et al. [35], with a significance level of 95 %.

For HAlk quantification the three different wakame samples were analyzed in triplicate and the concentrations were extrapolated from the standard-addition calibration curves.

Results and discussion

CE-MS method optimization

Figure 1 shows the chemical structure and the molecular mass of the studied HAlks. Because they are structurally quite similar, the CE separation of HAlks is not straightforward. All HAlks are based on a β-carboline structure (9H-pyrido[3,4-b]indole) with at least one amino ionizable group, and differ in only one substituent and/or in saturation, which does not result in substantial molecular-charge or size differences. These properties make separation of HAlks an interesting challenge for CE. Elsewhere [36], we described a theoretical method to optimize the electrophoretic separation of HAlks on the basis of their dissociation constants, the pH of the BGE, and the electroosmotic flow (EOF). The method was useful to select the optimum pH for the separation of a mixture of HAlks by CE-UV. A BGE with 50 mmol L^{-1} Tris (pH 7.8) was used, and addition of 20 % (v/v) methanol resulted in improved peak shape, peak capacity, and separation resolution. However, these BGE do not have the volatility necessary to generate an efficient electrospray in MS detection, to enable optimum sensitivity and signal stability and avoid the formation of salt deposits in the interface or at the entrance of the mass spectrometer [26, 27, 33, 34]. Acidic BGEs containing acetic acid, formic acid, and moderate amounts of ammonia are usually preferred for CE-MS in positive-ion mode. However, the sheath-flow interface also enables good sensitivity in positive-ion mode using neutral or slightly basic volatile BGEs because it is mainly the sheath-liquid pH, and not only the ionization state of the analyte resulting from the BGE pH, which determines the optimum spray efficiency in a given ESI mode [37].

On the basis of the separation optimized by CE-UV using the Tris BGE (pH 7.8) [36], we selected some BGEs based on ammonium acetate (NH_4CH_3COO) and ammonium dihydrogen phosphate–ammonium hydrogen phosphate ($NH_4H_2PO_3$–$(NH_4)_2HPO_3$) at different ionic strengths (from 10–50 mmol L^{-1}) and methanol contents (from 0 to 20 % (v/v)). In the ammonium phosphate BGEs, a sufficiently low ionic strength was maintained (10 mmol L^{-1}) to ensure an appropriate volatility. Although the best separation efficiency was obtained with ammonium phosphate at 25 mmol L^{-1} ionic strength with 10 % (v/v) methanol, followed by ammonium acetate at 25 mmol L^{-1} ionic strength with 10 % (v/v) methanol, the detection sensitivity in positive-ion mode was 10 times higher with the ammonium acetate BGE. Using the ammonium acetate BGE, different sheath-liquid flows (from 1 to 5 μL min^{-1}), use of isopropanol or methanol as organic modifier, and use of acetic or formic acid at different concentrations (from 60 to 100 % (v/v) and from 0.05 to 1 % (v/v), respectively) as acidic additive were studied. The best sensitivity and spray stability were obtained at 3 μL min^{-1} sheath-liquid flow with 60:40 (v/v) isopropanol–water and 0.05 % (v/v) formic acid. Figure 2a shows the total ion electropherogram (TIE) of a 1 μg mL^{-1} standard mixture of the six HAlks. As can be observed, all the HAlks were separated except harmine (peak 3) and harmol (peak 4), which were slightly separated at approximately 6.4 min (R_s=0.5). The extracted ion electropherograms (EIE) of each HAlk (Fig. 2b) enabled us to clearly distinguish the successful separation of the six alkaloids on the basis of the different m/z values of their singly charged molecular ions.

Using these optimized conditions, the method was validated using an external calibration procedure followed by repeatability and reproducibility experiments. The quality data are

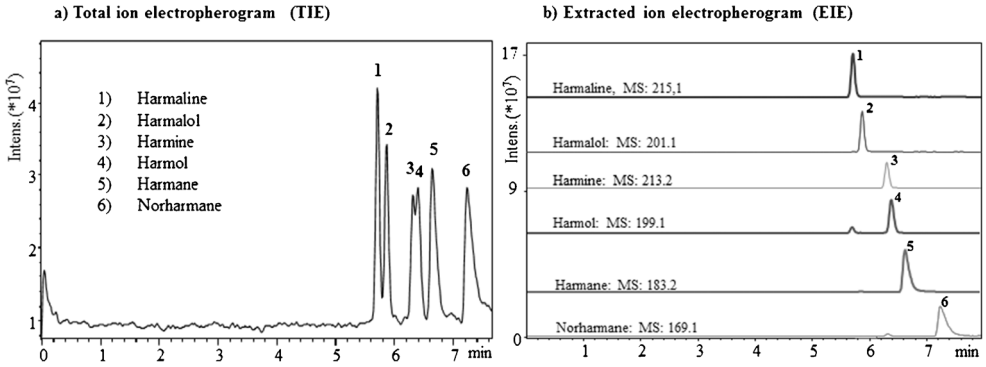

Fig. 2 (**a**) Total ion electropherogram (TIE) of a standard mixture of the HAlks at 1 μg mL^{-1}. (**b**) Extracted ion electropherograms (EIE) of the singly charged molecular ion of each HAlk

summarized in Table 1. In agreement with the structural similarities of the HAlks, the linearity ranges (r^2>0.99), the linear regression slopes, and the LODs and LOQs were similar in all cases. LODs ranged from 5.9 ng mL^{-1} for harmol to 1.2 ng mL^{-1} for harmine, harmane, and norharmane. Although most available reports are focused on method development and optimization [23–25], the only work reporting LODs for these alkaloids is that of Posch et al. [27], who obtained LODs of 4.2 ng mL^{-1} for norharmane and 13.7 ng mL^{-1} for harmane using CE-MS, whereas in this work we obtained LODs of 1.2 ng mL^{-1} for both these analytes. An additional work analyzing these HAlks is that of Huhn et al. [26], who used CE-LIF-MS; despite LODs not being calculated, their electropherograms revealed detectability of HAlks in solutions of 40 nmol L^{-1}. These values correspond to concentrations ranging from 7 ng mL^{-1} to 9 ng mL^{-1}, whereas the LODs obtained in this work range from 1.2 to 5.9 ng mL^{-1}. Furthermore, the obtained values are within the range of LODs reported for other alkaloids analyzed by CE-MS, including strychnine (120 ng mL^{-1}) and brucine (130 ng mL^{-1}) [38] or hyoscyamine (1000 ng mL^{-1}) and scopolamine (100 ng mL^{-1}) [39]. Several authors have also reported that the LODs for alkaloids obtained by CE-MS are tenfold lower than those obtained by CE-UV (approximately 1000 ng mL^{-1}) [38, 40, 41]. However, the LODs obtained by CE-MS are slightly higher than those reported for HPLC with fluorescence and MS detection, e.g. 0.5 ng mL^{-1} and approximately 0.3 ng mL^{-1}, respectively, for harmine, harmaline, harmol, and harmalol [7, 9]. The higher LODs compared with HPLC-UV are one of the main disadvantages of microscale separation techniques, including CE. The main advantage of CE in the analysis of HAlks is the reduction of analysis time to half that required in HPLC, and solvent and sample consumption are also substantially minimized. Furthermore, the

Table 1 Quality data for the external and standard-addition calibration curves of the six HAlks, including linearity ranges, limits of detection (LOD), and limits of quantitation (LOQ). Repeatability (ten replicates) and reproducibility (three days, ten replicates per day) studies were performed at a concentration of 1 μg mL^{-1} (%RSD, relative standard deviation)

HAlk	Linearity		LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Repeatability, %RSD (n=10)		Reproducibility, %RSD (n=10, 3 days)	
	Range (μg mL^{-1})	y=a+bx (r^2>0.99)			t_m	Area	t_m	Area
External calibration								
Harmaline	0.008–10	$3.4\times10^7x-2.8\times10^4$	2.5	8.0	1.3	10	3.1	17
Harmalol	0.006–10	$6.0\times10^7x-1.8\times10^6$	1.8	6.0	1.1	7.3	2.9	10
Harmine	0.004–10	$5.2\times10^7x+1.5\times10^5$	1.2	4.0	1.1	2.5	3.2	9.7
Harmol	0.020–10	$5.4\times10^7x-1.2\times10^6$	5.9	20	0.9	6.4	3.6	9.1
Harmane	0.004–10	$5.4\times10^7x+4.2\times10^4$	1.2	4.0	1.0	8.9	3.8	9.9
Norharmane	0.004–10	$5.8\times10^7x-3.3\times10^5$	1.2	4.0	1.0	9.9	4.5	12
Standard-addition calibration								
Harmaline	0.014–16	$3.4\times10^6x-2.8\times10^6$	5.1	14.1	1.2	12	3.4	12
Harmalol	0.013–16	$5.2\times10^6x-1.2\times10^6$	4.0	13.0	1.1	10	2.5	11
Harmine	0.007–16	$7.9\times10^6x+7.9\times10^5$	2.3	7.0	1.0	9.3	2.6	12
Harmol	0.033–16	$4.9\times10^6x-1.2\times10^4$	10.0	33.3	0.8	9.1	3.35	10
Harmane	0.006–16	$8.7\times10^6x+2.8\times10^5$	1.8	6.0	1.1	9.1	3.2	10
Norharmane	0.005–16	$1.3\times10^7x-5.3\times10^5$	1.5	5.0	1.0	6.7	3.3	8.1

repeatability and reproducibility values were in perfect agreement with those obtained by other authors for the analysis of HAlks by HPLC [9]. Repeatability of peak areas and migration times were good, and %RSD was in the range 2.5–10 % for peak areas and 0.9–1.3 % for migration times, which are the typical values for CE-MS [42–44]. Reproducibility was also satisfactory, and %RSD values were in the range 9.1–17 % for peak areas and 2.9–4.5 % for migration times.

Sample-pretreatment optimization

To analyze the HAlks in commercial wakame samples, a sample pretreatment was necessary. Because β-carbolines are alkaloids with at least one weak basic group (pK_{a1} within the range from pH 6.8 to 9.5), the pH can be used to determine their ionization degree. Alkaloids are usually extracted either with a low polarity solvent at basic pH, in which they are expected to be in a neutral form, or using a polar solvent at low pH, in which they are expected to be positively charged. However, two of the HAlks used in this work, harmol and harmalol, contain hydroxyl groups in their structure (Fig. 1), which contribute a negative charge at pH close to the typical pK_a of these groups. In basic solutions these two HAlks are not expected to be in a neutral form, but instead have a negatively charged form. In consequence, it should be expected that poor recovery of harmol and harmalol will be obtained on their extraction with non-polar solvents at basic pH. It was verified in practice that a basic extraction with dichloromethane, maintaining a pH of 10 by use of ammonia, obtains very poor recoveries. Therefore, no further trials were done using this condition.

An alternative and widely used strategy to extract alkaloids is the use of polar organic solvents at acid pH [4, 45]. Using this strategy, we studied different variables of the extraction step, specifically the incubation time and temperature, type and content of organic solvent, and type and content of acid. The extraction temperature was evaluated from 50 °C to 90 °C, and the best extraction efficiencies were obtained in all cases at 80 °C. The organic solvents tested were ethanol and methanol at contents in the range 40–80 % (*v*/*v*), whereas the acid media was generated with formic or acetic acid at concentrations of 1 mol L^{-1}, 3 mol L^{-1}, and 10 mol L^{-1}, and with hydrochloric acid at 1 mol L^{-1} and 3.5 mol L^{-1} (for more information see Electronic Supplementary Material (ESM), Fig. S1–S3). In all cases the extraction was clearly improved using 50 % (*v*/*v*) methanol and 3.5 mol L^{-1} HCl, the most acidic condition. In contrast, the weak and volatile organic acids provided lower extraction efficiencies. Having optimized the extraction-mixture composition, the next step was the optimization of the incubation times. Figure 3 shows a plot of the recoveries as a function of the incubation time. As can be observed, for lower incubation times the recoveries tended to increase, reaching a maximum (approximately 100 %) for all the HAlks at 6 h and minimum (approximately 40 %) for harmol at 0.66 h (40 min). After more than 6 h incubation (e.g. 12 or 24 h), recoveries significantly decreased in all cases. In addition to the highest recoveries, an incubation time of 6 h provided higher consistency between the recoveries obtained for the different analytes and higher accuracy, as can be observed from the small standard deviations shown as error bars in Fig. 3.

CE-MS analyses of compounds present at very low levels in real samples require elimination of all potential interferences from the matrix. The main reason is to prevent lack of reproducibility caused by adsorption of matrix components on the inner capillary wall and generation of spray instability or even breakdown [34]. For small analytes, a typical procedure consists of removing the high-molecular-mass compounds by centrifugal filtration using 3 KDa MWCO filters. However, the filtration step must be evaluated to ensure it does not reduce recovery efficiencies. Experiments based on analysis of standard solutions and samples revealed that ultrafiltration maintained the recoveries of approximately 100 % for the different HAlks and also lead to cleaner electropherograms. Therefore, an ultrafiltration step was included in the standard procedure after the extraction.

Figure 4a shows the extracted ion electropherograms (EIE) of harmalol (201.0 *m*/*z*) for (i) a 0.1 μg mL^{-1} standard mixture of the six HAlks, (ii) a wakame extract spiked at 0.1 μg mL^{-1} with the standard mixture, and (iii) a wakame extract. As can be seen, the electrophoretic profile of the wakame extract is complex, but the presence of harmalol can be easily detected from comparison of the three electropherograms. Furthermore, as observed in Fig. 4b, c, which show the full-scan and MS–MS mass spectra of the electrophoretic peak corresponding to harmalol in the wakame extract (Fig. 4a, electropherogram iii), the MS information and comparison of spectra confirm the identification. The full-scan mass spectrum (Fig. 4b) shows the molecular ion of harmalol with the characteristic isotopic distribution of a singly charged ion, and the MS–MS mass spectrum (Fig. 4c) contains the molecular ion of a characteristic fragment (the MS–MS transitions observed for the other HAlks are indicated in the Fig. 4 caption).

Analysis of HAlks in wakame samples

Once the sample pretreatment was optimized, validation of the complete procedure was performed by means of the standard-addition calibration method to study possible matrix effects. Repeatability was characterized by the standard deviation (S.D.) of a ten-replicate batch analyzed on the same day. Reproducibility, or inter-day repeatability, was also characterized, by means of the S.D. between batches obtained on three different days. These experiments were performed on sample matrixes spiked with the analytes, and the quality data are summarized in Table 1. As can be observed, the linearity

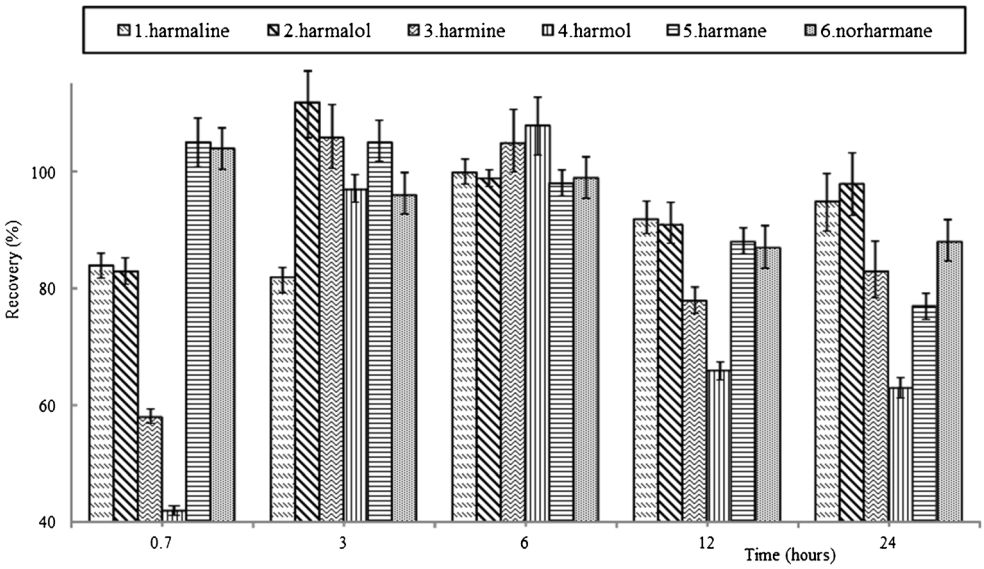

Fig. 3 Recoveries of HAlks at different incubation times, calculated by spiking the extract at a concentration of 1 μg mL^{-1} before and after sample incubation. (Extraction mixture: 50 % (*v*/*v*) methanol and 3.5 mol L^{-1} HCl. Extraction temperature: 80 °C). At 6 h the dispersion of the average value is represented with vertical bars ranging from −*s* to +*s*, *s* being the standard deviation

ranges were slightly different from the values obtained with the external calibration curve, and the LODs and LOQs were approximately two times higher. Moreover, the sensitivity given by the linear regression slopes clearly decreased (Table 1). Additionally, the statistical comparison of the slopes obtained in the calibration curves of each different HAlk revealed significant differences between the six harmala alkaloids. These results indicated a matrix effect on the HAlks, which needed to be addressed to obtain accurate quantification in wakame samples. With regard to peak-area and migration-time repeatability and reproducibility, the values were again good and were in the range 6.7–12 % and 8.1–12 % for peak areas and 0.8–1.2 % and 2.5–3.4 % for migration times, respectively, which were similar to those obtained before with the external calibration curve.

The quantification of the six HAlks in the wakame samples from three different manufacturers, two from Galicia (Spain) (wakames 1 and 2) and one from Brittany (France) (wakame 3), was performed taking into account the above-mentioned matrix effects and hence using the standard-addition calibration method. Three of the six studied HAlks were detected in the wakame samples (i.e. harmalol, harmaline, and harmine). As explained before for harmalol (Fig. 4), the full-scan mass spectra and MS–MS spectra of harmaline and harmine were useful to unambiguously confirm their presence in the sample before quantification. Only harmalol was found at a similar concentration in the samples from the three different manufacturers ($c_{average}$±S.D.: 24±2, 24±1, and 22±1 μg kg^{-1} dry algae). Harmaline (17±1 μg kg^{-1} dry algae, wakame 2) and harmine (7±1 μg kg^{-1} dry algae, wakame 1), were only found in some of the wakame samples from the Atlantic coast of Galicia.

The results revealed differences in the alkaloid content depending on the wakame sample. Another interesting finding

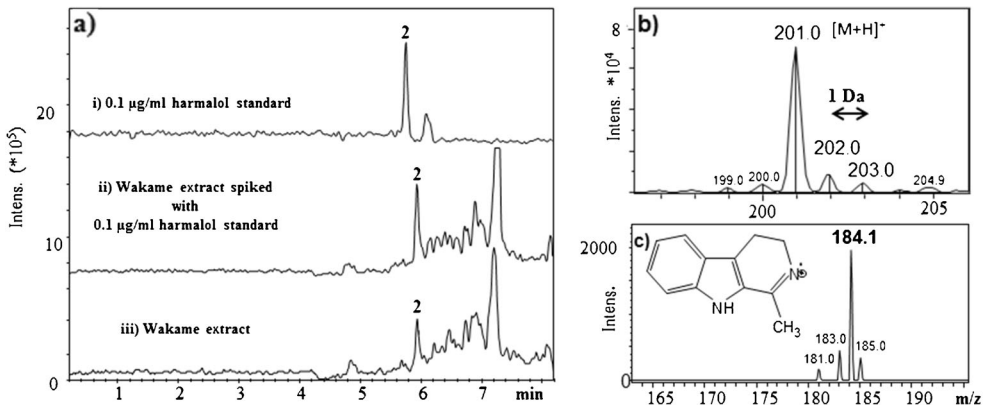

Fig. 4 (**a**) EIE of harmalol (201.0*m*/*z*) when analyzing (**i**) 0.1 μg mL^{-1} standard mixture solution of the six HAlks, (**ii**) wakame 1 extract solution spiked at 0.1 μg mL^{-1} with the standard mixture solution, and (**iii**) wakame 1 extract solution. (**b**) Full scan and (**c**) MS–MS mass spectra of the electrophoretic peak corresponding to harmalol from the analysis of the wakame extract solution (**iii**). The MS–MS transitions confirming the HAlks were (in *m*/*z*): (1) harmaline 215.1>173.9, (2) harmalol 201.0>184.1, (3) harmine 213.2>198.0, (4) harmol 199.1>171.0, (5) harmane 183.2>167.1, (6) norharmane 169.1>N/D

was that harmalol was the main HAlk in all cases, when it is widely accepted that harmalol and harmol are the main metabolites of harmine, harmaline, and harmane [7, 21, 22]. The concentrations of harmalol, harmaline, and harmine found in wakame were very different from the values that can be found in seeds of *Peganum harmala* (Syrian rue) (0.6 % harmalol, 0.003 % harmol, 5.6 % harmaline, and 4.3 % harmine (*m*/*m*)) [4] or in an Ayahuasca tea (0.06 mg harmaline mL^{-1} and 0.90 mg harmine mL^{-1}) [7], that is typically prepared from the cortex and stems of *Banisteriopsis caapi* and leaves of *Psychotria viridis* (containing the hallucinogen *N*,*N*-dimethyltryptamine (DMT)). Taking into account that maximum-daily-intake (MDI) values for HAlks have not been defined, and the typical MDI for harmane and norharmane indicated before, the alkaloid contents found are low enough to indicate that regular wakame consumption is safe for adults. Some manufacturers recommend a maximum intake of 1–2 g dry wakame per day on the basis of the presence of other bioactive components or potential contaminants (iodine, heavy metals, arsenic, etc.).

The concentrations obtained were lower than the environmental and dietary exposures to harmane and norharmane resulting from cigarette smoking or coffee drinking, but greater than the levels reported under some conditions for meat. Levels of norharmane and harmane were found to be up to 9.34 and 1.41 μg g^{-1}, respectively, in ground and instant coffee and up to 9 and 3 μg g^{-1}, respectively, per cigarette in sidestream smoke from filter cigarettes [10, 12]. In meat extracts, concentrations are up to several hundred ng g^{-1} for both substances [10]. Safe exposure levels for HAlks have not been defined, but maximum daily intake (MDI) of the main HAlks has been estimated to be 4 and 1 μg kg^{-1} body weight for norharmane and harmane, respectively [13].

Conclusions

In this work, a CE-MS method for separation of six HAlks in less than 8 min was successfully developed and validated. The identity of the different compounds was confirmed from their MS–MS characteristic fragmentation patterns. The method was applied to the determination of HAlks in commercial wakame (*Undaria pinnatifida*) samples, after optimizing a high-recovery extraction sample pretreatment. Using the standard-addition calibration method, the existence of matrix effects in the determination of these alkaloids was proved. The validated method results in LODs ranging from 1.5 to 10 ng mL^{-1} for these samples, with excellent repeatability and reproducibility. Harmalol, harmine, and harmaline were present in natural wakame at concentrations ranging from 7 to 24 μg kg^{-1} of dry algae. Further work must be done to fully characterize and quantify the major and minor bioactive components of modern foodstuffs in the western diet, including algae, to manage safety concerns and other problems related to public health resulting from the recreational use of some plant extracts.

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