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Comparison of UV absorption and electrospray mass spectrometry for the high-performance liquid chromatographic determination of domoic acid in shellfish and biological samples

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ABSTRACT

Domoic acid, a neurotoxic amino acid produced by the marine diatom *Nitchia pungens* multiseries, was determined in samples of anchovies, razor clams, mussels, crab, rat serum, urine and feces by HPLC with UV absorption and electrospray (ESI) mass spectrometric (MS) detection. Shellfish samples were extracted with methanol–water followed by clean-up of the extracts with solid-phase extraction cartridges (strong anion or strong cation exchange). An aliquot of the fraction containing the domoic acid was analysed by HPLC. HPLC column size, mobile phase composition and flow-rate were selected so that essentially the same conditions could be used for both HPLC–UV and HPLC–ESI-MS with selected ion monitoring (SIM) determinations. These included the use of acetonitrile–water–formic acid as the mobile phase, at a flow-rate of 0.2 ml/min (split 13:1 for HPLC–ESI-MS-SIM, 10 μl/min to the mass spectrometer). The results indicated that extracts found positive by the HPLC–UV method could be readily confirmed directly by HPLC–ESI-MS-SIM without additional sample treatment down to levels of 0.1 μg/g of domoic acid. This study demonstrates the use of HPLC–ESI-MS-SIM for the routine confirmation of domoic acid in a wide variety of samples.

INTRODUCTION

Domoic acid (Fig. 1) is a neurotoxic amino acid which was first isolated from the red alga *Chondria armata* [1,2]. It was identified as the toxic substance in mussels from eastern Canada that caused a poisoning episode resulting in several deaths [3]. Since then, several methods for the determination of domoic acid in shellfish and plankton have been reported [4–6], all involving HPLC with UV absorption detection. Several chemical confirmatory procedures have also been developed [7–9] based on the formation of either UV-absorbing or fluorescent derivatives.

The sample preparation procedure for domoic acid in shellfish is fairly simple, requiring minimal sample clean-up after the initial extraction [4,5,10,11]. This is more than adequate to enforce the guideline level of 20 μg/g (ppm) domoic acid in shellfish set by the Canadian Department of Health and Welfare. However, for certain applications these methods may not be adequate. For example, more sensitive methods might be useful for studying the uptake of domoic acid by bivalve molluscs, in toxicology feeding studies or for the prediction of potential accumulation of domoic acid in shellfish well in advance of any serious contamination. During the course of some research on the chemical

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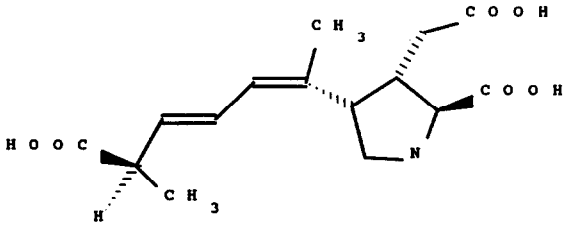
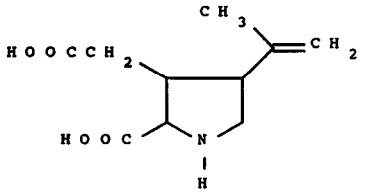


Domoic Acid



Kainic Acid

Fig. 1. Structures of domoic acid and kainic acid.

confirmation of domoic acid, we found that the use of two solid-phase extraction (SPE) cartridges (an anion-exchange followed by a reversed-phase cartridge) was very effective in removing co-extracted material which interfered in the derivatization reactions [7,9]. The strong anion-exchange clean-up procedure developed by Quilliam *et al.* [6] is even simpler and applicable to most shellfish samples for the direct determination of domoic acid at sub-μg/g levels. The chemical derivatization procedures, although useful, do not provide unequivocal proof that domoic acid is present in a sample. In this respect, HPLC–mass spectrometry (MS) has a significant advantage. Domoic acid and other seafood toxins have been studied by Quilliam and Pleasance's group [12–15] using HPLC–electrospray (ESI) MS. Their work clearly demonstrated the potential of the technique for the confirmation of domoic acid and other seafood toxins in shellfish extracts.

In this paper we describe investigations into the use of HPLC–ESI-MS for the routine confirmation of domoic acid in a variety of sample extracts. The aim was to determine how easily and quickly the confirmation could be made and to compare the results with values obtained by HPLC–UV analysis. The timeliness of HPLC–ESI-MS confirmation is particularly important in cases where regulatory guidelines are exceeded or human illness is involved so that appropriate action can be taken quickly.

EXPERIMENTAL

Reagents

Domoic acid standard solutions were prepared in doubly deionized water from a calibration solution (DACS, 0.89 μg/ml; National Research Council of Canada, Halifax, Canada). All solvents and chemicals were of HPLC or analytical-reagent grade. All solutions of standards and samples were refrigerated when not in use.

Liquid chromatography–UV detection

The HPLC system consisted of an Eldex Model 9600 ternary gradient pump, a Rheodyne Model 8125 injector with a 20-μl sample loop, a reversed-phase LC-18 (Supelco) column (150 × 2.1 mm I.D., 5 μm) and a diode array detector (Hewlett-Packard Model 1040A) set to monitor at 242 nm. The mobile phase consisted of 0.2% (v/v) formic acid plus 12% (v/v) acetonitrile in water (pH 3.0). The flow-rate was set at 0.5 ml/min.

Liquid chromatography–mass spectrometry

The HPLC system consisted of a Beckman Model 112 pump, a Rheodyne Model 8125 injector with a 20-μl (for seafood) or a 50-μl loop (for serum, urine and feces) and a reversed-phase Deltabond ODS (Keystone) column (200 × 2.1 mm I.D., 5 μm). The mobile phase consisted of 0.1% (v/v) formic acid + 12% (v/v) acetonitrile in water and was pumped isocratically at a flow-rate of 0.20 ml/min. In order to accommodate the low flow-rate of the electrospray interface, the LC effluent was split. A splitter was constructed by connecting two pieces of fused-silica tubing with different internal diameters (0.12 and 0.35 mm I.D.) to a tee-union (Valco). The length of each tube was adjusted to facilitate a suitable splitting ratio of between 5:1 and 13:1. The end of the larger diameter tubing was connected to a UV detector (Micromeritics Model 788) operating at a wavelength of 254 nm such that UV chromatograms

could be concurrently recorded. The smaller-diameter tubing carrying a flow-rate of 40–10 μl/min was directly connected to the pneumatic-assisted electrospray interface, which was constructed in our laboratory with a similar design to a commercial model (API 3; Sciex, Toronto, Canada). As shown in Fig. 2, a zero-dead-volume tee-union (Valco) was used to hold two pieces of coaxial stainless-steel tubing, the inner 27-gauge (0.205 mm I.D. × 0.406 mm O.D.) tubing was 45 mm long and the outer 21-gauge (0.406 mm I.D. × 0.813 mm O.D.) tubing was 37 mm long. The inner tubing protruded *ca.* 1 mm from the tip of the outer stainless-steel tubing. The 0.17 mm O.D. (0.12 mm I.D.) fused-silica tubing delivering the LC effluent passed through the inner stainless-steel tubing and protruded 0.5 mm from its tip. A nitrogen nebulizing gas [40 p.s.i. (275.8 kPa)] was delivered through the side-arm of the tee-union such that it flowed through the gap between the inner and outer stainless-steel tubing. The tee-union was connected to a high-voltage power supply (Bertan Series 125) with a limiting current of 50 μA. The optimum voltage was usually 5–6 kV. The splitter and the electrospray probe assembly were mounted on a solid PTFE block [or other electrical insulation material, about 1/4 in. (0.635 cm) thick] which in turn was mounted on a three-dimensional micro-manipulator (Brinkman) so that the position of the electrospray probe could be reproducibly adjusted in the x, y and z directions. The position of the electrospray probe tip was about 1–2 cm away from the counter electrode (*i.e.*, the atmospheric pressure ion source "interface plate") and was 0.5–1 cm off-axis from the ion-entrance orifice. It was found that the noise could be minimized by setting the angle of the probe to 30–45° with respect to the mass spectrometer axis.

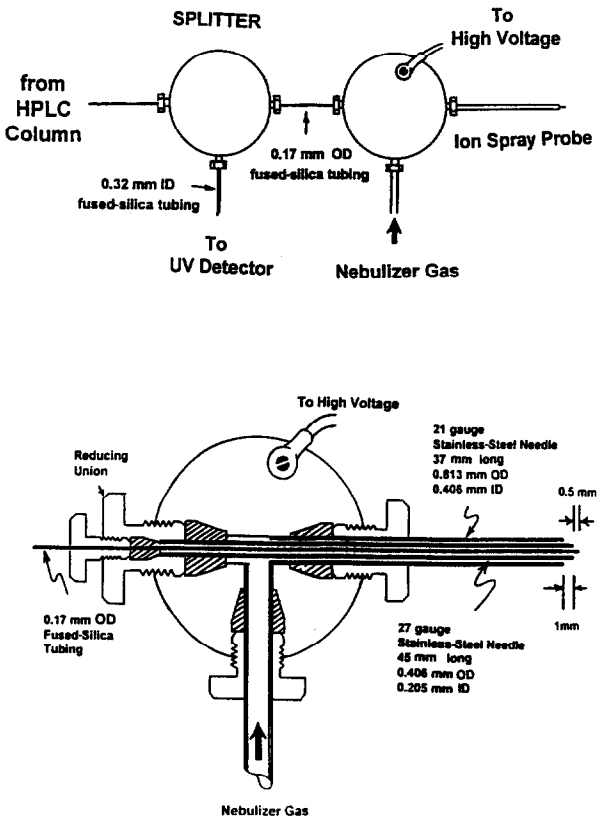


Fig. 2. Top: schematic diagram showing the construction of the electrospray interface. Bottom: details of the probe tip and nebulizer gas connection.

The mass spectrometer employed was a TAGA 6000E triple quadruple mass spectrometer (Sciex) equipped with an atmospheric pressure ionization ion source. It was operated in a single quadruple mode for maximum sensitivity. Daily operating parameters (including the position of the electrospray probe, voltages of focusing lens) were optimized by using arginine as a reference standard. Instrumentation control, data acquisition and data processing were software (Sciex) controlled by a DEC PDP-11 computer. All quantitative analyses were performed in the selected ion monitoring (SIM) mode. Three ions were simultaneously monitored, at m/z 312, 214 and 205, corresponding to the $[M+H]^+$ ion of domoic acid, kainic acid (as an internal standard) and tryptophan, respectively. The dwell time was set at 100 ms for seafood and 750 ms for the serum, feces and urine analyses.

Extraction of shellfish

The extraction procedure was similar to those described elsewhere [6,9] with combinations of methanol and water as the extraction solvent. A 10-g amount of homogenized tissue (Sorval homogenizer) was mixed with 10 ml of deionized water in a 50-ml centrifuge tube for 1 min using a

vortex mixer. A 20-ml volume of methanol was added and the contents were mixed again for 1 min on the vortex mixer. The mixture was centrifuged (5 min at 3000 rpm (700 *g*) or until a clear supernatant was obtained) and the supernatant removed to a clean tube. A 10-ml volume of methanol was added to the residue and the contents were mixed and centrifuged again as mentioned above. The clear supernatant was removed and combined with the first. The final volume was adjusted to 50 ml and represented a sample concentration of 0.2 g/ml.

Preparation of rat serum, urine and feces samples

Serum and urine samples were diluted ten-fold with 50% (v/v) methanol water before SPE clean-up. For each clean-up, 2.0 ml of serum extract (or 2.5 ml of urine extract) were passed through the SPE cartridges. Feces samples (2 g) were extracted using the same volumes of methanol–water (50:50, v/v) as used for the shellfish samples. A 2.0-ml aliquot of the supernatant was used for SPE clean-up.

Strong anion-exchange clean-up

The clean-up is essentially the same as that described elsewhere [6]. A 5-ml volume of shellfish extract (or 2.0 ml of serum extract, 2.5 ml of urine extract or 2.0 ml of feces extract) was passed through a 3-ml Supelclean LC-SAX SPE cartridge (Supelco, USA) [which was preconditioned with 6 ml of methanol followed by 3 ml of deionized water and 3 ml of methanol–water (50:50, v/v)]. The effluent was discarded and the cartridge washed with 5 ml of acetonitrile–water (10:90, v/v), which was also discarded. Domoic acid was eluted with 3 ml of acetonitrile–water–formic acid (10:88:2, v/v/v). A 20-μl volume of this solution was analysed by HPLC.

Strong cation-exchange clean-up

This clean-up procedure has been described elsewhere [7]. An aliquot of sample extract (acidified to pH 3–4) was added to a 3-ml phenylsulfonic acid strong cation-exchange (SCX) SPE cartridge (Bond Elut SCX, Baker, USA) (pre-conditioned with 6 ml of methanol followed by 6 ml of 0.1 *M* hydrochloric acid) and the effluent discarded. The cartridge was washed with 3 ml of deionized water and the effluent discarded. Domoic acid was eluted with 6 ml of 0.7 *M* hydrochloric acid directly on to a 3-ml reversed-phase C_{18} SPE cartridge (Baker) (preconditioned with 6 ml of methanol followed by 6 ml of 0.7 *M* hydrochloric acid). All acid passing through the reversed-phase SPE cartridge was discarded and the cartridge was then washed with 3 ml of deionized water which was discarded. Domoic acid was eluted with 4 ml of 20% (v/v) acetonitrile in 1% (v/v) aqueous acetic acid. A 20-μl volume of this solution was injected into the HPLC system.

RESULTS AND DISCUSSION

The methanol–water extraction worked well for all sample types examined. Some adjustments to the sample mass:extraction solvent ratios were required to obtain extracts suitable for clean-up with both the urine and feces samples. Recoveries from both spiked and naturally incurred shellfish samples were usually greater than 90% over a range of 0.2–40 μg/g domoic acid with good repeatability similar to that observed earlier for shellfish [12]. The recoveries at the 1.0 μg/g level from spiked urine (84%) and feces (86%) were lower. There were some differences in the effectiveness of the two SPE clean-up procedures employed for HPLC with UV detection. Both performed well for all seafood samples (mussels, razor clams, crab meat, anchovies) and the serum samples studied (detection limits were in the range 0.05–0.1 μg/g). The SAX SPE procedure was used for these samples on a regular basis as only one SPE cartridge was required as opposed to two cartridges for the SCX SPE clean-up. However, the SAX SPE clean-up was not as effective as the SCX SPE clean-up for urine and feces samples. Fig. 3 compares chromatographic results for extracts of rat feces spiked at 1 μg/g using the SCX clean-up. The extracts cleaned up with SAX SPE required a modification of the mobile phase (from 12 to 10% acetonitrile, see Experimental) to separate domoic acid from interfering co-extractives in both feces and urine samples. Detection limits for urine and feces

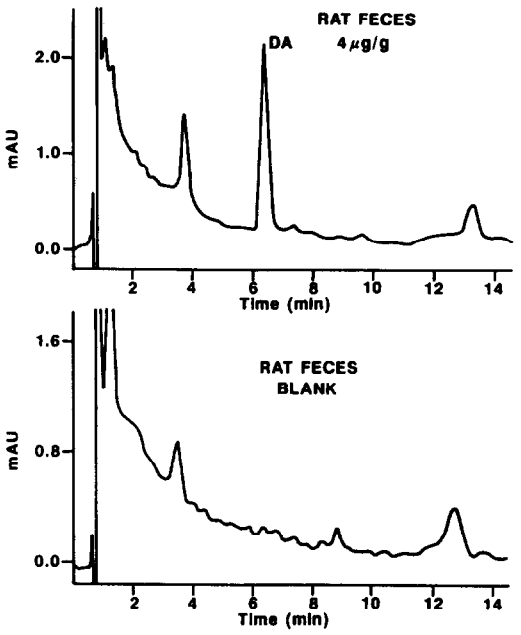


Fig. 3. Chromatograms of extracts of blank and spiked (1 μg/g domoic acid) rat feces samples cleaned up using SCX/C_{18} SPE cartridges. DA = Domoic acid.

samples were about 0.1–0.2 μg/g with the SCX cleanup.

Fig. 4 shows the electrospray mass spectrum of domoic acid in the positive-ion mode. Similar to that reported by Quilliam *et al.* [12], the electrospray mass spectrum displays a large peak at *m/z* 312 that arises from the protonated molecule and no significant fragmentation. Sodium attachment produces an ion at *m/z* 334. The dominant protonated molecule was chosen in the selected ion monitoring.

The use of kainic acid as an internal standard proved to be useful in providing more reliable quantitative results. Although it is not an ideal compound (the first choice would be a stable isotopically labeled domoic acid, but none is commercially available) and it does not share a common structure with domoic acid (compare the structures in Fig. 1). Its availability in our laboratory was the prime reason why we chose this compound as an internal standard to compensate for variations in the splitting ratio of the LC effluent. Fig. 5 shows the SIM responses from an injection containing 38.5 ng of domoic acid and 50 ng of kainic acid, using a dwell time of 100 ms for each ion. The minimum detection limit (MDL, based on an *S/N* ratio of 5:1) for the domoic acid under these conditions was estimated (by extrapolation) to be 1.5 ng per injection or equivalent to 0.1 μg/g in the samples. Extending the dwell time to 750 ms in later serum, urine and feces analyses improved the *S/N* ratio by a factor of 4–6 thus routinely attaining the MDL at 0.4–0.3-ng levels (some-

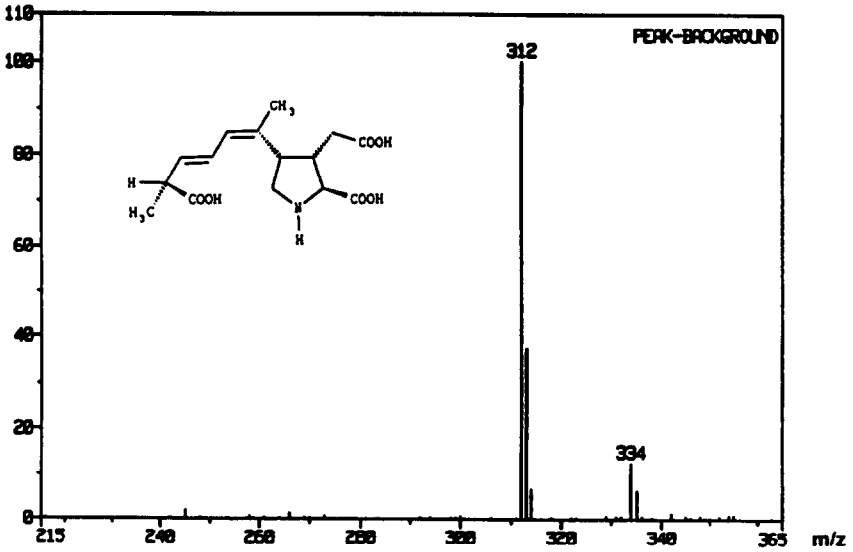


Fig. 4. Electrospray mass spectrum of domoic acid in the positive-ion mode. Scanning range: *m/z* 100–400. Y-axis, relative intensity, %.

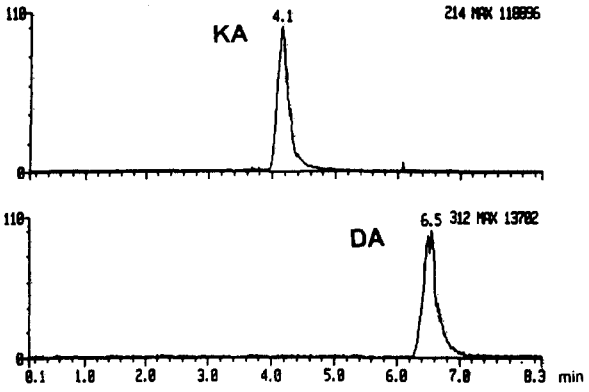


Fig. 5. SIM mass chromatograms of an injection containing 38.5 ng domoic acid (DA) and 50 ng kainic acid (KA) using a dwell time of 100 ms. Injecton volume, 20 μl. Y-axis, relative intensity, %.

times even down to 0.1 ng). When a splitting ratio of 10–15:1 was employed, absolute minimum detection limits at the low-picogram level could be achieved. This compares favorably with that reported by Quilliam *et al.* [12] using a commercial version of an electrospray interface and a newly improved mass spectrometer (Sciex Model API 3). The calibration graph showed linear responses within the working concentration range (1–140 ng). The same linear range was observed for the HPLC–UV analyses.

Table I compares results obtained by HPLC–UV detection with those obtained by HPLC–ESI-MS-SIM for a variety of sample extracts. As can be seen, the agreement between the two techniques is good over more than a 100-fold concentration range (0.4–41.2 μg/g of domoic acid). Also, the analyses were carried out over several days, indicating a good day-to-day correlation betweeen the two methods. The HPLC–ESI-MS-SIM results clearly confirm the findings obtained by HPLC–UV detection. Figs. 6 and 7 compare chromatograms for razor clams and crab meat obtained with the two detection methods. The first example shows a sample contaminated at the level of 16.5 μg/g near the Canadian guideline of 20 μg/g, whereas the second

TABLE I

COMPARISON OF RESULTS OBTAINED BY HPLC–UV AND HPLC–ESI-MS-SIM METHODS FOR DOMOIC ACID IN BIOLOGICAL SAMPLES

Sample	Domoic acid (μg/g)	
	HPLC–UV[a]	HPLC–ESI-MS-SIM[a]
Razor clam A[b]	41.2	43.1
Razor clam B[b]	16.6	16.5
Anchovy[b]	10.5	12.6
Crab meat[b]	0.4	0.5
Blank mussel (Prince Edward Island, Canada)	ND[c]	ND[c]
Blank mussel (Nova Scotia, Canada)	ND	ND
Spiked mussel (Prince Edward Island, Canada)	6.1	6.5
Spiked mussel (Nova Scotia, Canada)	5.1	6.2
Blank urine	ND	ND
Spiked urine A	3.7	2.7
Spiked urine B	0.4	0.3
Blank feces	ND	ND
Spiked feces A	2.7	3.4
Spiked feces B	0.6	0.7
Blank serum	ND	ND
Spiked serum A	8.5	12.5
Spiked serum B	0.4	0.5

[a] Single determinations.
[b] Naturally incurred residues.
[c] ND = Not detected.

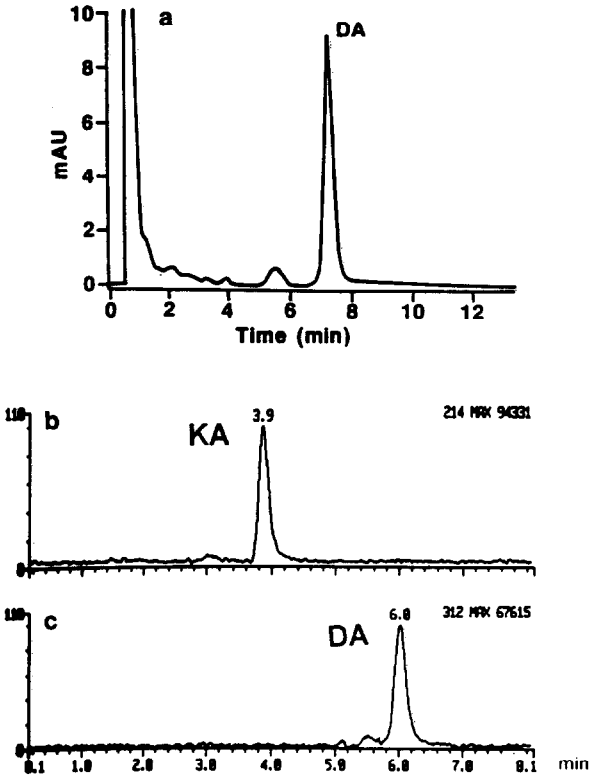


Fig. 6. Comparison of chromatograms from (a) HPLC–UV detection and (b, c) HPLC–ESI-MS-SIM analyses of a contaminated razor clam sample (16.5 μg/g). Injecton volume, 20 μl; dwell time, 100 ms. KA = Kainic acid; DA = domoic acid. Y-axis, relative intensity, % (b and c).

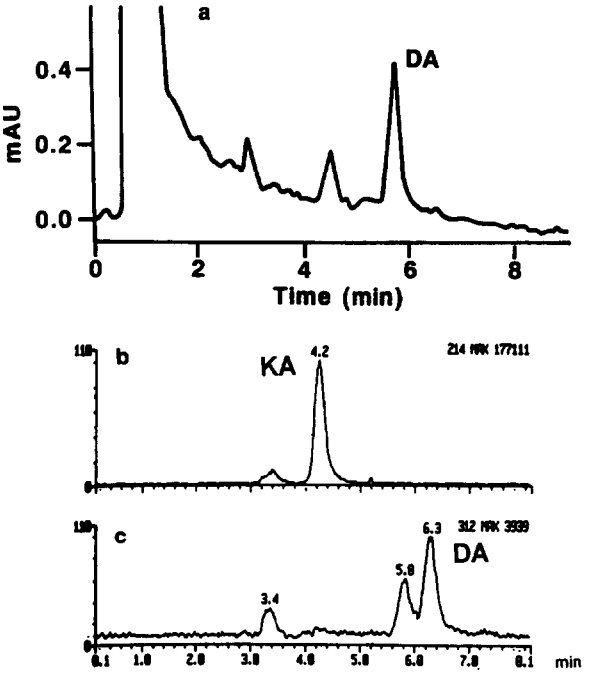


Fig. 7. Comparison of chromatograms from (a) HPLC–UV detection and (b, c) HPLC–ESI-MS-SIM analyses of a contaminated crab meat sample (0.53 μg/g). Injecton volume, 20 μl; dwell time, 100 ms. KA = Kainic acid; DA = domoic acid. Y-axis, relative intensity, % (b and c).

example (contaminated with 0.53 μg/g of domoic acid) shows that contamination even 40 times below the guideline can be easily detected and confirmed by the HPLC–ESI-MS-SIM method. Minor interferences become more apparent at low levels. It is not certain whether or not the small peaks (retention times 3.4 and 5.8 min in Fig. 7) that eluted before the domoic acid (retention time 6.3 min) are isomers of domoic acid, although several domoic acid isomers have been previously reported to be present in shellfish and plankton [12]. Perhaps MS–MS will be helpful in establishing the identity of these peaks.

Extending the analysis of domoic acid to biological samples is beneficial in the area of toxicological research but it is also more demanding in the development of analytical methodology. Both the sensitivity and selectivity requirements are usually higher owing to the lower concentrations and the more complex nature of the sample matrix. In both respects, HPLC–ESI-MS-SIM proves to be a valuable tool in these applications. For example, tryptophan is known to be a potential interferent with UV detection especially if no SPE clean-up is employed. However, the compound is easily distinguished from domoic acid by MS because of their mass differences. Monitoring of this compound was added in the later analyses of monkey urine, serum and feces by HPLC–ESI-MS-SIM. It was found that the matrix from urine and feces imposed no particular problem during the MS determination. As shown in Fig. 8, ng/g levels can be easily detected. However, serum samples appear to contain a high concentration of some strong ionic compounds that eluted earlier than the kainic acid and domoic acid. The ion current was so strong that it exceeded the limiting current and automatically shut off the high-voltage power supply, as indicated by the negative

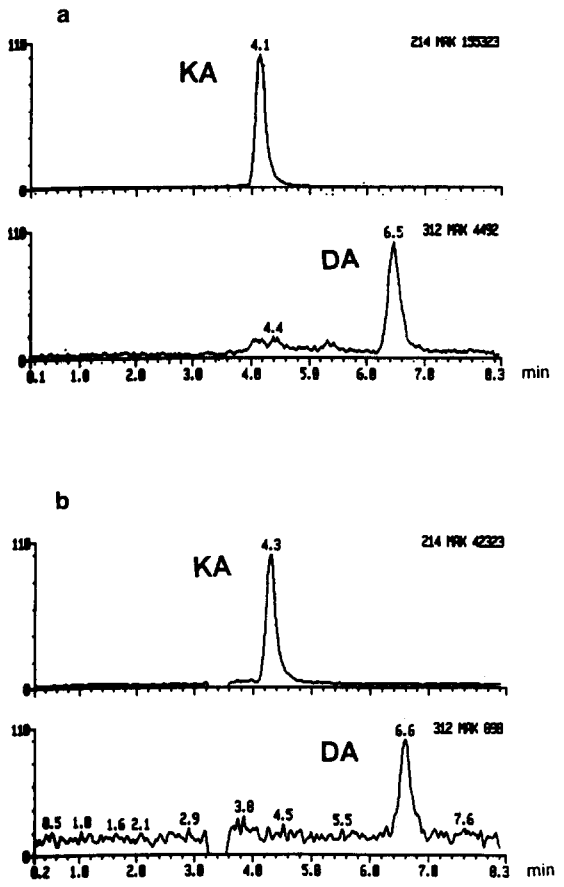


Fig. 8. SIM mass chromatograms from (a) a spiked rat urine sample (0.18 ppm) and (b) a spiked rat serum sample (0.14 ppm). Injection volume, 50 μl; dwell time, 750 ms. KA = Kainic acid; DA = domoic acid. Y-axis, relative intensity, %.

dip in the mass chromatogram in Fig. 8. A manual reset of the high-voltage power supply was required to restore the high voltage on the electrospray probe before the elution of the analytes of interest. It is not certain whether the tailing of these strong electrolytes has any effect on the ionization of the later-eluting kainic acid. If so, it may lower the response of the kainic acid and thus affect the domoic acid determination.

CONCLUSIONS

The results presented in Table I clearly demonstrate that MS confirmation is particularly useful at levels near the detection limit of the HPLC–UV method, as unambiguous results were obtained by the former owing to the substantially greater selectivity and somewhat better sensitivity. It has been successfully applied to the analysis of various seafood and biological samples.

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