RECENT UPDATES OF HPLC-UV AND LC-MS METHODS FOR DOMOIC ACID DETECTION: A COMPARISON REVIEW

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Abstract

Shellfish poisoning is caused by the consumption of molluscan shellfish meat contaminated with marine biotoxins produced by diatoms or dinoflagellate. Under European legislation, domoic acid (DA), the main constituent of amnesic shellfish poisoning, is monitored to protect the shellfish consumer.

The aim of this study is to compare most often used analytical methods for domoic acid determination. In this regard extraction procedures, chromatographic conditions and quantitation results are commented. Phycotoxin profiling of mussels and plankton from Bulgarian coast is planned. This detailed review is focused on DA detection methods as it is the most common detected toxin in investigated area up to now. DA is analyzed using either the HPLC-UV method of Quilliam et al. (1995) or LC with mass spectrometric (MS) detection.

Reviewing published data in recent years on both methods and quantitation results allows a comparison between them to be made. The HPLC-UV method detection limits ranged between 0.1 and 1 μg of DA for gram of tissue, depending on the sensitivity of the UV detector. HPLC-UV method is recommended by the European Commission as the reference method. Main disadvantage of this method is the labor-intensive purification step which is required for decreasing the matrix effect. LC with mass spectrometric (MS) detection is extensively used worldwide for DA allowing both effective seafood control and monitoring of phytoplankton in terms of the simultaneous analysis of different groups of marine biotoxins including domoic acid. For ensuring consumer protection uptake, distribution, transformation, and elimination of ASP phenomena should be studied. This requires very sensitive and selective analytical methods for DA determination. Although it is a high cost technique LC-MS guarantees better sensitivity but also a great selectivity on the determination of DA in the mussel tissue.

Keywords: *shellfish poisoning, domoic acid, pectenotoxin-2, yessotoxin, mussels*

1. INTRODUCTION

Seafood products are an excellent food with important nutritional value (Merdzhanova, et al., 2016; Stancheva, et al., 2017), but in certain circumstances, represent a suitable substrate for the proliferation of microorganisms pathogens (Stoica, 2014; Yilmaz & Bilgin, 2005; Kacar, 2011; Pomykala, et al., 2012). In addition, through the food chain, they can accumulate and metabolize various types of molecules, more or less complex, known by the name of marine biotoxins (Visciano, et al., 2016). The accumulation of biotoxins by humans through consumption of contaminated shellfish products can lead to different disorders, indicated as shellfish poisoning (Deeds, et al., 2008).

Amnesic shellfish poisoning (ASP) is caused by consumption of shellfish that have accumulated domoic acid, a neurotoxin produced by some strains of phytoplankton (Jeffery, et al., 2004). Clinical signs of acute ASP in humans consist of gastrointestinal distress, confusion, disorientation, seizures, permanent short-term memory loss, and in the most severe cases death (Perl, et al., 1990). Recently discussed is the repetitive exposure to low doses of DA. These are DA levels below those that cause signs of intoxication listed above. Increased toxin susceptibility and impaired mitochondrial function in laboratory studies, as well as potential memory deficits in humans among others are signs linked with chronic low level DA exposure (Grattan, et al., 2016; Hiolski, et al., 2014; Lefebvre, et al., 2012).

Marine toxins can cause human intoxications and important economic losses in seafood-producing areas being considered as a food safety risk. To avoid this, regulatory authorities fix maximum levels of these compounds in mollusks and fish flesh to prevent unsafe products being placed in the market. Control systems include accurate analytical methods to officially detect and quantify toxins. These methods are considered as reference, although alternative analysis can be used if they provide equivalent levels of protection. Since 2004 DA is regulated in EU and a threshold of 20 mg kg^{-1} shellfish meat is set (EC, 2004). HPLC-UV is recognized as official method for DA determination in shellfish (EC, 2005). Routine testing involves homogenization, extraction and chromatographic analysis (Saeed, et al., 2017).

Many updates and improvements of the official method are published resulting in significant time and costs savings for testing labs. Most significant contribution of these innovations is lowering the detection limit. This advantage is important in sense of monitoring not only the acute but also chronic exposure to domoic acid in food consumed.

Aim of this study is to review and compare methods currently used to analyze and quantify domoic acid in shellfish as well as in seawater (phytoplankton samples) as the latter are known to produce the neurotoxin.

2. MATERIALS AND METHODS

The review is based on scientific papers published in referenced journals in the last ten years. A background information for the review is provided and a summary of the types of analytical methods under development and currently being used in domoic acid analysis field. Literature searches were undertaken to collate information on:

- Sample preparation techniques
- Chromatographic methods on domoic acid determination

Literature searches began with a structured electronic search using the following key words:

- Domoic acid, ASP;
- Mussel, shellfish, seawater, plankton
- Extraction, clean-up
- Analytical methods, chromatographic determination, HPLC-UV, LC-MS/(MS) and combinations of them.

Papers that were identified through electronic searching were assessed for relevance by initially reviewing the abstracts. Additional papers were assessed using the reference list of reviewed publications, and through searches that used slight variations on the above keywords (McLeod, et al., 2015). Furthermore, only papers are included that provide information at least of one of the set performance parameters: extraction step, purification step, chromatographic determination details, LOD and/or LOQ, information on DA concentration determined.

3. RESULTS AND DISCUSSION

3.1. Background information

Analytical methods for marine toxins generally involve a liquid chromatographic (LC) step that separates mixtures of toxin congeners from each other and the other extractives in shellfish samples using a solvent mixture (mobile phase) flowing through a column packed with a stationary phase. An aliquot of sample extract (or toxin calibration standard) is injected onto the column and the effluent is coupled to a detection system, designed to exploit the spectroscopic properties of the toxins (ultraviolet, fluorometric and/or mass spectrometry) (McLeod, et al., 2015). Main extraction method used is liquid-liquid. This is due to the fact that domoic acid (DA) is a cyclic amino acid with three

carboxylic groups that cause its high polarity and hydrophilicity (Costa, et al., 2010). Methanol aqueous solution (1 : 1) is most often used as extracting agent of the homogenized shellfish meat. In this way, the best recovery and extract stability are achieved (Quilliam, et al., 1995). The main disadvantage is the presence of several interferences, which can introduce false positives. A purification procedure on strong anion exchange (SAX) solid phase extraction (SPE) cartridges is therefore necessary to reduce the errors (Quilliam, et al., 1995). The latter provides selective isolation of domoic acid and related compounds from interfering substances, as well as preconcentration to facilitate analysis of trace levels (Barbaro, et al., 2016).

EU reference method for domoic acid determination and most often used for regulatory purposes is HPLC with UV absorbance detection. Chromatographic conditions include HPLC column: 25 cm long, 4.6 mm i.d. packed with 5–10 µm C18 bonded silica gel, column temperature 40°C; mobile phase flow rate in the range 1.0–1.5 ml min^{-1}; injection volume 20 µl. Retention time of domoic acid should be 7–15 min. HPLC mobile phase is 100 ml acetonitrile, 400 ml water and 1.0 ml trifluoroacetic acid (TFA), diluted to 1 L with water. The detection of domoic acid is facilitated by its strong absorbance at 242 nm (Quilliam, 2003a; Vale & Sampayo, 2001).

Reported HPLC-UV detection limits for domoic acid are about 10–80 ng ml^{-1} or 0.1 and 1 μg DA g^{-1} tissue depending on the sensitivity of the UV detector, the method of extraction and clean-up. If crude extracts (either acidic or aqueous methanol) are analysed without clean-up, the practical limit for quantitation is about 1 mg kg^{-1}. This is suitable for most regulatory laboratories concerned with detecting contamination levels greater than 20 mg kg^{-1} (Quilliam, et al., 1989a).

Some examples of studies using the same chromatographic technique but differing in sample preparation are provided below.

Domoic acid analysis of shellfish samples with strong anion exchange and HPLC-UV determination from the Croatian coast (Arapov, et al., 2016) showed results below the established LOD of 0.1025 µgDA g^{-1} shellfish meat. Peneva et al. (2011) (Peneva, et al., 2011) studied DA presence in Bulgarian and Turkish shellfish without SAX cleanup and with HPLC-UV determination. DA concentration in positive samples ranged 0,02 - 0,55 mg kg^{-1}. DA concentrations in *M. edulis* approaching 2 mg kg^{-1} were found in samples from Scapa Bay, Scottland (Bresnan, et al., 2017). Domoic acid was extracted and quantified by HPLC with UV as described above with minor modifications (Hess, et al., 2001). The method limit of detection was 0.25 µg DA g^{-1}shellfish flesh.

As HPLC-UV is the method most commonly used for routine regulatory purposes, a combined LC-MS method is most desirable for confirmation of peak identity (Quilliam, 1996; Quilliam, et al., 1989b; Lawrence, et al., 1994; Food Safety Authority, 2016). Chromatograms of the two methods are shown on Figure 1.

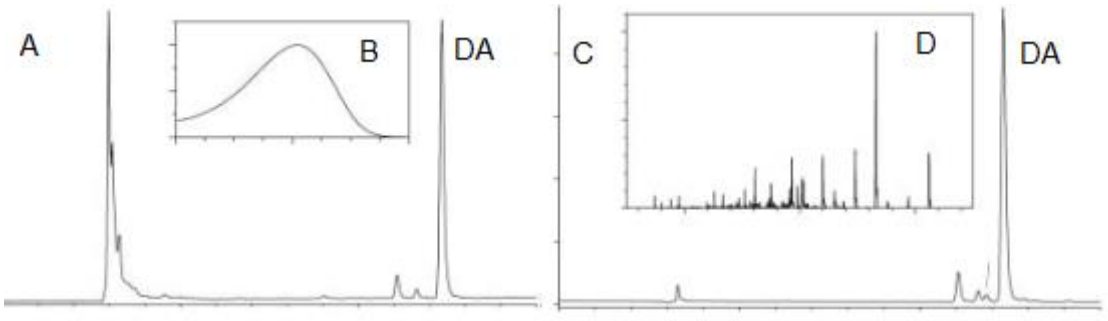

Figure 1. A, HPLC-UV and **C**, LC-MS chromatograms of the SAX-cleaned extracts of a razor clam sample contaminated with 80 mg kg^{-1} domoic acid. Insets B and D show respectively UV and MS-MS spectra of DA (Quilliam, 2003a)

With LC-MS very selective detection of domoic acid and its isomers is possible. Confirmation of identity can be achieved by scanning the mass spectrum with high front-end voltages that induce fragmentation or by performing MS-MS to generate a fragment ion spectrum such as that shown in the inset (Fig. 1 D).

Liquid chromatography coupled with mass spectrometry (LC-MS) guarantees the best sensitivity but also a great selectivity on the determination of DA in the mussel tissue (Quilliam, 2003b; Ciminiello, et al., 2005; Pardo, et al., 2007; Pleasance, et al., 1990). Hess et al. (2001, 2005) compared LC-MS and HPLC-UV, underlining that the latter technique could yield a false positive when the samples contain interfering species. In the literature, one of the main problems in the DA determination of mussel samples is the matrix effect and a labor-intensive anion exchange solid phase extraction is usually carried out for an efficient sample cleanup (Hess, et al., 2001; Hess, et al., 2005).

Sample preparation includes a single dispersive extraction with 20 ml of aqueous 80% methanol (ratio 1:4) at 20,000 rpm with a homogeniser for 1 min. After a 10 min centrifugation at 4000 rpm, the supernatant is filtered into a screw-cap autosampler vial with disposable syringe filter. The equivalent of 1.0 mg extract (5 µl) is injected on the column without any further clean-up (Vale & Sampayo, 2001).

Various LC-MS interfaces for the analysis of ASP toxins have been investigated, including continuous-flow fast atom bombardmen (FAB), thermospray, atmospheric pressure chemical ionization and electrospray interfaces. Electrospray ionization was found to be best suited for analysis of domoic acid (Quilliam, et al., 1989b; Quilliam, 1996; Lawrence, et al., 1994).

The determination of DA carried out by reversed phase liquid chromatography with MS/MS detection. LC is performed on a reversed phase C18 column and LC parameters: temperature – 25◦C, flow0,8 ml/min, run time 25 min gradient. Mass spectrometric detection of DA is performed in the SRM mode with two transitions and MS parameters: 312 > 266 (quantifier), 312 > 161 (qualifier); The LOD (S/N = 3) of DA is 40 pg on-column using an AB Sciex API 2000 triple quadrupole mass spectrometer (Luckas, et al., 2015).

Some examples of LC-MS determination of domoic acid are given below:

DAs were extracted with a water/ methanol mixture (50/50; v/v) and analysed by LC-MS/MS. Mean DA concentrations varied 264.4 - 274.1 ng g^{-1} shellfish meat after censored data treatment (Picot, et al., 2013). Maximum DA concentration in bivalves from estuarine / Laguna production areas in 2003/2006 in Portugal were established in 2004 – 79 mgDA g^{-1} shellfish meat. Determination method used includes LC–MS analysis of methanolic extract (Vale, et al., 2008). Domoic acid was present in all sand crab (*Emerita)* samples and ranged from 2.2 to 23.7 µg g^{-1} of tissue; while only one sample exceeded the 20 ppm (20 µg g^{-1}) FDA and California Department of Public Health imposed quarantine limits for domoic acid toxicity in harvested shellfish (Shanks, et al., 2016).

3.2. Updates in sample preparation

In recent studies several updates in the sample preparation (Table 1) and the detection method (Table 2) were made resulting in lowering the detection limits and time, labor and consumables saving. Tissue homogenates can be extracted with 50% methanol followed by a highly selective strong anion exchange solid phase extraction and a derivatized with dansyl chloride (DNS-Cl) to form the dansyl derivative of domoic acid (DNS-DA). Reaction times were reported very rapid and proceeded under ambient conditions to yield stable derivatives. A study of the collision-induced dissociation of ESI-produced protonated DNS-DA was carried out to identify the most sensitive transitions to use in development of a selected reaction monitoring detection method. Compared with un-derivatized DA, DNS-DA showed a 5-fold increase in sensitivity of MS/MS detection and improved retention on a reversed phase LC stationary phase (Beach, et al., 2016).

Magnetic solid-phase extraction (MSPE) of domoic acid (DA) is an innovative and promising technique. Thereby magnetic microspheres (e.g. with Fe_3O_4@SiO_2@UiO-66 core) are used as sorbents for rapid and efficient extraction of polar analytes from complex biological samples (Zang, et al., 2015). Zhang et al. (2016) demonstrated thet ferrite magnetic nanospheres are promising sorbents for

efficient extraction of highly polar analytes from high ionic strength solutions. A simple, rapid and sensitive method for determination of trace levels of domoic acid (DA) in seawater was developed, based on a magnetic solid-phase extraction (MSPE) followed by high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS). Five kinds of ferrite magnetic nanospheres (MFe_2O_4; M = Fe, Co, Ni, Cu and Zn) were prepared and first used as sorbents for MSPE of DA and removal of salt interference. The developed method showed low limit of detection (2.5 pg mL^{-1}; S/N = 3:1), low limit of quantification (5.0 pg mL^{-1}; S/N = 10:1), and good recoveries (86.0–98.1%) in seawater samples. (Zhang, et al., 2016)

A dual Solid Phase Extraction (SPE) cartridge assembly was applied for the extraction of target compounds from water. Optimized SPE parameters included cartridge material, initial sample pH, sequence of the cartridges in the SPE assembly as well as composition and volume of the elution solvent. The method was validated, providing acceptable mean recoveries and reproducibility for most analytes. Limits of detection were at the ng L^{-1} level (Zarvou, et al., 2017).

Two consecutive extractions with methanol (2 × 10 mL) and no purification steps of a small aliquot of mussel tissue (20 mg) is proposed by Barbaro (2015). Due to a reduction of the extraction of the interfering species and the application of very sensitive and selective hydrophilic interaction liquid chromatography HILIC-MS/MS method. The procedural method was validated through the estimation of trueness, extract yield, precision, detection, and quantification limits of analytical method. The sample preparation was also evaluated through qualitative and quantitative evaluations of the matrix effect. These evaluations were conducted both on the Da-free matrix spiked with known DA concentration and on the reference certified material (RCM). The estimated LOD is very low - 9 ng g^{-1}.

Table 1. Recent updates in sample preparation methods for DA extraction

Sample preparation method	**Reference**	**Matrix**	**Chromatographic method**	**Improvement**
Standard method • the crude extract • clean-up on strong anion exchange (SAX) solid phase extraction (SPE)	(Quilliam, et al., 1995)	Mussels tissue	HPLC-UV	1. extracted from shellfish tissues by homogenization with methanol-water (1:1, v/v) 2. filtration of • the crude extract or • after clean-up on strong anion exchange (SAX) solid phase extraction (SPE) cartridges (manual)
Derivatization with dansyl chloride (DNS-Cl)	(Beach, et al., 2016)	Mussel tissue	LC- MS/MS	good recovery (83% - 107%) quantification of low levels of DA in shellfish as an early warning sign method detection limit of 1.1 μg DA kg^{-1} mussel tissue

Magnetic solid-phase extraction (MSPE) with Fe_3O_4@SiO_2@UiO-66 core	(Zang, et al., 2015)	Mussel tissue	HPLC–MS/MS	low limit of detection (1.45 pg mL^{-1}; S/N = 3:1), low limit of quantification (4.82 pg mL^{-1}; S/N = 10:1),
Ferrite magnetic nanospheres (MFe_2O_4; M = Fe, Co, Ni, Cu and Zn)	(Zhang, et al., 2016)	seawater	HPLC–MS/MS	low limit of detection (2.5 pg mL^{-1}; S/N = 3:1) low limit of quantification (5.0 pg mL^{-1}; S/N = 10:1) good recoveries (86.0–98.1%)
Dual Solid Phase Extraction (SPE) cartridge assembly	(Zarvou, et al., 2017)	Seawater	LC–MS/MS	multi-class cyanobacterial and algal toxins in water Limits of detection -at the ng L^{-1} level
No purification	(Barbaro, et al., 2016)	Mussel tissue	HILIC-MS/MS	Fast, without cleanup steps method detection limit (9 ng g^{-1})

3.3. Improvements in chromatographic determination

Andjelkovic et al. (2012) reported a DA range 0.53–1.67 mg kg^{-1}. Extraction was performed with aqueous 50% methanol and purification through SPE-SAX columns. Liquid chromatography was performed on a HPLC-UV system with C18 column (300×3.9 mm, 10 μm) with isocratic gradient of the mobile phase containing 87% aqueous solution of 8.5% phosphoric acid adjusted to pH 2.5 and 13% of 100% acetonitrile. This solution was prepared freshly before each analysis. The injection volume was 20 μl and analysis time was set at 20 min. Detection wavelength was set at 242 nm. Calibration was performed by a full set of calibration standards of DA (1–25 mg ml^{-1}). Under these conditions, the detection limit was 0.8 μg g^{-1} tissue (Andjelkovic, et al., 2012).

Table 2. Recent updates in chromatographic methods for DA determination

Chromatographic method	**Reference**	**Matrix**	**Modification**	**Advantage**
HPLC-UV	(Andjelkovic, et al., 2012)	mussels	Improved pH by switching TFA with phosphoric acid	• detection limit - 0.8 μg/g tissue
LC-MS/MS	(Krock, et al., 2008)	mussels	C8 phase	• multi-call algal toxins analysis • suitable for plankton samples
LAESI-MS	(Beach, et al., 2016)	shellfish tissue	laser ablation electrospray ionization high-resolution mass spectrometry	• run time of 12 s; • routine, high-throughput screening of DA

LC-MS/MS	(McCarron, et al., 2014)	shellfish	hybrid triple quadrupole linear ion trap mass spectrometer	• reduced amount of sample introduced to the mass spectrometer • multi-call algal toxins analysis • spectral library
LC-TOF/MS	(Rossi, et al., 2016)	shellfish	Time of flight technique	• detection of also isomers of domoic acid • Limit of detection is about 0.00125 mg/kg
HPLC-Fl	(Pocklington, et al., 1990)	seawater	Precolumn derivatization with 9-fluorenylmethyl-chloroformate to form the FMOC derivative	LOD • 0.5 ng ml^{-1} (isocratic elution) • 15 pg ml^{-1} (gradient elution)

Domoic acid was present in all analysed mussel samples from the Black Sea, Bulgaria. Concentration ranged 0.493 – 0.919 μg DA g^{-1} hp. The extraction was performed with methanol and the domoic acid concentration was detected on LC-MS/MS system (Peteva, et al., 2017). Measurements were carried out in positive-ion mode by multiple reaction monitoring (MRM) experiments. Mass spectral experiments were performed on triple-quadrupole mass spectrometer equipped with ion source coupled to LC liquid chromatograph. After injection of 5 μL of sample, separation of toxins was performed by reversed-phase chromatography on a C8 phase. The analytical column (50×2 mm) maintained at 20 °C. The flow rate was 0.2 mL min^{-1} and gradient elution was performed with two eluents, where eluent A was water and eluent B was acetonitrile-water (95:5 v/v), both containing 2.0 mmol L^{-1} ammonium formate and 50 mmol L^{-1} formic acid. The chromatographic run was divided into three periods:

1) 0–8.75 min for domoic acid,
2) 8.75–11.20 min for gymnodimine and spirolides, and
3) 11.20–18 min for okadaic acid, dinophysistoxins, pectenotoxins, yessotoxin, and azaspiracid.

Measurements were carried out in positive-ion mode by multiple reaction monitoring (MRM) experiments (Krock, et al., 2008; Krock, et al., 2007).

The suitability of laser ablation electrospray ionization high-resolution mass spectrometry (LAESI-HRMS) for high-throughput screening or quantitation of DA in a variety of shellfish matrices was assessed. The method was first optimized for use with HRMS detection. Domoic acid was selectively analyzed directly from shellfish tissue homogenates with a run time of 12 s. The limits of detection were between 0.24 and 1.6 mg DA kg^{-1} tissue, similar to those of LC/UV methods. The precision was between 27 and 44% relative standard deviation (RSD), making the technique more suited to screening than direct quantitation. LAESI-MS showed good agreement with LC/UV and LC/MS and was capable of identifying samples above and below 5 mg DA kg^{-1} wet shellfish tissue, one quarter of the regulatory limit. These findings demonstrate the suitability of LAESI-MS for routine, high-throughput screening of DA. This approach could result in significant time savings for regulatory labs carrying out shellfish safety testing on thousands of samples annually (Beach, et al., 2016).

LC/MS methodology for the analysis of domoic acid and lipophilic toxins in shellfish was developed using a hybrid triple quadrupole linear ion trap mass spectrometer. For routine quantitation a scheduled selected reaction monitoring (SRM) method for the analysis of domoic acid, okadaic acid, dinophysistoxins, azaspiracids, pectenotoxins, yessotoxins, gymnodimines, spirolides, and pinnatoxins was developed and validated. The method performed well in terms of LOD, linearity, precision, and trueness. Taking advantage of the high instrument sensitivity, matrix effects were mitigated by reducing the amount of sample introduced to the mass spectrometer. Optionally, samples can be analyzed using information dependent acquisition (IDA) methods, either in positive or negative mode, which can provide an extra level of confirmation by matching the full product ion spectra acquired for a sample with those from a specially constructed spectral library (McCarron, et al., 2014).

Domoic acid (DA) in shellfish is determined by using LC-TOF/MS, which is really sensitive and provides a higher resolution allowing for the detection of also all the isomers of domoic acid. The limit of detection is about 0.00125 mg kg^{-1}. The quantitation limit for domoic acid by this method is, at least, 0.005 mg kg^{-1} (Rossi, et al., 2016).

A very sensitive procedure, based on reaction with 9-fluorenylmethylchloroformate to form the fluorenylmethoxycarbonyl (FMOC) derivative and HPLC analysis with fluorescence detection, has been developed for monitoring of domoic acid in marine matrices such as seawater and phytoplankton (Pocklington, et al., 1990).

HPLC-Fl study from the Marmara Sea (Dursun, et al., 2016) on plankton net samples revealed DA range from 1.16 to 5.25 μg DA ml^{-1} in Dec, 2010 and 0.96–3.85 μg DA ml^{-1} in February, 2011 with limit of detection (LOD) 0.2 ng DA ml^{-1}.

4. CONCLUSIONS

Chromatographic methods are a desirable component of the overall state-of-art framework for marine toxins. Their variety allows a choice depending on the purpose of the study: monitoring, screening, determination of ecological status or establishment of emerging treat.

This study is part of a project which major objective is to determine marine toxins - PSP, ASP and DSP in samples of wild and cultivated Black Sea shellfish and plankton. Very few marine toxins from Bulgarian coast are investigated (Kalinova, et al., 2015) and therefore a multi-toxin method seems to be most useful. The multi-class algal toxin LC-MS/MS method by Krock (2008) is suitable for the purposes of the project, because of the advantages listed, time-test and reliability. Furthermore, the results on domoic acid levels will be used to develop an in-house validated method using available equipment HPLC-UV.

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