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EU REFERENCE METHODS FOR DETECTION OF MARINE BIOTOXINS IN SEA FOOD

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Abstract

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Marine biotoxins can be found in marine shells as mussles, rapana and scallops, and could cause different poisoning symptoms. Black Sea mussel *Mytilus galloprovincialis* is the only one marine aquaculture produced nowadays in Bulgaria. Its safe production has an impact on the market as well on the human health.

This review discusses current analytical methods for those toxins that are legally regulated in Europe, namely domoic acid, saxitoxin, okadaic acid, yessotoxin, pectenotoxin and azaspiracids, and all their analogues and how are applied in Bulgaria.

Due to legislative changes that were implemented in 2011, most legally required methods are chromatographic. Saxitoxin and domoic acid are monitored by high-performance liquid chromatography with fluorescent and ultraviolet detection, respectively. All other toxins are monitored nowadays by means of chromatographic separation and mass spectrometry detection.

Key words: marine biotoxins, sea food, detection methods, official control
List of abbreviations: RDA – recommended daily allowance; PUFA – polyunsaturated fatty acids; SFA – saturated fatty acids; CONTAM – Panel on Contaminants in the Food Chain; EFSA – European Food Safety Authority; HABs – harmful algal blooms; TEFs – Toxicity equivalency factors; EU – European Union; PSP – Paralytic shellfish poisoning; STXs – saxitoxins; ASP – amnesic shellfish poison; DA – domoic acid; DSP – diarrhetic shellfish poisoning; OA – okadaic acid; DTXs – dinophysistoxins; YTXs – yessotoxins; PTXs – pectenotoxins; AZAs – azaspiracids; EC – European Commission; LC – liquid chromatography; MS/MS – tandem mass spectrometry; HPLC – high-performance liquid chromatography; UV – ultraviolet; FD – fluorescent detection; NeoSTX – Neosaxitoxins; GTX – Gonyautoxin; dc – decarbamoyl; i.p. – Intraperitoneal; CE – capillary electrophoresis; NRL – National Reference Laboratory; SD – standart derivation; SM – shellfish meat; EURLMB – Laboratory for Marine Biotoxins; OCLs – Official Control Laboratories; UPLC – Ultra Performance Liquid Chromatography; MLTs – marine lipophilic toxins

Introduction

The total aquaculture production for 2012 in Bulgaria is 7557.14 tons. Aquaculture production in Bulgaria (in tonnes) is dominated by fish production, followed by the production of molluscs (Black Sea mussel *Mytilus galloprovincialis*), which is the only one marine species cultivated (NSP, 2014).

Production and consumption of Black sea mussel should be accounted due to its nutritional characteristics – Black Sea mussels are a good source of fat soluble nutrients. Wild and farmed samples provided considerable amounts of vitamin D_3. 100 g of analyzed samples

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supplies 300 to 600% of the RDA (recommended daily allowance) (5 μg per day for Bulgaria) (Stancheva and Dobreva, 2014). Regarding the n-6/n-3 and PUFA/SFA ratios mussels are good source of the identified biologically active substances. Recent study by Dobreva et al. showed that the consumption of Black Sea mussels could decrease the incidence of coronary heart disease in humans (Dobreva et al., 2015).

At present there are 38 farms for cultivating Black sea mussel and their total production is about 850 tonnes for 2012. Negative impact on their production process has the anthropogenic pollution, which leads to significant euthrophication . A consequence is a formation of an algal bloom. (NSP, 2014). Some toxic microalgae (dinoflagellates, diatoms) are usually present in the plankton in low concentrations, but under some environmental conditions they can rapidly multiply and turn the bloom to harmful. These microalgae produce phycotoxins (marine biotoxins, algal toxins), which accumulate in the digestive organs of filter-feeding shellfish, zooplankton and herbivorous fishes and through the trophic chain can produce human intoxications (Botana et al., 2013). CONTAM Panel concluded that processing of shellfish could lead to an approximate 2-fold increase in the concentration of lipophilic marine biotoxins (OA-, AZA- PTX- and YTX-group toxins) in shellfish meat. Shellfish that contains levels of lipophilic toxins below the regulatory limits may reach, after processing, levels that are higher than the regulatory limits (EFSA, 2009).

Episodes of harmful algal blooms (HABs) previously regarded as sporadic and localised phenomena and restricted to certain geographic areas have started to show up as recurrent and ubiquitous in any coast of the world and sometimes cause great damage to the local fishing and marine aquaculture industry and also to the natural communities of marine organisms (Vieites and Cabado, 2008).

It is obvious that reliable methods should be used for detection and quantification of phycotoxins in sea food. The methods must be validated according to international guidelines to ensure adequate results and to protect public health and reduce their economic impact.

This paper is aiming to present and discuss the analytical methods for detecting phycotoxins under European Union legislation. Some examples of already conducted toxin profiling with their results are given. The maximum values of phycotoxins present in sea food are also indicated.

Toxicity equivalency factors (TEFs) are being applied to express the detected analogues as equivalents. TEFs are cited in order combined toxicity of the different analogues to be shown.

EU legislation for marine biotoxins

Based on their chemical properties phycotoxins can be divided in two groups hydrophilic and lipophilic.

Currently regulated in the European Union (EU) legislation hydrophilic marine toxins are PSP toxins (saxitoxin – group (STXs)), ASP toxins (domoic acid (DA)). Regulated lipophilic toxins are DSP (okadaic acid (OA) and dinophysistoxins (DTXs)), yessotoxins (YTXs), pectenotoxins (PTXs)) and azaspiracid -group toxins (AZAs).

To protect public health, maximum concentration limits for some algal toxins in bivalves, as well as official control programs, have been set up in many countries. In the European Union, Commission Regulations (EC) No 853/2004 and Commission Regulations (EU) 786/2013 establish the permitted limits of lipophilic marine biotoxins in live bivalve molluscs. Table 1 presents the classification and current EU reference values of regulated marine toxins.

Table 1

Classification and EU reference values for regulated marine toxins (Commission Regulation, 2004; Commission Regulation, 2013)

	Toxin group	Current EU limits in shellfish meat (A)
Hydrophilic toxins	STX	800 μg PSP.kg^{-1} SM
	DA	20 mg DA.kg^{-1} SM
Lipophilic toxins	OA , DTXs and PTX	160 μg OA.kg^{-1} SM
	YTX	3.75 mg YTX .kg^{-1} SM
	AZAs	160 μg .kg^{-1} SM

Toxicity equivalence factors (TEFs) have been used to convert the concentrations of the OA-, AZA-, YTX-, STX- and PTX-group toxins respectively into OA, AZA1, YTX, STX and PTX2 equivalents in order to allow for the combined toxicity of the different analogues (EFSA, 2009). The TEF values adopted by the CONTAM Panel, based on acute toxicity following i.p. administration to mice, are presented in Table 2.

The limited toxicological information does not allow the setting of robust TEFs for the oral route for any of the toxin groups. Even for the i.p. route, the available toxicity data are very limited for the AZA-, YTX- and PTX-group toxins. Further toxicological data are needed for the establishment of robust TEFs for the oral route of administration for all toxin groups. The assumption of dose additivity should be assessed following exposure to combinations of toxin analogues and milligram amounts of purified toxins should be produced for this purpose.

In 2006 European Commission published the adopted Lawrence method as alternative official method to detect

Table 2
Toxicity equvalence factors, as defined by EFSA

Analogue	TEF	Results expression
OA	1	µg OA equivalents kg^{-1}
DTX1	1	µg OA equivalents kg^{-1}
DTX2	0.6	µg OA equivalents kg^{-1}
PTX1	1	µg PTX equivalents kg^{-1}
PTX2	1	µg PTX equivalents kg^{-1}
YTX	1	µg YTX equivalents kg^{-1}
hYTX	1	µg YTX equivalents kg^{-1}
45-OH-YTX	1	µg YTX equivalents kg^{-1}
45-OH-hYTX	0.5	µg YTX equivalents kg^{-1}
AZA1	1	µg AZA equivalents kg^{-1}
AZA2	1.8	µg AZA equivalents kg^{-1}
AZA3	1.4	µg AZA equivalents kg^{-1}
STX	1	µg STX equivalents kg^{-1}1
NeoSTX	1	µg STX equivalents kg^{-1}
GTX1	1	µg STX equivalents kg^{-1}
GTX2	0.4	µg STX equivalents kg^{-1}
GTX3	0.6	µg STX equivalents kg^{-1}
GTX4	0.7	µg STX equivalents kg^{-1}
GTX5	0.1	µg STX equivalents kg^{-1}
GTX6	0.1	µg STX equivalents kg^{-1}
C2	0.1	µg STX equivalents kg^{-1}
C4	0.1	µg STX equivalents kg^{-1}
dc-STX = 1	1	µg STX equivalents kg^{-1}
dc-NeoSTX	0.4	µg STX equivalents kg^{-1}
dc GTX2	0.2	µg STX equivalents kg^{-1}
dc GTX3	0.4	µg STX equivalents kg^{-1}

paralytic shellfish poisoning (PSP) toxins (Commission Regulation, 2006).

The total content of amnesic shellfish poison (ASP) of edible parts of molluscs (the entire body or any part edible separately) must be detected using the high-performance liquid chromatography (HPLC) method or any other recognised method according Commission Regulation (EC) No 2074/2005 of 5th December 2005 (Commisssion Regulation, 2005).

In 2011 the European Regulation (EU) 15/2011 established that official controls of lipophilic marine toxins should be performed with analytical methods based on liquid chromatography (LC) with tandem mass spectrometry (MS/MS) detection (Commission Regulation, 2011). It is is now considered the reference technique, and from 2015 LC–MS/MS is mandatory for official controls (Rúbies et al., 2015).

Materials and Methods

Analytical methods for detection of paralytic shellfish poisoning (PSP)

Table 3 provides information about origin, chemical structure and poisoning symptoms of PSP. STX-group toxins cause PSP in humans. More than 30 different STX analogues have been identified of which STX, NeoSTX, GTX1 and dc-STX seem to be the most toxic ones (Scientific opinion, 2009).

The chemicals methods used to determine PSP toxins are fluorimetric assays, HPLC with fluorimetric detection (either pre-column or postcolumn oxidation), LC/MS and capillary electrophoresis (CE) methods.

The HPLC methods are widely used to quantify PSP toxins present in seafood samples, e.g. Gribble et al. (2005), Pitcher et al. (2007) etc., and they are also useful to provide the PSP profile because chromatographic methods are identification methods as well. These toxins have only a weak chromophore group, and it must be modified before detection: when they are oxidised in alkaline solution, a purine is formed, andbecomes fluorescent at acidic pH. This reaction can be either a pre-column one or a post-column one, and obtained purines are monitorised with an FL detector (Botana et al., 2013).

Table 3
Characteristics of regulated marine biotoxins (Hallegraeff, 2004 ; EFSA, 2009)

PSP	Tetrahydropurine	Alexandrium, Gymnodinium catenatum, Pyrodinium bahamense	Within 30 min: tingling sensation or numbness around lips, gradually spreading to face and neck; prickly sensation in fingertips and toes; headache,dizziness, nausea, vomiting, diarrhoea
ASP	Kainoid	Pseudonitzschia	After 3–5 hours: nausea, vomiting, diarrhoea, abdominal cramps.
DSP	Polyether, spiro-keto assembly	Dinophysis, Prorocentrum	After 30 min to a few hours (seldom more than 12 hours): diarrhoea, nausea, vomiting, abdominal pain
PTX	Polyeter, ester macrocycle	Dinophysis	Considered not to pose any health risk
YTX	Ladder-shaped polyether	Protoceratium reticulatum, Gonyaulax spinifera, Lingulodinium polyedrum	
AZA	Polyether, second amine, 3-spiro ring	Azadinium, Amphidoma languida	Diarrhoea

The Lawrence method is official method to detect PSP toxins and approved by the European Union (EU) for monitoring these toxins (AOAC, 2005; Commission Regulation, 2006). It is based on the pre-column oxidation of PSP toxins with hydrogen peroxide and sodium periodate followed by fluorimetric detection. It was validated for the determination of STX, neoSTX, GTX2,3, GTX1,4, dcSTX, GNTX5(B1), C1,2 and C3,4 in molluscs (mussels, clams, oystersand scallops).

In 2014 the UK NRL for marine biotoxins screened for the presence of PSP in sehellfish using pre-column oxidation and HPLC with FD (an adopted Lawrence method). Cleaning was carried by C18 rasins solid phase exctraction cartridges. Peroxide and/or periodate oxidations are applied to cleaned extracts derived from an acetic acid extraction. The identification of the presence of PSP toxins relied on matching the retention time of any oxidation products in samples with those of the corresponding reference standarts. By identification a semi- quatitative screen analysis of samples is required, followed by quantitative testing (UK-NRL for Marine Biotoxins, 2014).

Precise and accurate quantitation of the class of PSP toxins remains challenging and is best achieved by combining LC-FD and LC–MS/MS methods (Krock et al., 2007). The PSP toxin profile of the cultured Chilean strain of *A. Catenella* is determined by LC-FD. Following toxins were present in decreasing order of molar percentage of total content: C1/C2 (57%), GTX1/GTX4 (24%), B1 (=GTX5) (13%), GTX2/GTX3 (3%), neoSTX (2%) and B2 (=GTX6) (1%).

Identification and confirmation of the toxin components was also done with minor modifications by the HILIC-LC/MS method. The HILIC-LC/MS method confirmed the identity of the key toxins C1, C2, GTX1 and GTX4 in the Chileanstrain of *A. catenella*, and also traces of toxins C3 and C4, which were not detectable with the LC-FD method were detected. The authors presumed this is due to different toxin response factors and detection limits of the respective methods. The detection limit for GTX3 is lower by the fluorescence method (1, 19 ng. ml^{-1}) than by mass spectrometer (0.22 ng. ml^{-1}), but this pattern is reversed for the N1-hydroxy carbamoyl toxins such as GTX1/GTX4 (resp. 0.02/0.03 ng. ml^{-1} – 0.01/0.15 ng. ml^{-1}). (Krock et al., 2007)

Analytical methods for detection of amnesic shellfish poisoning (ASP)

Organisms that produce ASP, chemical structure of ASP, as well as poisoning symptoms can be seen in Table 3. Main representative of ASP toxins is domoic acid.

The Quilliam method, which is based on HPLC-UV, is the regulatory method for determination of domoic acid in shellfish in laboratories (AOAC International, 2000; Quilliam**,** 2003). The procedure includes extraction with methanol-water (1:1) and clean-up on strong anion exchange solid phase extraction cartiges.

The detection of domoic acid is facilitated by its strong absorbance at 242 nm. The HPLC-UV detection limit for domoic acid is about 10–80 ng.ml^{-1}, depending on the sensitivity of the UV detector. The detection limit in tissue is dependent upon the method of extraction and cleanup. If crude extracts (either acidic or aqueous methanol) are analysed without cleanup, the practical limit for quantitation is about 1 mg.kg^{-1}(ppm). This is suitable for most regulatory laboratories concerned with detecting contamination levels greater than 20 mg.kg^{-1} (Quilliam , 2003).

Although the detection limit of DA can be improved by using different injection volumes in the LC-UV, the matrix complexity may result in some degree of uncertainty about the peak's identity at low DA levels (< 3 ng.ml^{-1}), when asensible UV spectrum cannot be obtained (Mafra et al., 2009). In this case, confirmation by LC-MS is sometimes necessary and should also be required whenever DA is reported in a new geographical area. LC-MS/MS is perhaps the most important and legally accepted confirmatory tool, providing a highsensitivity, accuracy and selectivity for DA and its isomers in crude extracts (McNabb et al., 2005).

There are studies on algal production of DA to track the toxin in seawater, as an early alert for toxin accumulation in marine organisms, are available for remote, subsurface detection (Doucette et al., 2009).

Following the EU legislation HABs were studied in the Bizerte Lagoon (Tunisia) in 2013 and presence of domoic acid was proved (Souad et al., 2014). ASP quantification was performed following the Quilliam (1995) method. ASP toxicity for mussels was relatively rare. Average concentration of ASP measured during the survey is 5.81±26.34 µg.100 g^{-1} for mean concentration ± SD and 180.80 for maximum concentration. EU ASP limit is 20 mg DA.kg^{-1}SM.

In Bulgaria a research about the presence of PSP indicated values below the limit of quantification (Kalinova, 2015).

In the period 2009–2011, 15 samples of *Mytilus galloprovincialis* were studied for domoic acid using the method, described in BDS EN 14176 with HPLC-UV. In only 8 of the samples concentration of domoic acid is 0.02–0.55 mg.kg^{-1}. These concentrations are not dangerous because even 0.55 mg.kg^{-1} is about 40 times less than the reference limit (20 mg.kg^{-1}) (Peneva et al., 2011).

These results are not representative, as some farms only provide samples for toxicity testing once a year. This could lead to potential shellfish intoxications by ASP and PSP during the remaining period of the year (Kalinova, 2015).

Analytical methods for detection of lipophilic marine toxins

An overview of producers, chemical characteristics and symptoms of poisoning are presented in Table 3.

EU reference method for lipophilic shellfish toxins detection is an instrumental chemical method utilising liquid chromatography with tandem mass spectrometry (LC MS/MS). This provides a highly specific and quantitative determinationof the full range of regulated lipophilic toxins (McNabb et al., 2005) – okadaic acid group toxins: OA, DTX1, DTX2, DTX3 including their esters, pectenotoxins group toxins: PTX1 and PTX2, yessotoxins group toxins: YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX; azaspiracid Shellfish Poisoning: AZA-1, AZA-2 and AZA-3;

The method (AESAN, 2015) is based on the extraction of OA, PTX, AZA and YTX group toxins with 100% methanol from homogenised tissue. Extracts are then filtered and directly analysed by liquid chromatography with tandem mass spectrometric detection (LC – MS/MS) in order to investigate the presence of free OA, free DTX1 and free DTX2, PTX1, PTX2, AZA1, AZA2, AZA3, YTX, homo YTX, 45 OH YTX and 45 OH homo YTX . To determine the total content of OA group toxins, an alkaline hydrolysis is necessary from methanolic extract prior to LC - MS/MS analysis with the aim of converting any acylated esters of OA and/or DTXs to the parent OA and/or DTX1 or DTX2 toxins . After hydrolysis (either acidic or basic), extracts are filtered and analysed by LC – MS/MS. Chromatographic separation is performed by gradient elution.

Number of single laboratory validation studies has been published and the method has been validated by the European Union Reference Laboratory for Marine Biotoxins (EURLMB) within its network of National Reference Laboratories (NRLs) and Official Control Laboratories (OCLs).

However, most LC-MS/MS methods are focused on the analysis of the 12 toxins that are legislated in the EU. For these toxins TEFs have been established (Alexander et al., 2008) (Table 2).

Hence, LC-MS/MS methods detect different marine toxin groups in a short period of time (Stobo et al., 2005; McNabb, 2008; Gerssen et al., 2011) and they are classified according to the chromatographic solvents used. Basic or acid mobile phases can be used with an elution gradient. The method with both basic and acid mobile phases was also adjusted to the new technologies, UPLC-MS/MS, and more than 20 analogues were separated in only 6.6 min (Fux et al., 2007). One advantage of the acid against basic chromatography is that the first one facilitates good separation of acidic OA analogues by suppressing the ionisation of the carboxyl groups and preventing deleterious ion exchange interactions with residual silanol groups in the stationary phase (Suzuki et al., 2011). Nevertheless, the chromatography of compounds included in the YTX group can be problematic under acidic conditions (Gerssen et al., 2009a). Detection limit for lipophilic toxins achieved by the LC-MS/MS approach are low and toxins can be detected at levels below the current regulatory limit (Table 4). For both acid and basic chromatography conditions, these detection limits are lower for toxins ionised in the positive mode than in the negative one (Blay et al., 2011; van den Top et al., 2011).

Since the late 1980s, the North-central Adriatic Sea has frequently experienced blooms of harmful algal species, producing marine lipophilic toxins (MLTs) which accumulate in mussels. A 2-year LC-MS/MS study (2012–2014) of the MLT profile in mussels from the North-central Adriatic Sea in the context of the presence of toxic phytoplankton concentrations in seawater was performed (Bacchiocchi et al., 2015). Okadaic acid increased in mussels from all areas during the summer and autumn-winter periods with a rising trend between 2012 and 2014. Yessotoxins (YTXs) content in mussel increased sharply in the autumn-winter periods even exceeding the legal limit. Traces of Azaspiracid 2 (AZA-2) were observed often in mussels during the study period, confirming for the first time the presence of this biotoxin in Mediterranean seafood.

Table 4

LODs of two types of liquid chromatography (LC) for the detection of lipohilic marine toxins: (1) chromatography under acidic conditions and (2) chromatography under basic conditions (Botana et al., 2013)

LODs	OA: 10 µg/kg	Kilcoyne and Fux, 2010	OA: 5 µg/kg	Kilcoyne, 2010
	AZA-1: 0.3 µg/kg		AZA-1: 0.5 µg/kg	
	OA: 15 µg/kg	Villar-Gonzalez et al., 2007)	AZA-1: 2 µg/kg; AZA-2: 2 µg/kg	van den Top et al., 2011
	PTX-2: 10 µg/kg		AZA-3:3 µg/kg	
			OA: 3 µg/kg	
			DTX-1: 5 µg/kg	
			DTX-2: 3 µg/kg	
			PTX-2: 2 µg/kg	
			YTX: 16 µg/kg	

Analysis included methanol extraction, NaOH hydrolysis and HCl neutralisation. The method of Gerssen (Gerssen et al., 2009b) was used for LC–MC/MC determination.

In Bulgaria any analysis of lipophilic toxins including DSP toxins are being performed at all due to the lack of experts, expertise and equipment (Kalinova, 2015).

Conclusions

Marine biotoxins present a danger for aquacultures of each country developing this sector. Relaible and approved methods should be used for the determination of phycotoxins in food. LC-MS can be used for all of the marine biotoxin groups. Toxins of DA and saxitoxin groups can also be detected by HPLC respectively with UV and Fl detection.

In Bulgaria aquaculturing increases its impact on economy and in last years Bulgarian consumers recognise more the nutritional value of sea food. Therefore sporadic evaluation of phycotoxins content of the most consumed aquaculture *Mytilus galloprovincialis* is reckless and precarious. There is a plain indication of emerging expertising and initiation of phycotoxins proofing process. Following the EU legislation a respectful profile of Black Sea biotoxins will be revealed.

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