Food Science and Applied Biotechnology, 2019, **2**(2), 166-174

UFT Academic Publishing House, Plovdiv

Food Science and Applied Biotechnology
e-ISSN: 2603-3380

Journal home page: www.ijfsab.com
https://doi.org/10.30721/fsab2019.v2.i2

OPEN ACCESS

Research Article

Empirical determination of conversion factor for representing phycotoxin levels in whole mussel *Mytilus galloprovincialis* meat

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Abstract

Mussels accumulate marine biotoxins (phycotoxins) produced by certain phytoplankton species. In EU limits for toxin concentration (e.g. domoic acid, okadaic acid, yessotoxins etc.) are set beyond that mussels are safe for consumption. Marine biotoxins tend to accumulate in the digestive gland (hepatopancreas) of the mussel. Consequently, this tissue is preferred for toxin concentration determination. Normally the whole shellfish is consumed and therefore the occurrence data for phycotoxins need to be expressed in terms of whole shellfish meat. A theoretical factor of five is used to convert the value to whole shellfish meat. The aim of this study was to determine an empirical factor in order to convert phycotoxin levels from hepatopancreas to whole shellfish meat of the main marine aquaculture product in Bulgaria - mussels *Mytilus galloprovincialis*. Wild and cultivated mussels were collected from the north Black Sea coast of Bulgaria in 2017. In total 13 mussel samples were studied whereas of each sample subsamples of hepatopancreas only and whole mussel meat were prepared. Phycotoxins were extracted for all types of phycotoxins by means of liquid-liquid extraction and their concentration was determined by LC-MS/MS. Yessotoxins appeared in most of the samples and therefore seemed most suitable for empirical conversion factor determination. It was calculated as the ratio between YTX levels in hepatopancreas and whole shellfish meat. The mean defined value was 5.36. Determination and application of empirical conversion factor is important e.g. when using very low toxin levels for chronic exposure assessment. An empirical conversion factor is also useful if different species are investigated. Moreover, its application on phycotoxin levels in hepatopancreas could give representative results and avoid false negative results if studying whole shellfish sample.

Keywords: lipophilic marine biotoxins, YTX, shellfish, exposure assessment

Abbreviations: ArfD – acute reference dose; AZA – azaspiracids; DA - domoic acid; hp –hepatopancreas; DSP - diarrhetic shellfish poisoning; LC-MS - liquid chromatography - mass spectrometry; LOD - limit of detection; OA - okadaic acid; PTX – pectenotoxins; sm - shellfish meat; SRM - selected reaction monitoring; TDI - tolerable daily intake; YTX – yessotoxins.

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Article history:
Received 7 June 2018
Reviewed 4 August 2018
Accepted 13 December 2018
Available on-line 14 May 2019

https://doi.org/10.30721/fsab2019.v2.i2.43

Introduction

Marine biotoxins (phycotoxins) are toxic metabolites produced by some species of unicellular algae developing during natural phenomena known as harmful algal blooms. They tend to accumulate in filter feeding bivalves – mussels, oysters etc. Phycotoxins are grouped in different classes – hydrophilic, e.g. domoic acid (DA) and lipophilic, e.g. okadaic acid (OA), azaspiracids (AZA), yessotoxins (YTX), pectenotoxins (PTX), etc. (Ferron et al. 2016). Some groups of toxins are known to cause human sickness after being consumed (European Food Safety Authority (EFSA), 2008a; EFSA, 2008b EFSA, 2009). The toxicological information for YTXs includes mainly studies on their acute toxicity in mice (Rodríguez et al. 2015). YTXs are potent cytotoxins (Paz et al. 2007), able to cause damage of the myocardium (Aune et al. 2003; Tubaro et al. 2004). Furthermore, YTX affects Ca^{2+} influx in human lymphocytes (de la Rosa et al. 2001), produces a Ca^{2+}-dependent decrease of cyclic adenosine monophosphate (Alfonso et al. 2003; Pazos et al. 2005), slows down the disposal of an E-cadherin degradation product (ECRA100) (Callegari and Rossini 2008), and causes neurotoxicity in rat cerebellar neurons (Pérez-Gómez et al. 2006). Related YTX symptoms and toxicity effects after oral admission are rather unknown in humans, so consumption of YTX-contaminated shellfish still remains an unidentified health risk (Tubaro et al. 2010). Consumption of contaminated shellfish products may not result in an acute intoxication, but it is not yet clear what would be the effect of their long-term exposure. Furthermore, seafood contamination by yessotoxins, also at low doses and for a long period of time, can result in cardiomyocytes with “loose packing” of myofibrils and aggregated rounded mitochondria (Sossa et al. 2013). The black mussel *Mytilus galloprovincialis* is a preferred sea food on the Bulgarian north coast and often offered in restaurants and on markets (not published). Shellfish aquaculture is widespread along its entire coastline with 23 sampling sites for *Mytilus galloprovincialis* farming (Bulgarian Agency for Food Safety (BAFS 2016)). Mussel production is increasing recently (Ministry of Agriculture and Food 2017). Yessotoxins (Georgieva et al. 2018) and other phycotoxins - domoic acid (Peteva et al. 2017; Peneva et al. 2011) have been reported in shellfish samples from the Bulgarian coast of the Black Sea. In Bulgaria there is little evidence that marine biotoxins pose an acute public health risk, though there have been occasional cases of suspected DSP poisoning reported following the consumption of recreationally harvested shellfish (recreational harvesters and medical practitioners, oral communication). However, these compounds remain an important quality control issue largely because of their potential of acute (Pulido et al. 2016) and chronic effects (Hiolski et al. 2014; Kolrep et al. 2017). Most of the phycotoxins found in plankton have also been found in contaminated shellfish tissues, in which they are relatively persistent and are concentrated in the digestive gland (hepatopancreas) (MacKenzie et al. 2002; O'Driscoll et al. 2014; Mafra Jr. et al. 2015). The amount of toxin accumulated by different bivalve species depends upon their ingestion rates of toxic cells, which, in turn, is a function of their particle capture efficiency, clearance rates, and capacity for selective feeding, as well as processes regulating toxin assimilation or elimination, such as digestion (i.e. absorption efficiency), affinity for the toxic compounds, toxin transformation (i.e. metabolism and conjugation), and excretion (Bricelj and Shumway 1998). The distribution of a toxin across different organs of the same animal is very difficult to study due to problems of cross-contamination between organs. The digestive gland has been shown to contain particularly sticky fluids that can adhere to tissues, even during washing steps (cited by Hess et al. 2005). Therefore, this tissue is preferred in some studies for investigating the toxin content (Edebo et al. 1988; Aasen et al. 2005). Normally whole shellfish is consumed and therefore the occurrence data for phycotoxins need to be expressed in terms of whole shellfish meat. If hepatopancreas was analyzed a factor of 5 is usually used to convert the value to whole shellfish meat. This factor, though not representing exactly all individual mollusks, is considered to represent a good approximation (EFSA 2008a; 2008b; 2008c;

2009). This recalculation in whole shellfish meat is necessary in order to estimate a value that is comparable with the legislative limits, e.g. ARfD, tolerable daily intake (TDI) etc. for any phycotoxin are legislated in μg.g^{-1} whole shellfish meat/bodyweight and μg.g^{-1} shellfish meat/day, respectively (EFSA 2008a; EFSA, 2008b; EFSA, 2008c; EFSA 2009). The half-life time for YTXs was estimated as 20 to 24 days in blue mussels (Aasen et al. 2005) and 49 days in GreenshellTM mussels (MacKenzie et al. 2002) which is much higher than the depuration rate of domoic acid (Novaczek et al. 1992). This lower depuration rate makes YTXs more suitable for studies of toxin level conversion from hepatopancreas to whole shellfish meat. For example, Aasen et al. (2005) and Mackenzie et al. (2001) made toxin determination in hepatopancreas samples. They calculated levels of yessotoxin(s) in whole meat of *M. edulis* - Norwegian blue mussels and *M. galloprovincialis* from the North Sea, respectively, on the assumption that YTX(s) are localized in the digestive glands, and that digestive glands on average account for 20% by weight of the flesh.

Liquid chromatography coupled to mass spectrometric detection (LC–MS) is becoming a prime technology because of its high sensitivity and specificity (Quilliam 1998; Quilliam et al. 2002; Goto 2001). Tandem mass spectrometric (LC–MS/MS) methods for marine biotoxins in shellfish are currently being developed and validated following performance criteria developed by regulatory authorities (Burrow and Seamer 2001; Özden et al. 2006). For a specific as well as a sensitive detection of individual YTXs LC-MS/MS techniques have been developed. The group of YTXs is routinely incorporated in multi-toxin methods for the detection of lipophilic biotoxins, that are used in some laboratories (Krock et al. 2008).

Materials and Methods

Field sampling. A sampling program for collecting mussels was initiated. This provided the raw materials for the experimental analyses reported here.

Cultivated mussels were sampled from farming sites (Kavarna Bay) and wild - from recreational sites (Balchik and Galata) on the North Bulgarian Black Sea coast (Table 1, Figure 1). Sampling was performed at various intervals during harvesting campaigns in 2017.

Table 1. Sampling data

Sample number	Sampling site	Period	Mussel type
1	Balchik	May	wild
2	Kavarna	April	cultivated
3	Kavarna	March	cultivated
4	Balchik	May	wild
5	Kavarna	June	cultivated
6	Kavarna	July	cultivated
7	Balchik	April	wild
8	Kavarna	September	cultivated
9	Kavarna	August	cultivated
10	Galata	April	wild
11	Galata	June	wild
12	Kavarna	June	cultivated
13	Balchik	July	wild

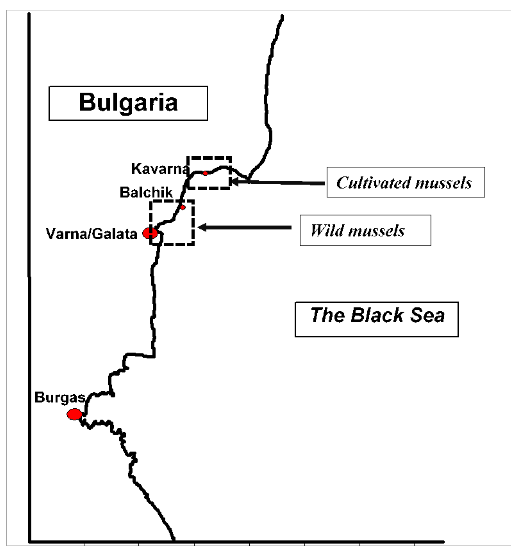

Figure 1. Map of sampling locations

Sample extractions. Mussels (≈1 kg) of approximately same size class (≈5.2 cm) were shucked and drained. Thereafter, each flesh sample was divided in two subsamples. The first one (≈200 g) (Subsample 1) was homogenized using a blender. Mussels in second subsample (≈200 g) were dissected and divided into hepatopancreas (≈50 g) and remaining flesh portions. Hepatopancreases were homogenized (Subsample 2) and the rest was discarded. Subsamples 1 and 2 homogenates were extracted by high-speed blending (POLYMIX®PT 1200E, KINEMATIKA AG, Germany). About 20.00 g homogenate of subsample 1 and 4.00 g homogenate of subsample 2 (Table 2) were extracted separately with methanol/water (9:1 v/v) for 10 min and subsequently twice with methanol/water (8:1 v/v) for 5 min by maintaining the overall sample to solvent ratio 1:4 (Hess et al. 2005; García-Mendoza et al. 2014). The extracts were degreased with hexane and centrifuged (4500 x g for 15 min). Supernatant (1.00 ml) was filtered through syringe filter (0.45 μm pore size, ⌀ 25.00 mm, Minisart, Sartorius, Germany). The extracts were transferred into autosampler vials and kept frozen at -20°C until analysis.

LC-MS/MS analyses. LC-MS/MS determination of YTXs was performed according to multi-toxin method developed by Krock et al. 2008. Mass spectral experiments were performed on a SCIEX-4000 QTrap, triple quadrupole mass spectrometer equipped with a TurboSpray® interface coupled to an Agilent model 1100 LC. Measurements were carried out in positive-ion mode by selected reaction monitoring (SRM) experiments. Mass spectrometric parameters are summarized in Table 2. The SRM channel was monitored in window that covered the elution of the compound of interest (YTX) (parent > daughter ion). YTXs were quantified by comparison with authentic standard in concentration 100 pg.μl^{-1}. Standard solution was measured in triplicates: at the beginning of the samples series, after the twelfth sample and at the end of the samples series. A blank is always before and after the standard solution measured. An average value from the parent quantitative standard measurements was applied to estimate concentrations of the respective related compounds.

The limits of detection (LODs) (Table 2) for YTXs in both whole flesh and hepatopancreas were determined based on 3:1 signal-to-noise ratio.

Table 2. LODs for whole flesh and hepatopancreas subsamples and mass spectral parameters

Toxin	**YTX**	**YTX**
Subsample	whole flesh	hepatopancreas
Average weight of subsample [g]	20.07	4.09
LOD [pg/g]	0.36	2.29
Mass transition m/z	1160/965	
RT [min]	13.46	

Calculation of conversion factor. A factor of conversion represents the ratio between phycotoxin amount in hepatopancreas and in whole shellfish meat:

$$Fc = \frac{C_{hp}}{C_{sm}} \quad (1)$$

where:
Fc – factor of conversion
Chp - phycotoxin level in mussels hepatopancreas [pg.g^{-1} hp]
Csm – phycotoxin level in whole shellfish meat (sm) [pg.g^{-1} sm]

Phycotoxin levels in mussels hepatopancreas [pg.g^{-1} hp] and in whole shellfish meat (sm) [pg.g^{-1} sm] were calculated by using the following equation:

$$C = \frac{w}{m} \times V \quad (2)$$

where:
C - phycotoxin level in mussels hepatopancreas [pg.g^{-1} hp] or in whole shellfish meat (sm) [pg.g^{-1} sm]

w - phycotoxin concentration in mussels hepatopancreas [pg.µl^{-1} hp] or in whole shellfish meat (sm) [pg.µl^{-1} sm]
m – mass of hepatopancreas or whole shellfish meat extracted
V- extraction volume

Statistical analysis. Data obtained were analyzed by using Microsoft Office Excel 2010 Pro, a significance level (p) by comparing with the value of 0.05 was used.

Results and Discussion

In recent years, various scientists have published about phycotoxin detection and quantitation in shellfish samples. Alves-de-Souza et al. (2013) investigated seasonal variability of lipophilic marine toxins in bivalves from Southern Chile and detected high levels of yessotoxins in bivalves (51–496 ng.g^{-1}) and trace levels of PTX2. García-Mendoza et al. (2014) detected OA, PTX2 and dinophysistoxins (reaching 1647 µg.kg−1) in cultivated mussels (*Mytilus galloprovincialis*) from Baja California, Mexico. In Europe, on the Galician coast, Rodríguez et al. (2015) found that toxin profiles of studied mollusks comprised of OA and YTX.

YTXs in mussels. In total 13 hepatopancreas and 13 whole flesh samples were investigated (Table 3) whereas 10 hepatopancreas and 9 whole shellfish samples were positive for YTX. YTXs were present in the mussels with a range of 1053 – 10035 pg.g^{-1} hepatopancreas and 297 – 3211 pg.g^{-1} whole flesh.

Table 3. YTX levels in studied mussel samples

Type of sample	Number of samples	Positive for YTXs	Concentration range, pg YTX/g
Hepatopancreas	13	10	1053-10035
Whole shellfish meat	13	9	297- 3211

These values, both in hepatopancreas and in whole shellfish meat (sm) were found lower than reported in other recent studies (Haddouch et al. 2017; Schirone et al. 2018) and below the legislative limit of 3.75 mg.kg^{-1} sm (EFSA 2008c).

Calculation of conversion factor. A conversion factor is necessary when toxins were determined in hepatopancreas, but total toxin content should be expressed per whole sm in order to comply with the legislative limit of toxin level in shellfish. A theoretical conversion factor of 5 has been proposed by EFSA (EFSA 2008a; EFSA 2008b; EFSA 2008c; EFSA 2009). A conversion factor was calculated using eq.1. The results were divided in two categories – below 5 and above 5. The value 5 was used as separator, because this is the theoretical conversion factor. The results are presented in Table 4.

Table 4. Variability of YTX ratios in mussels (M. galloprovincialis)

	Sample №	group 1 [CF <5]	Sample №	group 2 [CF > 5]
Estimated conversion factor	2	1.82	1	6.00
	3	3.99	4	5.85
	6	3.71	5	5.49
	7	1.80	9	15.33
	8	4.24		
Mean	3.11		8.17	
Average CF	5.36			
Sources of variation	p-value 0.05			
	SD* 3.82			

**SD- standard deviation*

Analysis of the variation between the two categories showed that they are not significantly different ($p > 0.05$). This result confirmed that the two categories could be combined and an average Fc can be calculated. The study over a whole year (2017) showed an average ratio (Fc) of toxins in the digestive gland over toxins in the whole flesh in mussels *M. galloprovincialis* of 5.36 (Table 4). This result is comparable with average ratio of 5.20 that was found in the experiment on the Norwegian and Irish bulk samples of blue mussels (*M. edulis*) (Hess et al. 2005). Experiments with greenshell mussels

(*Perna canaliculus*) (MacKenzie et al. 2002) showed also an approximate ratio of 5 between YTX levels in hepatopancreas and in whole mussel meat. However, the large variations observed in the dataset (SD = 3.816) indicate that temporary differences may exist. This is not conclusive since the weight of the digestive gland also changes seasonally as a percentage of the total flesh weight (MacKenzie et al. 2002) and this has not been monitored during this routine analysis. Another reason is that the ratio depends on the time between exposure of shellfish to toxic plankton and analysis. If shellfish have recently fed on toxic plankton, the ratio will be higher in hepatopancreas than after longer times, when toxins have been transported to other tissues.

Table 5. Comparison of exposure estimation with theoretical and empirical factor of conversion

Case	**Phycotoxin**	**Contamination level in hepatopancreas reported (mean; range)**	**Reported exposure (theoretical factor applied)**	**Application of empirical Fc**	**Legislative limit (LL)/ArfD/ TDI** (EFSA, 2008a; EFSA, 2008b; EFSA, 2008c; EFSA, 2009	**Reference**
1.1	Yessotoxin – wild and cultivated mussels /summer 2017/	nr	0.000678 mg.kg^{-1} sm	0.000633 mg.kg^{-1} sm	LL 3.75 mg.kg^{-1}sm	(Georgieva, et al., 2018)
1.2			0.0031 µg.kg^{-1} bw	0.0029 µg.kg^{-1} bw	ArfD 25 µg.kg^{-1} bw	
5	Azaspiracids (AZA1) – bulk sample	2.24 mg AZA1 eq.kg^{-1} hp	0.45 mg AZA1 eq.kg^{-1}	0.34 mg AZA1 eq.kg^{-1} sm	LL 160 µg AZA1 eq.kg^{-1} sm	(Hess et al. 2009)
6	Okadaic acid esters	224.5 µg.g^{-1} hp	44.904 µg.kg^{-1} sm	41.884 µg.kg^{-1} sm	LL 160 µg OA.kg^{-1} sm	(Prassopoulou et al. 2009)

**nr- not reported, values are published in reports on project with incoming number 16012/2016, supported by Science Fund of Medical University Varna;*

Application of the estimated conversion factor. Generally, the approach applied by EFSA (EFSA 2008a; EFSA 2008b; EFSA 2008c; EFSA 2009) is to use a conversion factor of 5 for any marine toxins found in the hepatopancreas. Hereby estimated Fc of 5.36 for the YTX content in hepatopancreas and whole shellfish meat agrees with the value proposed by EFSA. Studies on contaminants (Georgieva et al. 2016) and bioactive compounds (Merdzhanova et al. 2016) in mussels *M. galloprovincialis* harvested from the Bulgarian coast showed comparable levels to the same mollusks from neighboring seas. However, in pollutant exposure estimation it is important to be very precise, especially when approaching or even reaching the legislative threshold of acceptance, e.g. maximum level of contaminant, above that it is not allowed the product to be put in the market or the acute reference dose (ArfD), the amount of a chemical in food that, that can be consumed in the course of a day or at a single meal with no adverse effects (Lawrence et al. 2011) etc. In these cases, it will be substantial to apply a specific approach that will give more accurate results and conclusions. In this regard this study could claim to be species and location specific.

As the estimated empirical factor is higher than the theoretical, when applying Fc results would be lower than in theoretical cases. In Table 5 results of applying the theoretical and empirical conversion factors are compared. The application of empirical Fc showed a negligible decrease in contamination level in cases 1.1 and 1.2. More significant is the decrease in cases 3 and 4. In case 3 (Hess et al. 2009) the authors calculated the contamination level in whole shellfish meat taking into account that hepatopancreas weight was 15.25 % of the whole mussel. Therefore, the estimated result is much lower that the theoretical. In case 4 (Prassopoulou et al. 2009), the authors converted the toxin level from hepatopancreas to whole shellfish using the theoretical factor of 5. If using the empirical factor, the result would be lower. This variation in the result is important in case of results close to the legislative norm, as false conclusions could have even economically important consequences including closures of recreational and farming sites due to high risk of intoxication.

Conclusions

Marine biotoxins are widespread distributed along coasts. Their occurrence is increasing in edible bivalve mollusks. Our study is placed in the context to make data available for the management of the risk of these toxins in shellfish. These results may justify the practice to only analyse the digestive gland, a step considered necessary to achieve adequate detection limits for phycotoxins in different analytical techniques.

Acknowledgements

This work was supported by Science Fund of Medical University Varna, Project Incoming number 16012/2016.

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