Assessment of the matrix effect in quantifying lipophilic toxins in seafood

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Abstract

Phycotoxin accumulation in seafood can cause human intoxication and significant economic losses in seafood-producing areas. To protect consumer safety, maximum permitted levels in bivalve molluscs have been set, with appropriate analytical methods for their determination and quantification. The reference method for lipophilic phycotoxins, commonly referred to as lipophilic toxins, is liquid chromatography tandem mass spectrometry (LC-MS/MS method), where coeluting components from the matrix can affect the efficiency of ionisation, resulting in erroneous quantification. In this study, the matrix effect was evaluated using the slope ratio analysis method, which involves comparing the slopes of the calibration curves in the matrix with those in pure solvent. The extent of the matrix effect was investigated in mussels, oysters, queen scallops and ascidians. The study covered all phycotoxins under Regulation EU 853/2004 for which certified standards are commercially available, including unregulated pectenotoxin 2. The results indicated that all lipophilic toxins were susceptible to this effect when LC-MS/MS was used for their determination. Significant ion suppression was evident for most analytes in all matrices, except for okadaic acid and dinophysistoxin 2 in bivalves, where significant ion enhancement was demonstrated, and dinophysistoxin 1 in oyster and scallop extract where no significant effect on ionisation was observed. Further analysis revealed no significant differences between the slope of mussel matrix-matched calibration and that of other bivalve matrices. Given this minor difference, the mussel matrix-matched calibration curve could be applied to minimise the matrix effect and to quantify phycotoxins in bivalve matrices analysed here, with the exception of the okadaic acid group in ascidians, which requires matrix-matched calibration prepared with the blank extract of these mentioned species. Given the risks phycotoxins pose to human health, ongoing analytical method development is necessary in this field to properly control food safety and ensure consumer health.

Key words: *lipophilic toxins; LC-MS/MS; matrix effect; bivalve molluscs; ascidians*

Introduction

Seafood is desirable in the human diet due to its high nutritional value. However, when seafood is harvested from areas where toxic species of phytoplankton are present, they can accumulate phycotoxins and become vectors of these toxins in the food chain. Organisms such as bivalve molluscs and ascidians are crucial vectors

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due to their feeding behaviour (filtration of large quantities of seawater) (Kvrgić et al., 2021a). Phycotoxin accumulation in seafood can cause significant economic losses in seafood-producing areas and, more importantly, these contaminants can cause human intoxication, that can even be lethal (Prakash et al., 1971; Yasumoto et al., 1978; Perl et al., 1990; Todd, 1993; FAO, 2004; Gestal Otero, 2008; Ryan et al., 2008).

Maximum permitted levels in bivalve molluscs are set by authorities to protect consumer safety, along with the analytical methods for their determination and quantification (Rodríguez et al., 2017). The reference method for detection of lipophilic toxins (LT) according to Commission Regulation (EU) 2019/627 is the EU-Harmonised Standard Operating Procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS (liquid chromatography tandem mass spectrometry) (EC, 2019). The method is suitable for determination of okadaic acid (OA) and dinophysistoxins (DTX1, DTX2), and their esters (DTX3), pectenotoxin group (PTX1 and PTX2), yessotoxin group (YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX), azaspiracid group (AZA1, AZA2, and AZA3) (EU-RL-MB, 2015), as well as the group of cyclic imines (CI) (spirolide - SPX1, gymnodimine - GYM, pinnatoxin - PnTX-G) (Kvrgić, 2021b, 2023).

In LC-MS/MS, coeluting components from the matrix such as lipids, phospholipids, proteins, and sugars may affect the efficiency of ionisation, especially in LC-MS/MS systems using electrospray ionisation. These components negatively affect the sensitivity, selectivity, precision, repeatability, and linearity of the method, so it is important to evaluate their influence as part of method validation (Trufelli et al., 2010; Cortese et al., 2020). The matrix effect (ME) appears as suppression or enhancement of the analyte signal, and can result in erroneous quantification (Matuszewski et al., 2003; Zhou et al., 2017; Cortese et al., 2020). ME cannot be predicted because it depends on various factors like the interaction of the analyte with co-eluting compounds, the influence of cross-contamination, high-concentrated standards, dissimilar influence of the matrix on different analytes, and dissimilar ionisation of the same analyte in different matrices (Cortese et al., 2020).

Based on published data, ME in LC-MS/MS methods for quantification of LT are widely recognised and represent a significant issue due to the lack of internal standards commonly used to reduce ME (Gerssen et al., 2009; Kilcoyne and Fux, 2010; Rúbies et al., 2015; Wang and Doucette, 2021; D'Amore et al., 2022). Various techniques are employed for ME evaluation, including post-column infusion, post-extraction spiking, and its modification known as "slope ratio analysis" (Bonfiglio et al., 1999; Matuszewski et al., 2003; Sulyok et al., 2007; Romero-González et al., 2011). Validation of LC-MS/MS methods for LT is predominantly conducted on commercially exploited bivalve species, such as Mediterranean mussels and European oysters in Croatia (Matić-Skoko et al., 2017). However, given the diversity of species, the most appropriate approach is to validate the method for all species in which LT are determined in a particular laboratory.

In this study, ME was evaluated using the slope ratio analysis method, which involves comparing the slopes of the calibration curves in the matrix (matrix-matched - MM) with those in pure solvent. When the slopes do not differ significantly, ME may be considered negligible (Matuszewski et al., 2003). A lower slope value for MM calibration indicates ion suppression, while a higher value suggests ionisation

enhancement (Zhou et al., 2017). This study aimed to investigate the extent of ME in the most commonly studied shellfish species: Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) and European oyster (*Ostrea edulis* Linnaeus, 1758), and in the less studied queen scallop (*Aequipecten opercularis* Linnaeus, 1758) and rarely explored edible ascidians of the *Microcosmus* spp. The evaluation covered all phycotoxins under Regulation EU 853/2004 for which certified standards are commercially available, including unregulated PTX2. Apart from assessing the intensity and specificity of ME on each analyte, interspecies differences in ME were also compared, aiming to determine the possibility of utilising one of the analysed species to construct a calibration curve that could be used for quantifying phycotoxins in others.

Materials and methods

Chemicals and analytical standards

Certified calibration solutions CRM-OA-d (10.4 ± 0.5 µmol/L), CRM-DTX1-b (10.4 ± 0.8 µmol/L), CRM-DTX2-b (4.7 ± 0.3 µmol/L), CRM-PTX2-b (5.13 ± 0.15 µmol/L), CRM-AZA1-b (1.54 ± 0.08 µmol/L), CRM-AZA2-b (1.43 ± 0.07 µmol/L), CRM-AZA3-b (1.43 ± 0.06 µmol/L), CRM-YTX-c (4.3 ± 0.2 µmol/L), CRM-hYTX (5.0 ± 0.3 µmol/L), were obtained from the National Research Council Canada, Institute for Marine Bioscience (Halifax, Canada). LC-MS grade methanol and acetonitrile, and formic acid and ammonium formate, were obtained from Honeywell (Seelze, Germany), ultrapure water was obtained from a Milli-Q water purification system (Millipore S.A.S., Molsheim, France).

Extract preparation

Uncontaminated bivalve and ascidian samples (previously analysed with the reference method for LT) were used to prepare blank methanolic matrix extracts intended for standard addition. Sample extraction followed the EU-Harmonised Standard Operating Procedure for the determination of lipophilic marine biotoxins in molluscs (EU-RL-MB, 2015) with minor modifications. After separation from the shell or tunic, 2 g homogenized soft tissue was extracted with 20 mL methanol. The modification of the original method involved mixing the extract with an aqueous mobile phase in equal proportions and filtration through 0.22 µm PTFE filters (Restek, Shanghai, China) to prevent the formation of possible precipitations in the LC-MS/MS. Extracts of each species prepared as previously described were spiked with certified standard phycotoxin solution at five concentration levels, ranging from 6 to 600 µg/kg (0.3 to 30 ng/mL) for the OA group and PTX2, 3 to 300 µg/kg (0.15 to 15 ng/mL) for the AZA group, and 75 to 1500 µg/kg (3.75 to 75 ng/mL) for the YTX group. The same procedure was conducted using methanol instead of sample extracts. Standard solutions in methanol were injected in parallel with extracts of shellfish and ascidians spiked with the standard phycotoxin solution in triplicate to construct MM calibration curves, and in solvent. The mean value of the calibration curve slopes in solvent was compared to the mean value of the slopes of matrix-matched calibration curves for each of the matrices. The same comparison was also performed between different matrices. The signal suppression/enhancement (SSE) due to matrix effects was calculated using the following equation (Equation 1).

Equation 1

$$SSE\ (\%) = 100 \times slope_{\text{MM calibration curve}} / slope_{\text{calibration curve in solvent}} \quad \textbf{(1)}$$

LC-MS/MS analyses

The determination of LT was carried out following the procedure for the determination of lipophilic marine biotoxins in molluscs (EU-RL-MB, 2015), with a modification in the mobile phase composition. LC-MS/MS analysis was performed using a 1290 Infinity UPLC system (Agilent Technologies, Singapore), coupled with a G6460 Electrospray Ionisation Triple Quad Mass Spectrometer (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was achieved on a Zorbax SB-C8RRHD 2.1×50 mm, 1.8 µm column with a Zorbax SB-C8 2.1×5 mm, 1.8 µm guard column (Agilent Technologies, Santa Clara, USA). An acidic mobile phase was employed, with the modification involving the use of 10 mM ammonium formate instead of the 2 mM concentration.

Data analysis

Construction of linear calibration curves and data analysis were performed using software Microsoft Excel 2019 MSO, Version 2301 Build 16.0.16026.2002 (Microsoft, SAD).

Results and discussion

The comparison of the slopes of the MM calibration curves with those in pure solvent indicates the presence of ME in all investigated matrices and for all analytes, albeit with varying intensity and manner with regard to ionisation. Ion suppression was evident for all analytes in all matrices (Figures

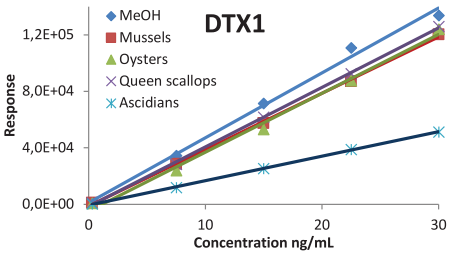

Figure 1. Dinophysistoxin 1 (DTX1) calibration curves

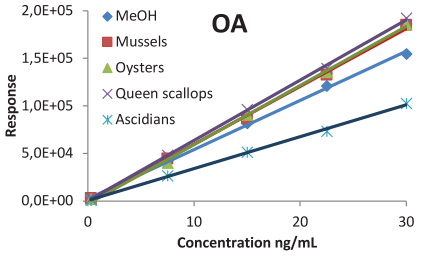

Figure 2. Okadaic acid (OA) calibration curves

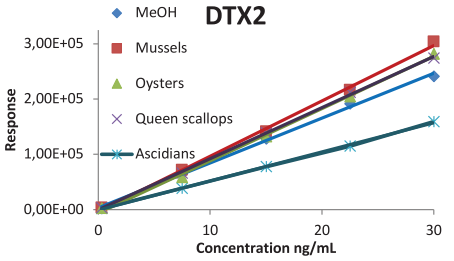

Figure 3. Dinophysistoxin 2 (DTX2) calibration curves

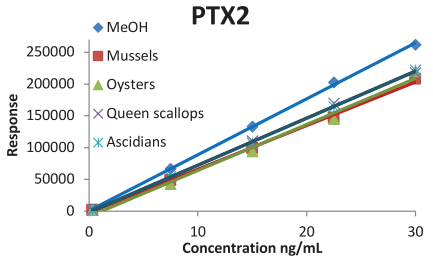

Figure 4. Pectenotoxin 2 (PTX2) calibration curves

1, 4–9), except for OA and DTX2 in bivalves, where ion enhancement was demonstrated (Figures 2 and 3). The extent of ME is presented in Table 1 as SSE values, calculated using Equation 1. A value higher than 100% indicates ionisation enhancement, while a value lower than 100% indicates ionisation suppression. Established deviations of SSE values were higher than 10% for all analytes in every matrix compared to calibration in solvent, indicating that the ME on ionisation is significant (NATA, 2012). The only exception was DTX1 in oyster and queen scallop extracts, for which ME was lower than 10%.

In the case of OA, ionisation enhancement ranged from 18% in mussel to 23% in scallop extract, while for DTX2, it ranged from 14% in scallop to 23% in mussel extract. In ascidian extract, ion suppression of 35% was recorded for both analytes, with maximal suppression of 62% recorded for DTX1 in the same matrix. Concerning PTX2, the largest ion suppression of 22% was established in mussel, while the lowest (16%) was observed in scallop and ascidian extract. When it comes to azaspiracids, similar ion suppression was noticed for AZA1 and AZA2, with the largest suppression in the scallop matrix (22% and 20% respectively) and the lowest in oyster extract (12% and 13%). However, for AZA3, the suppression was significantly higher, ranging from 26% in ascidian to 32% in scallop extract. Among analytes of the YTX

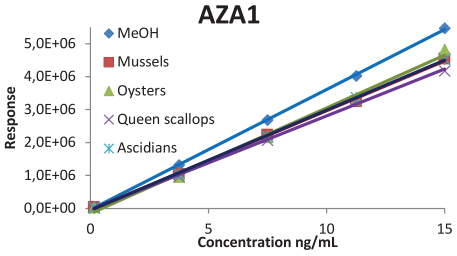

Figure 5. Azaspiracid 1 (AZA1) calibration curves

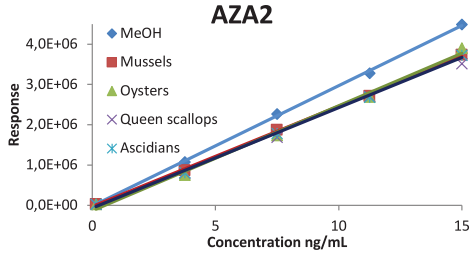

Figure 6. Azaspiracid 2 (AZA2) calibration curves

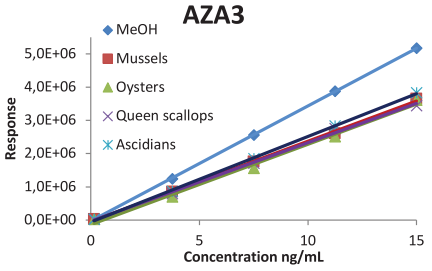

Figure 7. Azaspiracid 3 (AZA3) calibration curves

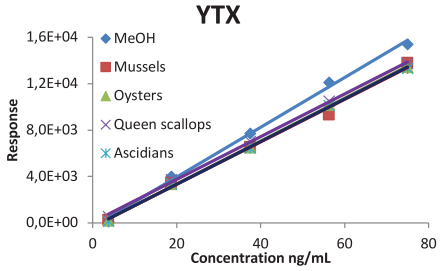

Figure 8. Yessotoxin (YTX) calibration curves

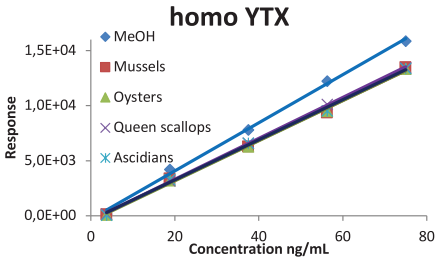

Figure 9. Homo yessotoxin (homo YTX) calibration curves

group, ion suppression was nearly equal in all matrices, ranging from 14% to 17%.

The obtained results indicate that the intensity of ME and its influence on ionisation are influenced by both the matrix and the chemical characteristics of the analyte, which is in accordance with previous studies. In their research on the influence of solid-phase extraction (SPE) on the reduction of ME in LC-MS/MS methods for LT, Gerssen et al. (2009) found that all LT are prone to ME, but to different extents. By applying the same method parameters for extraction, chromatographic conditions, and ionisation polarity as in the present study, those authors found the largest value of ion enhancement for OA in the great scallop (*Pecten maximus*) (103%) and for PTX2 in oysters (*Crassostrea gigas*) (40%), while in extract (*Mytilus edulis*), signal suppression of 39% was observed for OA. They established ion enhancement for YTX, which was most pronounced in mussel extract (25%), while in this study, signal suppression was recorded in all investigated matrices. Signal suppression was found for AZA1, but with substantial differences among matrices. García-Altares et al. (2013), in their validation study of the method for the determination of LT in various shellfish species, found different MEs in the ionisation of particular LT. Applying the same chromatographic

Table 1. Signal suppression/enhancement (SSE) of phycotoxins determined in mussel, oyster, scallop and ascidian extracts

Matrix	SSE	OA	DTX1	DTX2	PTX2	AZA1	AZA2	AZA3	YTX	Homo YTX
Mussels	SSE[a]/%	118	87	123	78	83	83	70	85	83
Oysters	SSE[a]/%	120	91	117	82	88	87	70	86	83
	SSE[b]/%	102	104	94	106	106	105	100	101	100
Scallops	SSE[a]/%	123	92	114	84	78	80	68	86	85
	SSE[b]/%	104	106	92	108	94	96	98	101	103
	SSE[c]/%	102	101	98	102	89	92	98	100	102
Ascidians	SSE[a]/%	65	38	65	84	83	83	74	86	83
	SSE[b]/%	55	43	53	108	100	100	106	100	100
	SSE[c]/%	54	42	56	102	95	96	107	99	100
	SSE[d]/%	53	41	57	100	106	104	109	100	98

SSE signal suppression/enhancement; OA okadaic acid, DTX dinophysistoxin; PTX pectenotoxin; AZA azaspiracid; YTX yessotoxin; [a]SSE in regard to solvent; [b]SSE in regard to mussel matrix-matched calibration; [c]SSE in regard to oyster matrix-matched calibration; [d]SSE in regard to scallop matrix-matched calibration

conditions as in our study, the authors established signal enhancement of OA, most prominently in mussel (*M. galloprovincialis*) and oyster (*C. gigas*) extracts, 65% and 54%, respectively. Ion suppression for AZA1 was evident in all matrices and for YTX and PTX2 in the majority of the investigated matrices.

With the exception of the OA group of phycotoxins in the ascidian matrix, the somewhat smaller ME presented here in our research could be explained by a minor modification of the original method, i.e., the dilution of the crude matrix extract with an aqueous mobile phase prior to instrumental analysis. Dilution of samples is a known method used to reduce ME (Qiu et al., 2020). In their research on the influence of the matrix of mussels (*M. galloprovincialis*), oysters (*Crassostrea* sp.), scallops (*Chlamys farreri*), and clams (*Ruditapes philippinarum*) on the ionisation of phycotoxins using the same method, the authors concluded that ME is only analyte-dependent. In contrast to our research, they found significant ion suppression of OA in all matrices, ranging from 55% to 76%, as well as ion enhancement in the case of PTX2 and YTX in the majority of matrices. Even though ion suppression for DTX1 was established in both studies, the authors reported it to be significantly higher in bivalves.

This study found that all LTs are susceptible to ME with the use of the LC-MS/MS method for their determination. Several studies, including Qiu et al. (2020), have explored strategies aiming to minimise ME in the LC-MS/MS analysis of LT. They recommended selecting $[M-H]^-$ over $[M+Na]^+$ precursor ions for OA and DTX1, as the former ion is less susceptible to ME. This difference in ion selection could explain the significant ME observed on OA in bivalves in the present study, where the $[M+Na]^+$ precursor ion was chosen. Other strategies for reducing ME on phycotoxin ionisation were evaluated, including solid phase extraction SPE (Gerssen et al., 2009; Kilcoyne and Fux, 2010; Cefas, 2011; Regueiro et al., 2011; Qiu et al., 2020; Wang and Doucette, 2021; D'Amore et al., 2022), the QuEChERS method (Rúbies et al., 2015; Wang et al., 2019), matrix solid-phase dispersion (Qiu et al., 2020), adjustment of chromatographic conditions (Fux et al., 2008; Kilcoyne and Fux, 2010; Qiu et al., 2020; Wang and Doucette, 2021), injection volume reduction (Wang and Doucette, 2021), extract dilution (Fux et al., 2008; Cefas, 2011; Qiu et al., 2020), standard addition (Ito and Tsukada, 2002) and MM calibration (García-Altares et al., 2013; Wang et al., 2019; Kvrgić et al., 2021b; Wang and Doucette, 2021). Although the application of these methods resulted in a reduction of matrix influence on the ionisation of LT, they have some disadvantages. Some are labour and resource-intensive, and most are only partially effective since they reduce ME for particular phycotoxins, while having no influence or even increasing it for others.

MM calibration has been shown to effectively reduce ME, though with certain disadvantages. It is impractical and requires larger quantities of standard solutions compared to calibration in solvent, particularly when analysing multiple shellfish species simultaneously, due to the varying effects on ionisation among species. To address this issue, one of the aims of this study was to explore the feasibility of using one matrix to construct the MM calibration, which could then be applied for quantification across all matrices. By comparing the slopes of MM calibration curves among mussel, oyster, scallop, and ascidian extracts, we determined that the SSE values for AZA1-3, YTX and homo YTX were within the

range of ±10% (Table 1), indicating that ME is negligible. However, exceptions were observed, particularly with the OA group, where significant ion suppression of OA, DTX1 and DTX2 was observed in the ascidian matrix compared to bivalve matrices. Given the minor difference of the ratio between the slope of mussel MM calibration and the slope of other bivalve matrices, the mussel MM calibration curve could be applied to minimise ME and quantify LT in bivalve matrices analysed in this study as well as in ascidians, with the exception of the OA group in ascidians.

Given the risks phycotoxins pose to human health, there is a need for ongoing development and improvement of reliable analytical methods to detect these contaminants. Considering the consequences of increasingly pronounced climate change, such as the emergence of new phycotoxins in areas and marine species where they were previously undetected, it is essential to also include these new compounds in analytical methods. Future research on strategies for overcoming ME should also encompass new compounds in diverse matrices. The validation of methods for the determination of shellfish toxicity levels should include even less prominent species to expand the applicability of the methods to species other than bivalves that accumulate phycotoxins. The toxic effects of phycotoxins underscore the necessity for ongoing national surveillance of their occurrence in marine species to ensure consumer safety.

Conclusion

This study demonstrated the impact of matrix interference in the LC–MS/MS analysis of LT in bivalve molluscs and ascidians. A significant effect on LT ionisation was observed in all investigated matrices. In order to compensate for matrix effects, a MM calibration curve could be used for quantitation purposes. To make it more feasible when analysing more than one species, a Mediterranean mussel matrix-matched calibration curve could be applied for matrix effect correction in the quantification of LT in European oysters, queen scallops and ascidians, with the exception of OA group in ascidians, which instead requires a matrix-matched calibration prepared with blank extract of the mentioned species. The unpredictability of ME on the ionisation of LT in the LC-MS/MS method underscores the importance of thorough examination in individual laboratories for every matrix and LT as part of the validation study. The analytical methods developed in this study are applicable for the analysis of lipophilic toxins in different seafoods, taking into account that these methods require constant development to better control food safety and to protect consumer health.

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Procjena utjecaja matriksa na kvantifikaciju lipofilnih toksina u plodovima mora

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Nakupljanje fikotoksina u plodovima mora može prouzročiti trovanja ljudi i znatne ekonomske gubitke u područjima koja se bave njihovim uzgojem. U cilju zaštite potrošača, zakonodavstvom su utvrđene najveće dopuštene količine (NDK) u dvoljušturnim školjkašima, kao i analitičke metode koje se primjenjuju u njihovom određivanju. Referentna metoda za određivanje lipofilnih fikotoksina ili lipofilnih toksina (LT) je tekućinska kromatografija u sprezi sa spektrometrijom masa (LC-MS/MS). Prilikom primjene ove metode, različite komponente matriksa mogu utjecati na ionizaciju analita i dovesti do pogrešne kvantifikacije. Cilj je ovog istraživanja bio ispitati utjecaj matriksa dagnji, kamenica, kapica i mješčićnica na ionizaciju LT usporedbom nagiba kalibracijskih pravaca u matriksu i otapalu, a odnosi se na fikotoksine za koje su dostupne certificirane standardne otopine i za koje su Uredbom EU 853/2004 utvrđene NDK, uključujući pektenotoksin 2 (PTX2). Rezultati istraživanja ukazuju na to da su navedeni LT podložni utjecaju matriksa ukoliko se primjenjuje LC-MS/MS metoda za njihovo određivanje. Uočena je značajna supresija ionizacije većine analita u svim matriksima, osim u slučaju okadaične kiseline (OA) i dinofizistoksina 2 (DTX2) u školjkašima, kod kojih je uočeno značajno pojačanje ionizacije te dinofizistoksina 1 (DTX1) za kojeg nije uočen značajan utjecaj matriksa na ionizaciju u ekstraktu kamenica i kapica. Usporedbom nagiba kalibracijskog pravca u ekstraktu dagnji s onima u ekstraktu ostalih školjkaša nisu utvrđene značajne razlike, stoga se kalibracijski pravac u ekstraktu dagnji može

primijeniti za kvantifikaciju LT i u ostalim vrstama obuhvaćenim ovim istraživanjem. Izuzetak su toksini OA skupine u mješčićnicama, za čiju je kvantifikaciju potrebno primijeniti kalibracijski pravac u ekstraktu navedene vrste. S obzirom na opasnost koju fikotoksini predstavljaju za zdravlje ljudi, neophodno je neprestano razvijati pouzdane analitičke metode za njihovo određivanje, koje će doprinijeti kontroli kvalitete hrane i sigurnosti potrošača.

Ključne riječi: *lipofilni toksini, LC-MS/MS, utjecaj matriksa, dvoljušturni školjkaši, mješčićnice*