CHALLENGES IN MYCOTOXINS DETERMINATION IN HIGHLY PIGMENTED FRUITS

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Abstract: Mycotoxins are food and feed contaminants. They exhibit high toxicity to living organisms. Therefore, their concentration in products intended for consumption should be strictly monitored. Due to the increasingly stringent standards related to mycotoxin content in food, the requirements for mycotoxin determination methods are constantly increasing. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is a gold standard considering the determination of mycotoxins and many other groups of compounds. However, this technique is burdened with some limitations connected to e.g. matrix effect (ME). Thus, we proposed polymeric materials that can be applied during the sample preparation step toward mycotoxins determination via LC-MS/MS. In this paper, we present the preliminary results on evaluating ME toward 8 mycotoxins before and after the clean-up of blackcurrant extract employing microspheres of 4-vinylpyridine crosslinked with trimethylolpropane trimethacrylate (poly(4VP-*co*-TRIM) or 1,4-dimethacryloyloxybenzene (poly(4VP-*co*-14DMB).

Introduction: Mycotoxins are secondary metabolites produced by various species of filamentous fungi. They are formed on various agricultural commodities both before and after harvest. Fruits are especially susceptible to being infected since they can be easily mechanically damaged by insects and animal bites, during unfavorable weather conditions or harvesting and postharvest handling. Mycotoxins exhibit high toxicity toward living organisms [1,2]. They can cause cancer, allergies, and organ toxicity [3] as well as many other abnormalities and malfunctions in humans and animals, e.g. hepatocellular carcinoma, abnormalities in fetal development, renal failure, interstitial nephropathy, and urothelial tumors [4]. Various mycotoxins exhibit hepatotoxic, neurotoxic, teratogenic, and nephrotoxic properties [4]. Due to their hazardousness, the content of mycotoxins in food and feed should be strictly monitored. Extremely high restrictions are applied to baby food [5-7]. Therefore, the relevant authorities constantly update the acceptable levels of toxins in various types of food [5-7]. Due to the high toxicity of mycotoxins as well as the restriction imposed by the relevant institutions and consequently the regulatory recommendations, new LC-MS/MS methods characterized by lower limits of detection and quantification as well as lower ME are in demand. Therefore, considering our previous findings [8], we propose polymeric materials that could be applied in the dispersive solid phase extraction (dSPE) step during the sample preparation protocol before the LC-MS/MS determination of mycotoxins in soft and highly pigmented fruits. We found that the polymeric materials effectively adsorbed polyphenolic compounds (especially flavonoids and anthocyanins) [8]. Their effectiveness was even greater than primary secondary amine and graphitized carbon black toward flavonoids removal [8]. Thus, studied polymers have the potential to be used to purify extracts of highly pigmented fruits before LC-MS/MS analysis.

Experimental: Microspheres were washed with acetonitrile (ACN, hypergrade) (4 times x 4 min (shaken), the ratio of polymeric material to ACN was 10 mg: 1 mL. Each time the mixture was centrifuged after shaking, and the organic solvent was discarded. Before further use, polymeric material was evaporated to dryness in the Genevac EZ-2 Elite Personal Evaporator to remove ACN residue. To determine the ME, 10 g of fruit pulp (blackcurrant) was vigorously hand-shaken with 10 mL of ACN for 1 min. Then, QuEChERS salt (1 g $MgSO_4$, 0.25 g NaCl, 0.25 g sodium citrate, 0.125 g sodium hydrogen citrate sesquihydrate) was added and the sample was mixed again for 1 min followed by centrifugation (10 min, 8228 × g) using the 5408 Eppendorf centrifuge. Then, 1 mL of the obtained extract was transferred into a tube containing 10 mg of poly(4VP-*co*-TRIM) or poly(4VP-*co*-14DMB) (dSPE step), shaken for 2 min, and centrifuged using the 5804 Centrifuge for 10 min at 16000 × g. A 600 μL of purified supernatant was transferred into a glass vial and evaporated until dry. The residue was dissolved in 600 μL of H_2O:0.2 mol/L NH_4HCO_3:CH_3OH (30:5:65, *v/v/v*), vortexed and sonicated. The obtained solution was fortified or not with a mixture of mycotoxins standards. ME was calculated using the equation: $ME[\%] = A/B \cdot 100$, where a is the average area of the analyte signal in the sample fortified before extraction, B is the average analyte signal in a standard solution prepared in a neat solvent [9]. The concentration levels of fortification (A and B) are presented in Table 1.

Table 1. Selected mycotoxins, their basic characterization, and the levels of fortifications in experimental samples.

Mycotoxins	CAS	Molecular formula	Levels of sample fortification [μg/kg]
Penicillic acid	90-65-3	$C_8H_{10}O_4$	25
Fusarenon X	23255-69-8	$C_{17}H_{22}O_8$	50
Neosolaniol	36519-25-2	$C_{19}H_{26}O_8$	25
Aflatoxin B_1	1162-65-8	$C_{17}H_{12}O_6$	3.125
Aflatoxin G_1	1165-39-5	$C_{17}H_{12}O_7$	6.25
Aflatoxin G_2	7241-98-7	$C_{17}H_{14}O_7$	3.125
Ochratoxin A	303-47-9	$C_{20}H_{18}ClNO_6$	25
T-2 toxin	21259-20-1	$C_{24}H_{34}O_9$	12.5

The prepared samples were centrifuged (10 min, 16000 × g) and filtered through 0.22 μm syringe hydrophilic filters. a sample was analyzed in triplicate using the 1290 series UHPLC system (Agilent Technologies, Santa-Clara, CA, USA) consisting of a degasser, binary pump, autosampler, column thermostat and controlled by Agilent MassHunter Acquisition software v.B.08. The apparatus was coupled with a 6460 triple quadrupole mass spectrometer (Agilent Technologies) equipped with an electrospray ion source (Agilent Jet Stream). For chromatographic separation, an HPLC column Shim-pack Scepter C18-120, 1.9 μm, 2.1 mm x 100 mm was used. The mobile phase components were: H_2O:0.2 mol/L NH_4HCO_3 (95:5, *v/v*) (solvent A) and CH_3OH:0.2 mol/L NH_4HCO_3 (95:5, *v/v*) (solvent B). The flow rate and the temperature were set to 0.25 mL/min and 40 °C. To obtain appropriate separation of the signals, the following gradient program was used: 0–2 min 5-20% solvent B, 2–4 min 20–40% solvent B, 4–10 min 40–95% solvent B, 10-12 min 95% solvent B, and postrun 2 min. The detection was performed using dynamic Multiple Reaction Monitoring (dMRM) mode (Table 2). Data were analyzed with Agilent MassHunter Quantitative Analysis software v.B.07.

Table 2. Settings of the dMRM method applied for selected mycotoxins determination in positive polarity.

Mycotoxins	RT [min]	Transitions	F [V]	CE [eV]
Penicillic acid	3.25	171 > 153 171 > 125	60 60	4 10
Fusarenon X	5.34	372.1 > 355 372.1 > 247	80 80	8 8
Neosolaniol	5.62	400.1 > 185 400.1 > 214.9	80 80	10 24
Aflatoxin B_1	7.96	313 > 285 313 > 270	100 100	24 34
Aflatoxin G_1	7.38	329 > 243 329 > 311	140 140	24 24
Aflatoxin G_2	7.07	331 > 313 331 > 285	80 80	30 30
Ochratoxin A	7.55	404 > 358 404 > 239	100 100	10 24
T-2 toxin	9.30	484.2 > 185 484.2 > 215	100 100	14 16

RT – retention time; F – fragmentor voltage; CE – collision energy

Results: Our study aimed to assess the ME during the determination of selected mycotoxins (Table 1) via LC-MS/MS analysis. We chose the blackcurrant matrix as a representative of a highly pigmented fruit. To the clean-up step, we applied two polymeric materials: poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB). The results from the preliminary studies indicated that studied polymers decreased ME during the quantitative determination of selected mycotoxins via LC-MS/MS. The reduction of ME was observed in the case of penicillic acid, aflatoxin B_1, aflatoxin G_1, aflatoxin G_2, and ochratoxin a (Table 3). In other cases, ME remained constant or slightly increased (fusarenon X, neosolaniol, T-2 toxin) (Table 3). The results are promising. Due to the disadvantages of commercially available dSPE material, searching for new effective materials for toxins determination is an up-to-date research goal. ME can be assessed considering the following division: negligible (ME > 90 and <110%), soft (80–90% and 110–120%); medium (50–80% and 120–150%); and strong (ME < 50% and <150%) [9]. Furthermore, ME values >100% and <100% mean ion enhancement and suppression, respectively. In terms of LC-MS/MS analysis of unpurified blackcurrant extract, medium and strong suppression of mycotoxin signals is observed. However, the application of poly(4VP-*co*-TRIM) or poly(4VP-*co*14DMB) improves the studied parameter. Moreover, the color of the acetonitrile extract of the blackcurrant after dSPE with poly(4VP-*co*-TRIM) or poly(4VP-*co*-14DMB) was reduced significantly in comparison to the unpurified extract.

Table 3. The ME of determined mycotoxins in samples with and without the dSPE step (n=2).

Mycotoxins	ME ± SD [%]		
	without dSPE step	poly(4VP-*co*-14DMB	poly(4VP-*co*-TRIM
Penicillic acid	8.73 ± 0.80	20.53 ±2.35	15.69 ± 0.63
Fusarenon X	12.83 ± 0.14	11.86 ± 0.40	12.20 ± 0.24
Neosolaniol	36.39 ± 0.27	30.91 ± 1.70	31.54 ± 0.39
Aflatoxin B_1	50.40 ± 1.48	61.98 ± 3.99	62.86 ± 1.84
Aflatoxin G_1	38.54 ± 0.76	53.36 ± 3.28	54.03 ± 1.30
Aflatoxin G_2	38.17 ± 1.71	45.67 ± 3.91	42.62 ± 2.44
Ochratoxin A	54.59 ± 0.97	73.00 ± 5.97	64.98 ± 3.51
T-2 toxin	74.37 ± 1.89	71.34 ± 4.68	72.40 ± 1.09

SD – standard deviation

Conclusions: The application of poly(4VP-*co*-TRIM) or poly(4VP-*co*-14DMB) as sorbents in the dSPE step improves the analytical performance (especially ME) of the LC-MS/MS method for the multi-mycotoxins determination. The reduction of ME of a greater number of studied mycotoxins is significant, thus our next step will be the optimization of various sample preparation steps to obtain the best analytical parameters that will meet the current recommendations for analytical methods.

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