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How different microfilters affect the recovery of eleven EU-regulated mycotoxins

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Microfiltration is a common step in liquid chromatography – tandem mass spectrometry (LC-MS), a method of choice in determining several mycotoxins in a solution at once. However, microfiltration may entail filter-analyte interactions that can affect the accuracy of the procedure, and underestimate exposure. The aim of our study was to assess how five different membrane materials for syringe filters (nylon, polytetrafluoroethylene, polyethersulphone, mixed cellulose ester, and cellulose acetate) affect microfiltration and recovery of EU-regulated mycotoxins, including aflatoxins B1, B2, G1, and G2, deoxynivalenol, fumonisins B1 and B2, zearalenone, T-2 and HT-2 toxins, and ochratoxin A. Polytetrafluoroethylene filters turned out to least affect microfiltration through mycotoxin loss, followed by more commonly used nylon filters, whereas the remaining three filter membrane materials had such a negative effect on recoveries that we found them incompatible with the procedure. Our findings clearly suggest that it is important to select a proper filter type that suits analyte properties and solution composition and to discard the first few filtrate drops to ensure the accuracy of the analytical procedure.

KEY WORDS: filter-analyte interaction; liquid chromatography-tandem mass spectrometry; underestimation; sample preparation

Reliable analytical methods for mycotoxin determination, preferably employing ultra-high-performance liquid chromatography coupled to (tandem) mass spectrometry (UHP)LC-MS/(MS) for unambiguous detection (1) are nowadays imperative to establish product safety and gather accurate mycotoxin occurrence data for proper assessment of the risk that these contaminants pose to public health.

Numerous methods have been developed over the years, from the enzyme-linked immunosorbent assay (ELISA) to chromatographic methods employing thin-layer chromatography (TLC), gas chromatography (GC), and liquid chromatography (LC), which use various detection methods and sample preparation procedures (2). The introduction of mass spectrometry (MS) to contaminant analytics brought the opportunity to determine a large number of chemically distinct compounds in a relatively short time and to gain a better insight into the real extent of mycotoxin contamination. In addition to greater reliability compared to the conventional detectors, these new methods have streamlined sample preparation, which saves time and lowers the overall cost of analysis (1, 3).

Preparation of samples containing several mycotoxins for liquid chromatography – tandem mass spectrometry (LC-MS/MS) often relies on the so-called *dilute-and-shoot* principle (1, 4–9) to avoid strong purification and to preserve the analytes as much as possible. As the name suggests, the dilute-and-shoot principle implies analyte extraction with a suitable solvent and dilution in appropriate ratio before injection into an LC-MS/MS system (1). Depending on the chromatography system used for separation and the pressure it uses, whether it is high-performance or ultra-high-performance liquid chromatography (HPLC or UHPLC, respectively), additional sample preparation steps are taken, such as microfiltration, usually with syringe filters, in order to protect the instrument from small particles, that is, to avoid clogging of LC tubing and to reduce the matrix effect, which is very important, since no further clean-up is applied before sample injection (7, 10, 11).

However, analytes can interact and stick with the filter membrane, which may result in their underestimation. These interactions between the analyte and the filter depend on the analyte's physicochemical properties, molecular weight, or ionisation state or on membrane structure, hydrophobicity, or hydrophilicity. All these considerations should drive the selection of appropriate filtrate membrane, which, in addition, should take into account sample

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medium properties and its compatibility with the membrane material (11, 12).

As far as mycotoxins are concerned, several hundred fungal metabolites have been discovered so far (9), yet only a few are regulated by law. In the European Union (EU), maximum permitted levels of mycotoxins are regulated by the Commission Regulation (EC) 1881/2006 (13) for food and Directive 2002/32/EC (14) for animal feed (Figure 1). When it comes to their determination, Malachová et al. (7) have found that filtration is not an option for their analytical procedure, as the loss of certain mycotoxins is too great. Kafouris et al. (8) and Kovač et al. (1), in turn, have reported that filtering solutions before instrumental analysis yields satisfactory results, conforming to method performance criteria.

The aim of this study was, therefore, to investigate how various filter membrane materials affect the recovery of the EU-regulated mycotoxins and to determine the extent of mycotoxin loss to dilute-and-shoot sample preparation procedure.

MATERIALS AND METHODS

Chemicals and materials

Certified analytical standards of the following mycotoxins were obtained from Romer Labs Biopure (Romer Labs, Tulln, Austria): AFB1, AFB2, AFG1, and AFG2 (2.0 g/mL for AFB1/AFG1, 0.5 g/mL for AFB2/AFG2), DON (100 g/mL), FB1 and FB2 (50 g/mL), ZEA (100 g/mL), T-2 (100 g/mL), HT-2 (100 g/mL), and OTA (10 g/mL). LC-MS-grade acetonitrile (ACN) and methanol (MeOH) were obtained from J. T. Baker (J. T. Baker, Deventer, The Netherlands). LC-MS-grade formic acid (FA) and LC-MS ammonium formate ($AFNH_4$) were supplied by Sigma-Aldrich (Sigma-Aldrich, Louis, MO, USA). Water (H_2O) was purified to the ultrapure grade with a Niro VV (Nirosta d.o.o., Osijek, Croatia) or a Purelab flex system (ELGA LabWater, Woodridge, IL, USA). Syringe filters (13 mm in diameter with a 0.22 μm membrane pore size) were obtained from the following suppliers: nylon (NY) from Kemolab d.o.o. (Veliko Polje, Croatia), polytetrafluoroethylene (PTFE) from Kefo d.o.o. (Sisak, Croatia), while polyethersulphone (PES), mixed cellulose ester (MCE), and cellulose acetate (CA) were obtained from Obrnuta faza d.o.o. (Pazin, Croatia).

Preparation of the multi-mycotoxin solution

The multi-mycotoxin standard solution was prepared by mixing the eleven mycotoxin standards with ACN, and then by diluting this mixture one hundred times with a solvent mixture of ACN/H_2O/FA (49.5/49.5/1, v/v/v) as shown in Table 1 to obtain analyte concentrations corresponding to the middle calibration point of the previously developed and validated multi-mycotoxin method by Kovač et al. (1). This solvent mixture composition for standard dilution matches the ratio of solvent components in real dilute-and-shoot without the matrix.

Microfiltration and mycotoxin recovery calculation

The multi-mycotoxin standard solution was microfiltered in such a way that the first three filtrate drops (filtered solution 1) and the following filtrate (filtered solution 2) were collected separately into two different vials and injected (in triplicate) into a UHPLC-MS/MS system. The unfiltered multi-mycotoxin solution was injected into the system as is (Figure 2). The whole procedure was performed in duplicate. To process the obtained data on peak areas for each compound, we took averages of repeated measurements and calculated the relative recovery (expressed with a percentage) for each mycotoxin and filter membrane material by comparing the average area of the filtered solution (1 or 2) with unfiltered standard solution according to Equation 1, as follows:

$$Relative\ recovery\ \% = \frac{Peak\ area_{\ filtered\ solution\ 1\ or\ 2}}{Peak\ area_{\ unfiltered\ solution}} \times 100 \quad (1)$$

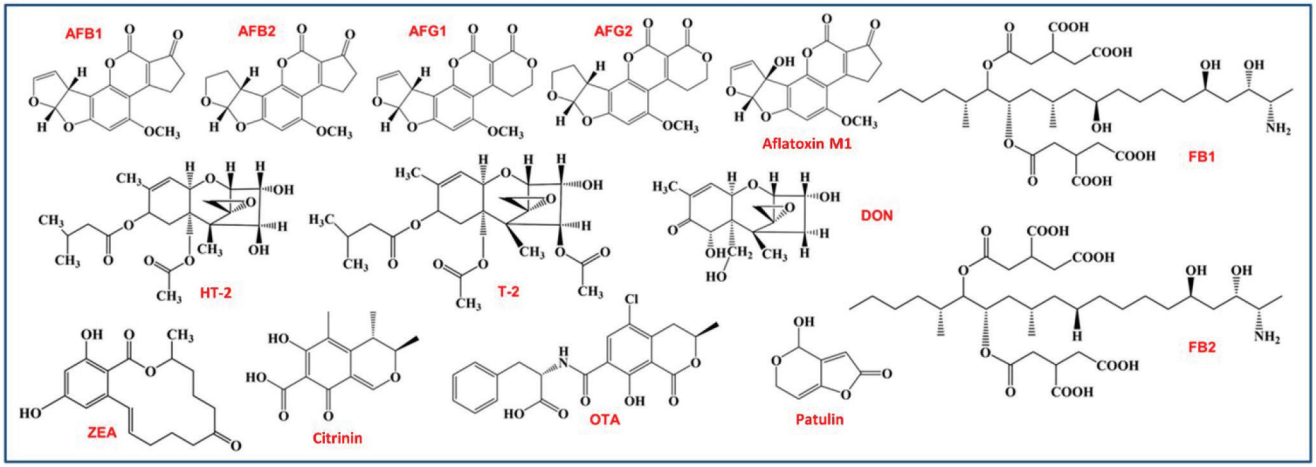

Figure 1 Chemical structures of EU-regulated mycotoxins (excluding ergot alkaloids)

Table 1 Preparation of the multi-mycotoxin standard solution

Analyte	Concentration of analytical standard (µg/mL)	Volume of analytical standard for preparation of 1000 µL of combined multi-mycotoxin standard solution* (µL)	Concentration of combined multi-mycotoxin standard solution (ng/mL)	Concentration of 100× diluted combined multi-mycotoxin standard solution (ng/mL)
DON	100	50	5000	50
AFB1	2.0	25	50	0.5
AFB2	0.5	25	12.5	0.125
AFG1	2.0	25	50	0.5
AFG2	0.5	25	12.5	0.125
FB1	50	75	3750	37.5
FB2	50	75	3750	37.5
ZEA	100	7.5	750	7.5
T-2	100	2.5	250	2.5
HT-2	100	2.5	250	2.5
OTA	10	2.5	25	0.25
Volume of combined multi-mycotoxin standard solution for preparation of 1000 µL of 100× diluted combined multi-mycotoxin standard solution** (µL)				10

*pure ACN; **ACN/H_2O/FA (49.5/49.5/1, v/v/v)

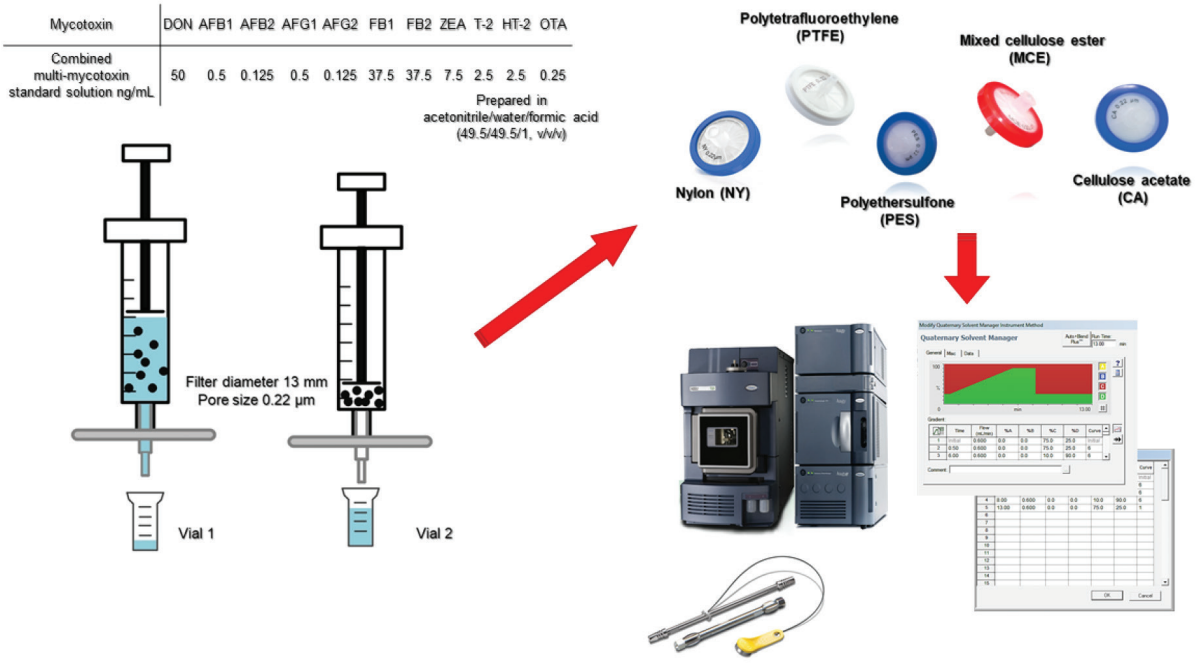

Figure 2 Microfiltrate preparation. Filtrate solutions (vials) 1 and 2 were injected into the UHPLC column one at a time

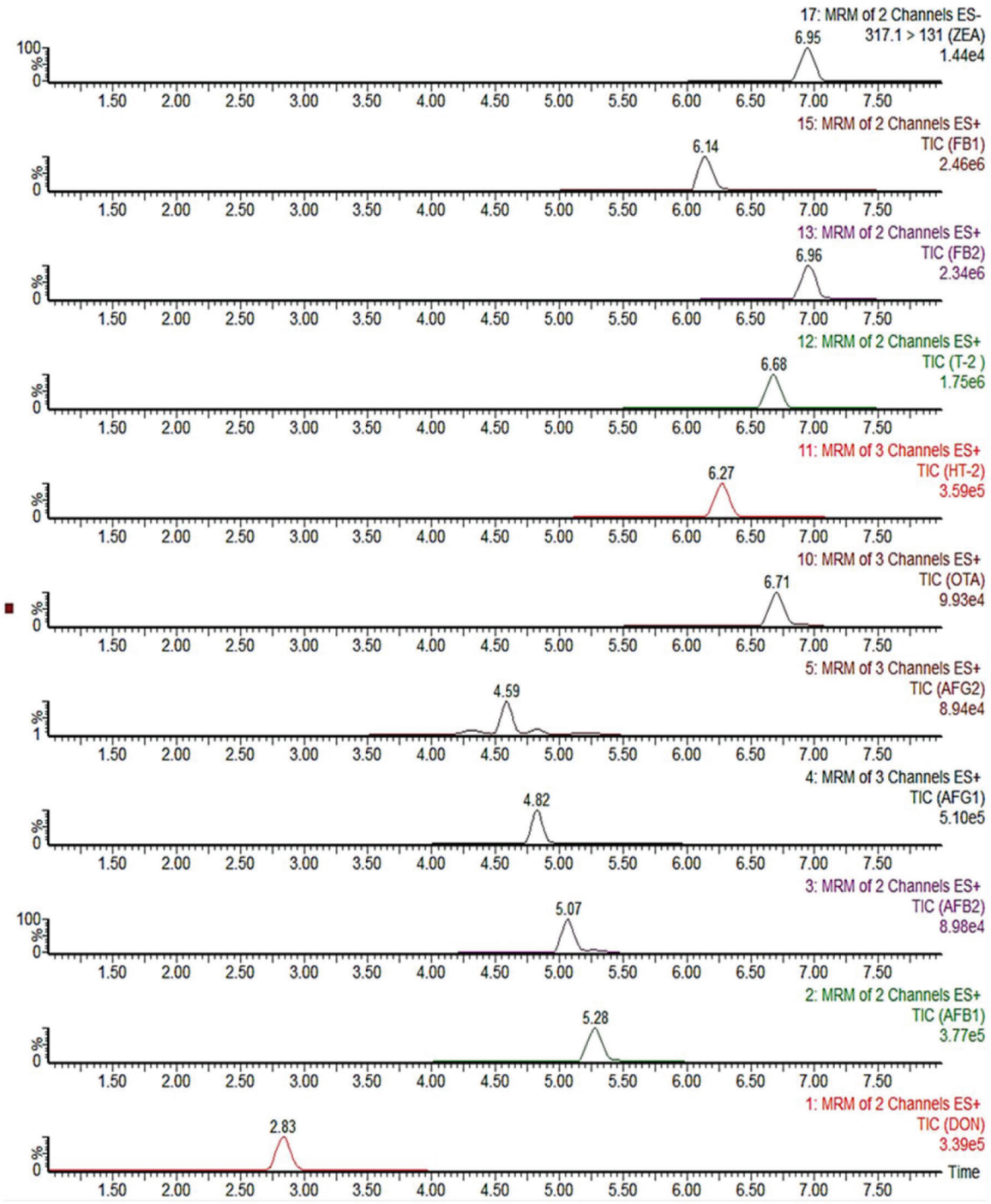

Figure 3 UHPLC-MS/MS chromatogram of the selected EU-regulated mycotoxins in the unfiltered multi-mycotoxin standard solution (AFB1/AFG1 0.5 ng/mL, AFB2/AFG2 0.125 ng/mL, DON 50 ng/mL, FB1/FB2 37.5 ng/mL, ZEA 7.5 ng/mL, T-2/HT-2 2.5 ng/mL and OTA 0.25 ng/mL) obtained with an Acquity H-class – Xevo TQ-S micro system

Table 2 MS/MS parameters for mycotoxin analysis

Mycotoxin	Precursor ion m/z	Cone voltage V	Collision energy V	Product ions m/z
AFB1	313.2 $(M+H)^+$	60	38	241.0
			23	285.3
AFB2	315.2 $(M+H)^+$	60	30	259.0
			25	287.0
AFG1	329.2 $(M+H)^+$	60	28	311.0
			24	243.0
AFG2	331.2 $(M+H)^+$	60	24	313.0
			28	245.1
DON	297.2 $(M+H)^+$	25	10	249.0
			10	203.2
FB1	722.4 $(M+H)^+$	50	40	334.3
			40	352.3
FB2	706.4 $(M+H)^+$	50	40	336.2
			40	318.2
ZEA	317.1 $(M-H)^-$	-58	30	131.0
			20	175.0
T-2	484.7 $(M+NH_4)^+$	25	20	185.0
			25	215.0
HT-2	442.6 $(M+NH_4)^+$	25	10	263.4
			15	215.3
OTA	404.1 $(M+H)^+$	30	24	239.0
			14	358.0

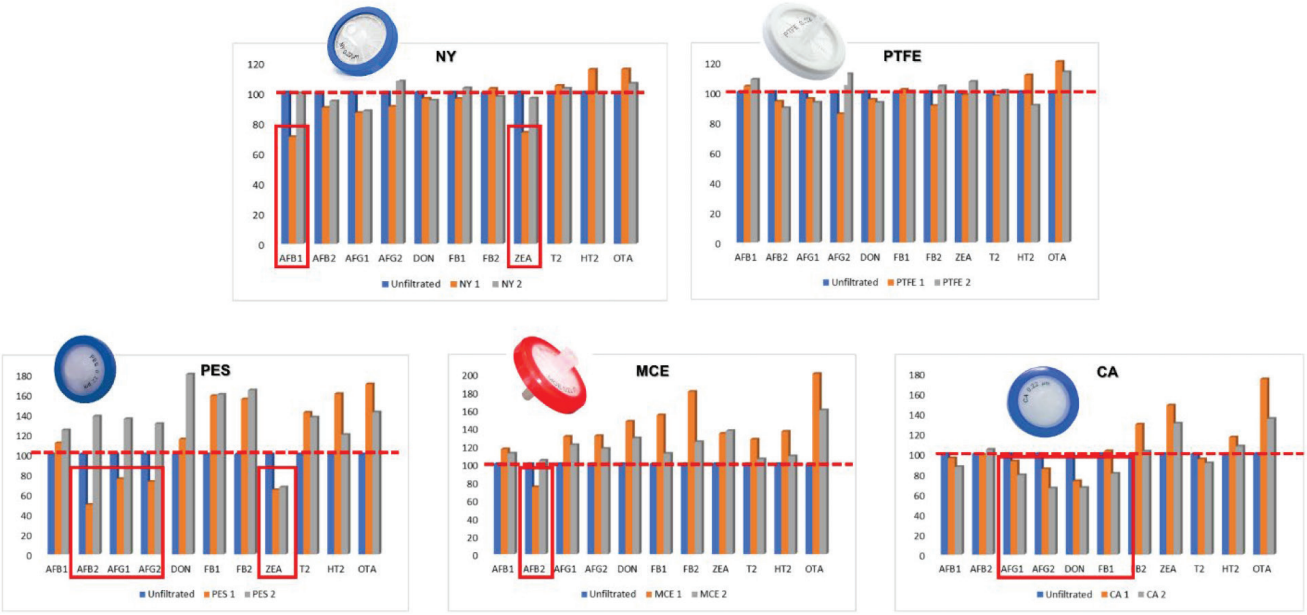

Figure 4 Recoveries of mycotoxins with various filter membrane materials in filtrate solution 1 and 2 relative to the recovery (dashed line) from the unfiltered multi-mycotoxin solution. Low recoveries indicating mycotoxin loss during filtration are framed in squares. CA – cellulose acetate; MCE – mixed cellulose ester; NY – nylon; PES – polyethersulphone; PTFE – polytetrafluoroethylene

UHPLC-MS/MS analysis

For instrumental analysis we used a UHPLC (Acquity H-Class, Waters, Milford, MA, USA) equipped with a quaternary pump system, coupled with a triple quadruple mass spectrometer (XEVO TQ-S micro, Milford, MA, USA) using a ZSpray electrospray interface (ESI). For chromatographic separation of the analytes we used a reverse phase column XBridge Peptide BEH C18 (150×3.0, 2.5 µm) (Waters, Milford, MA, USA) maintained at 50 °C, which we achieved with gradient elution of eluent A (aqueous solution of 5 mmol/L $AFNH_4$+0.02 % FA) and eluent B (MeOH) at a constant flow rate of 0.6 mL/min. The elution started with 75 % of eluent A in gradient, with a hold time of 0.5 min. Then we switched to 10 % of eluent A for 6 min, followed by equilibration to initial elution conditions after 2 min, giving a total run time of 13 min. The sample injection volume was set to 10 µL. Analytes were identified with the ESI-MS/MS working in the multiple-reaction monitoring (MRM) mode in positive and negative polarity, with two MS/MS transitions acquired per analyte (Table 2). The MS and MS/MS parameters used for analysis in both positive and negative ESI were as follows: capillary voltage 3.5 kV (+) and 2.5 kV (–), source temperature 150 °C, desolvation temperature 550 °C, desolvation gas flow 1000 L/h, and cone gas flow 50 L/h (both nitrogen). Argon gas was used for collision at a pressure of about 0.4 Pa in the collision cell. The obtained UHPLC-MS/MS chromatogram of the eleven mycotoxins in the unfiltered solution is shown in Figure 3.

Statistical analysis

Statistical data analysis was performed using Statistica 13.3. software (TIBCO Software Inc., Palo Alto, CA, USA). Significant difference was set to $p<0.05$. Before we ran statistical tests, we tested the data for normality of distribution using the Shapiro-Wilk test and for the homogeneity of variance using Levene's test. All variables that had normal distribution and homogenous variance (AFB1, AFG2, ZEA) were then tested with the *t*-test, and analysis of variance (ANOVA), while other variables were tested with the Mann-Whitney *U* test and Kruskal-Wallis H test. In the box and

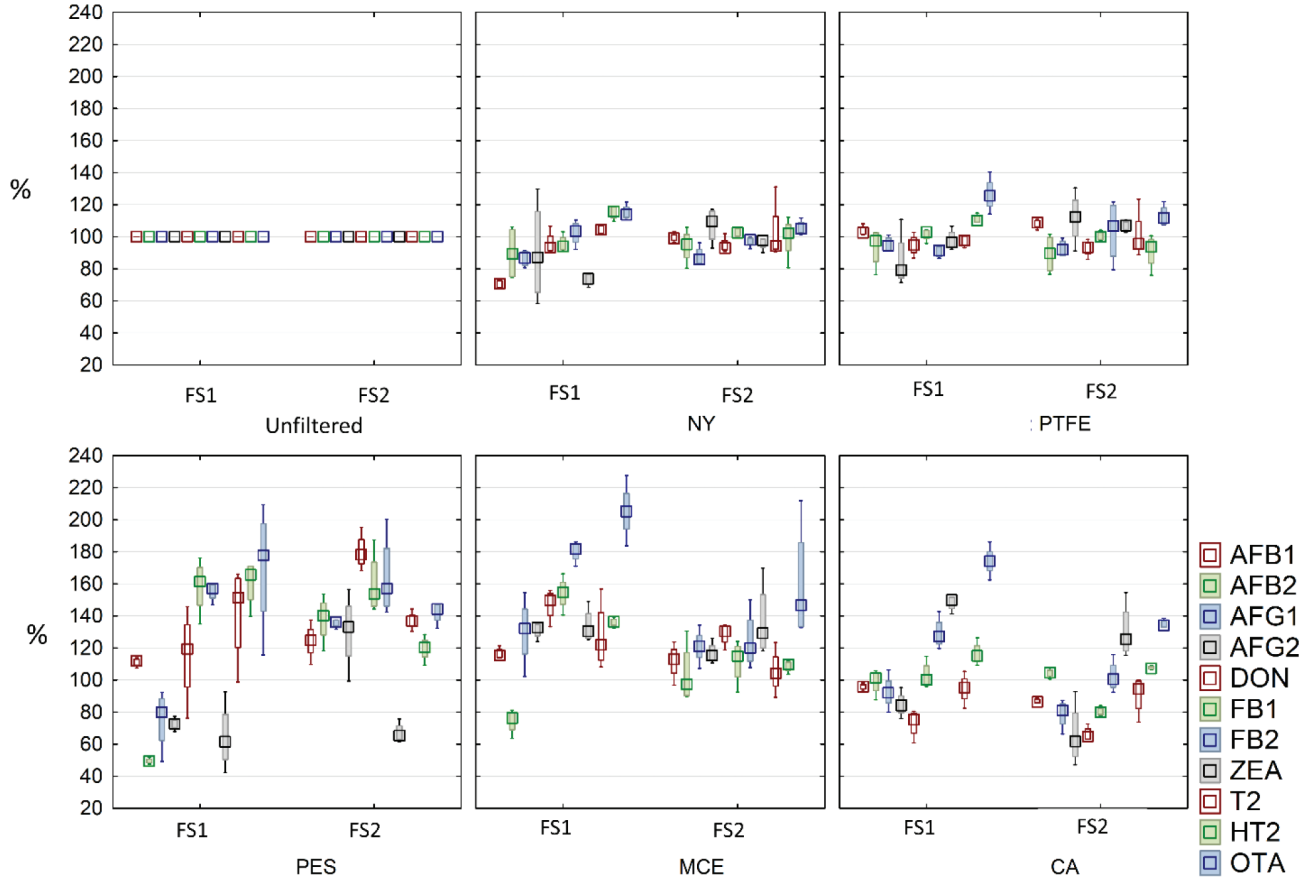

Figure 5 Relative recovery of the 11 mycotoxins in filtrate solutions 1 and 2 by filter membrane material. CA – cellulose acetate; FS1 – filtrate solution 1 (first three drops of the solution); FS2 – filtrate solution 2 (the rest of the solution); MCE – mixed cellulose ester; NY – nylon; PES – polyethersulphone; PTFE – polytetrafluoroethylene

whisker graphs medians are shown as central points, interquartile ranges (IQR) as boxes, and ranges as whiskers, since most data did not have normal distribution.

RESULTS AND DISCUSSION

Figure 4 shows mycotoxin recovery by filter membrane material. Mycotoxin loss was generally greater in the first filtrate solution than the second. Cellulose-based materials did not go well with acetonitrile in both filtered solutions, which confirms an earlier report by Carlson and Thompson (11).

However, the cellulose-based and polyethersulphone filters yielded unexpectedly high relative recoveries of up to 200 % for several, mainly *Fusarium* mycotoxins, regardless of the filtrate solution (1 or 2). This may be owed to a solvent-filtrate material interaction that may have modified the solution and produced an unexpected matrix effect, known to impact the method's performance (1). However, further research is needed to fully understand this phenomenon.

What we can see in more detail in Figure 5, is a very high recovery variation for the cellulose-based and polyethersulphone filters, regardless of which part of the filtrate was analysed, and much more even data distribution with the polytetrafluoroethylene and nylon filters. However, statistical analysis showed a significant difference between filtrate solution 1 and 2 recoveries only for AFB2 ($p=0.0039$) filtered through PES and MCE (49.32 % and 76.15 % in the filtrate solution 1, respectively vs 140.00 % and 97.35 % in the filtrate solution 2) and for HT-2 toxin ($p=0.00047$) filtered through PES (160.52 % in filtrate solution 1 vs 119.57 % in filtrate solution 2) (Figure 6).

On the other hand, recoveries significantly differed between filter types for AFB1 ($p=0.000$); AFG1 ($p=0.0003$); AFG2 ($p=0.003$); DON ($p=0.000$); FB1 ($p=0.000$); FB2 ($p=0.000$); ZEA ($p=0.000$); T-2 ($p=0.001$); HT-2 ($p=0.000$), and OTA ($p=0.000$), but not for AFB2.

Considering all these findings, PTFE turns out to least affect mycotoxin recovery during microfiltration, and NY material comes as the second best choice. It also turns out that discarding the first few filtrate drops should ensure a more reliable recovery. Other researchers, depending on the configuration of the analytical system, chose either to skip microfiltration or use a specific material of choice. Malachová et al. (7) did not use filtration in their HPLC analysis to avoid losses, whereas Kovač et al. (1), Frenich et al. (15), and de Santis (16) opted for NY and Carballo et al. (17) for PTFE. Some researchers who used microfiltration, such as Kafouris et al. (8), do not mention which type of filter membrane material they used.

CONCLUSIONS

Analyte loss to microfiltration following extraction can pose a serious problem to LC-MS quantitation. Although it can be skipped with some analytes and/or systems, there where it is required, the solution to the problem is to choose the membrane material, pore size, and filter size appropriate for specific analyte or solution properties. Furthermore, to ensure the accuracy of the analytical procedure, it may be necessary to discard the initial filtrate drops, as they only serve to saturate the filter membrane. Our study has revealed statistically significant differences in mycotoxin recoveries between filters, and has singled out polytetrafluoroethylene as the material of choice, followed by nylon. It has also pointed to some unexpectedly high recoveries, which deserves further attention.

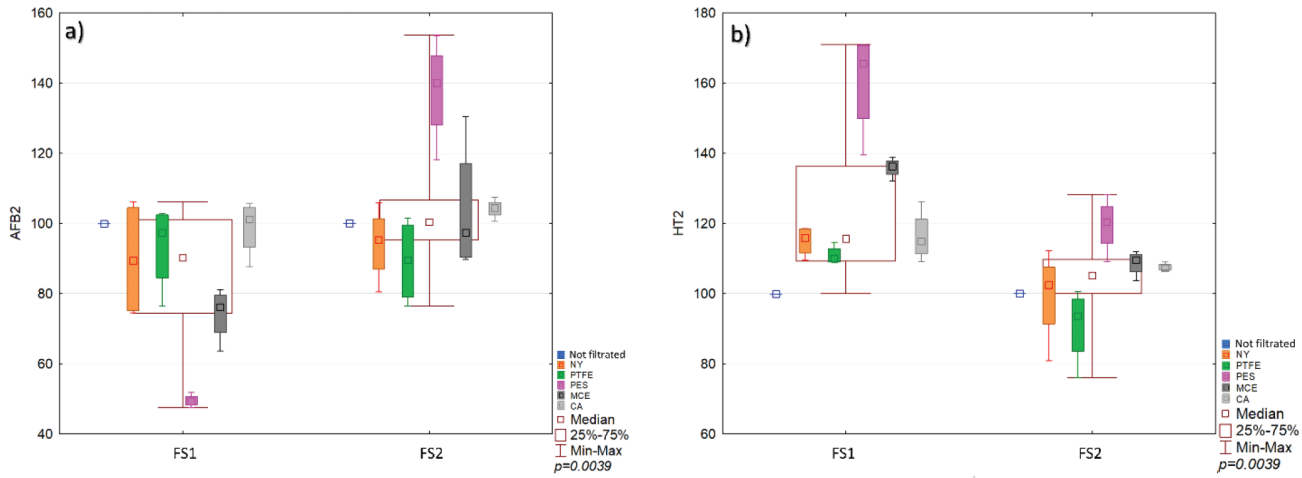

Figure 6 Relative recovery of the "outlier" mycotoxins AFB2 (a) and HT-2 (b) from filtrate solutions 1 and 2 by filter membrane materials. CA – cellulose acetate; FS1 – filtrate solution 1 (first three drops of the solution); FS2 – filtrate solution 2 (the rest of the solution); MCE – mixed cellulose ester; NY – nylon; PES – polyethersulphone; PTFE – polytetrafluoroethylene

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Utjecaj mikrofiltracije na iskorištenje odabranih mikotoksina reguliranih zakonom na razini Europske unije

Mikrofiltracija je čest korak u analitičkim postupcima u kojima se koristi tekućinska kromatografija – tandemska spektrometrija masa, koja je metoda izbora kada je riječ o jednoznačnom određivanju mikotoksina. Međutim, u slučajevima kada se primjenjuje mikrofiltracija, treba uzeti u obzir i moguće interakcije filtar-analit, koje bi mogle spriječiti točno određivanje pojavnosti mikotoksina, a time i izloženosti. U skladu s tim, u ovom istraživanju korišteno je pet različitih vrsta materijala membrane filtra (najlon, politetrafluoretilen, polietersulfon, miješani ester celuloze i celulozni acetat) za procjenu utjecaja mikrofiltracije na iskorištenje odabranih mikotoksina, reguliranih zakonodavstvom Europske unije, uključujući aflatoksine B1, B2, G1 i G2, deoksinivalenol, fumonizine B1 i B2, zearalenon, T-2 i HT-2 toksine te okratoksin A. Rezultati istraživanja otkrili su da politetrafluoretilenski filtri imaju najmanji utjecaj na gubitak mikotoksina tijekom filtracije, a uobičajeno korišteni najlonski filtri mogu utjecati na iskorištenje određenih mikotoksina ako se ne koriste pravilno. Ostala tri ispitana materijala filtarske membrane pokazala su se nekompatibilnima za ovu primjenu jer negativno utječu na iskorištenje većine analita. Rezultati istraživanja upućuju na nužnost odabira odgovarajuće vrste filtra tijekom postupka razvoja i validacije metode, prikladne za određeni analitički proces, a ovisno o svojstvima analita i sastavu otopine (uzorka), imajući u vidu nužnost odbacivanja prvih nekoliko kapi filtrata kako bi se osigurala točnost analitičkoga postupka.

KLJUČNE RIJEČI: interakcije filtar-analit; podcjenjivanje rezultata; priprema uzorka; regulirani mikotoksini; tekućinska kromatografija – tandemska spektrometrija masa