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Analytical Methods

Rapid screening of mycotoxins in liquid milk and milk powder by automated size-exclusion SPE-UPLC–MS/MS and quantification of matrix effects over the whole chromatographic run

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ABSTRACT

An automated, size-exclusion solid phase extraction (SPE)-UPLC–MS/MS protocol without pre-treatment of samples was developed to screen for four mycotoxins (OTA, ZEN, AFB_1, and AFM_1) in liquid milk and milk powder. Firstly, a mixed macropore-silica gel cartridge was established as a size-exclusion SPE column. The proposed methodology could be a candidate in green analytical chemistry because it saves on manpower and organic solvent. Permanent post-column infusion of mycotoxin standards was used to quantify matrix effects throughout the chromatographic run. Matrix-matched calibration could effectively compensate for matrix effects, which may be caused by liquid milk or milk powder matrix. Recovery of the four mycotoxins in fortified liquid milk was in the range 89–120% and RSD 2–9%. The LOD for the four mycotoxins in liquid milk and milk powder were 0.05–2 ng L^{-1} and 0.25–10 ng kg^{-1}, respectively. The LOQ for the four mycotoxins in liquid milk and milk powder were 0.1–5 ng L^{-1} and 0.5–25 ng kg^{-1}, respectively.

1. Introduction

Mycotoxins and their metabolites, particularly ochratoxin A (OTA), zearalenol (ZEN), aflatoxin B_1 (AFB_1), and aflatoxin M_1 (AFM_1), present a persistent potential risk to consumers because of carryover in cows' milk (Turner, Subrahmanyam, & Piletsky, 2009). Mycotoxin are toxic secondary metabolites produced by toxigenic fungi (Richard, 2007). Fungi are naturally opportunistic bio-deterioration agents of carbohydrate-rich agricultural commodities. These mycotoxins can cause serious illness and kill animals and humans. In Kenya, in 2004, 125 people died and almost 200 more required medical attention after eating aflatoxin-contaminated maize (Lewis et al., 2005). Thus, mycotoxin contamination can occur in crops and feeds as long as the conditions (humidity, temperature) are appropriate (Mantle, 2002; Visconti, Pascale, & Centonze, 1999). Studies on cows consuming feed containing relatively high levels of mycotoxin have shown the mycotoxin and its metabolites can transfer to milk (Gareis & Wolff, 2000; Yiannikouris & Jouany, 2002). In Europe, the AFM_1 in milk must not exceed 0.05 μg kg^{-1} [Commission Regulation (EC) No. 1881/2006]. Although many studies on detection of AFM_1 residue in milk have been reported, the presence of other mycotoxin residues in milk has been largely neglected. Thus, determination of various mycotoxins in liquid milk and milk powder is necessary to protect human health.

Complex matrices (mainly proteins and lipids) and low levels of mycotoxins are challenges that must be overcome by mycotoxin screening methods. A screening method for mycotoxins must be able to eliminate matrix interferences and determine mycotoxins with a high degree of accuracy and sensitivity. Elimination of interferences by proteins and lipids in chromatographic determination, generally, requires sample pre-treatment (de Zayas-Blanco, García-Falcón, & Simal-Gándara, 2004). Pre-treatment methods, such as extraction of aflatoxin M_1 using acetonitrile (ACN) and high-speed centrifugation (Hao Wang, Zhou, Liu, Yang, & Guo, 2011), matrix

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solid-phase dispersion–solid phase extraction (SPE) of eleven coccidiostats (Nász, Debreczeni, Rikker, & Eke, 2012), and microwave-assisted extraction–SPE of pesticides (Fang, Lau, Law, & Li, 2012), have been described in the literature for their ability to eliminate interferences in milk matrix. These pre-treatment techniques are performed by multistep manual operation and may suffer from serious drawbacks, such as low recovery, loss of the analyte, contamination, and prolonged analytical durations. Full automation and rapid detection are current trends in analytical chemistry. To minimise these limitations, Pereira et al. proposed an on-line high-performance liquid chromatography (HPLC)/ultraviolet (UV) clean-up method to determine sulfamethoxazole and trimethoprim in milk (Pereira & Cass, 2005). Compared with on-line LC/UV clean-up, on-line SPE coupled to LC/mass spectroscopy (MS) or LC/MS/MS is more sensitive and accurate. Few reports on the application of on-line SPE–LC/MS in the trace analysis of organic compounds in milk have been published.

The SPE columns reported thus far include anion exchange cartridges (Álvarez-Sánchez, Priego-Capote, Mata-Granados, & Luque de Castro, 2010), C_{18} HD cartridges (Kantiani et al., 2009), and OASIS HLB cartridges(Kuklenyik, Reich, Tully, Needham, & Calafat, 2004). The size-exclusion effect is mainly applied in chromatography separation. To the best of our knowledge, no successful application of automated size-exclusion SPE columns for milk sample cleanup has yet been reported.

Matrix effects impact the accuracy, precision, and robustness of LC–MS methods and are a growing concern in trace target analysis. These effects alter the signal intensity of a target analyte obtained by electrospray ionisation (ESI)–MS and thus decrease the accuracy of quantitative LC–MS/MS. While the mechanisms of matrix effects are poorly understood, two methods are commonly used to assess matrix effects: post-extraction spike (PES) and post-column infusion (PCI). For example, matrix effects during the detection of eight trichothecenes from wheat flour were evaluated by Rubert, James, Manes, and Soler (2012) using the PES method. Varga et al. (2012) also estimated matrix effects during MS quantification of 11 mycotoxins currently regulated in maize using the PES method. The PES method quantitatively assesses matrix effects by comparing the response of a target compound in neat solution standards with that of a spiked blank matrix sample that has been subjected to the same sample preparation process. Evaluation of matrix effects in a fully automated analysis method using the PES method is, therefore, not infeasible. The PCI method estimates matrix effects using permanent PCI of the target compound during chromatographic separation of the sample matrix and provides information about matrix effects over the entire chromatographic run (Stahnke, Reemtsma, & Alder, 2009). Despite this advantage, however, no evaluation of matrix effects in a fully automated SPE LC–MS/MS method using PCI has yet been reported.

The aim of this work was to develop a simple, accurate, and automated method for screening four mycotoxins in liquid milk and milk powder samples based on the on-line coupling of size-exclusion SPE to LC and ESI–MS. Mixed macropore silica gel (pore diameter, 300 Å), which is made of precisely classified high-purity silica coated with a thin film of silicone polymer where C_4 and NH_2 groups are introduced at a specific ratio, was applied as a size-exclusion SPE sorbent for simultaneous sample cleanup and extraction of the mycotoxins of interest. When a milk sample is injected into the SPE cartridge, proteins are eluted off from the cartridge immediately by the size-exclusion principle because the hydrophilic surface and small pores of the silica gel do not allow their retention in the cartridge. In contrast, low-molecular weight polar and nonpolar compounds are retained in the cartridge. Matrix effects over the whole chromatographic run time were studied using the PCI method.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade ACN was obtained from Thermo Fisher Scientific (Pittsburg, PA, USA). OTA, ZEN, AFB_1, and AFM_1 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Standard stock solutions of 1000 μg mL^{-1} mycotoxins were prepared by dissolving the mycotoxins in ACN. The solutions were kept at 4 °C in dark glass flasks until use. Deionised water was obtained using a Milli-Q water purification system (Millipore, Milford, MA, USA) and used to prepare all aqueous solutions.

2.2. Instrumentation

A schematic diagram of the automated size-exclusion SPE–ultra performance liquid chromatography (UPLC)–MS/MS system is shown in Fig. 1. The system was built on an Agilent 1100 two-dimensional LC (Palo Alto, CA, USA) coupled with ESI–MS (Thermo Scientific, San Jose, USA). The LC was equipped with a quaternary pump, a 7725i injection valve (valve 2), a 10-port valve (valve 1), an injection loop of 1 mL nominal volume, and a high-pressure gradient AP230 pump (Elite Co., Dalian, China), which was used for injection of samples into the size-exclusion SPE column. All stainless-steel parts of the UPLC system that came into contact with the sample were replaced by polyether ether ketone components. The SPE column was tailor-made with C_4 and NH_2 mixed macropore silica gel packed into a guard cartridge (10.0 mm × 4.0 mm i.d.). The C_4 and NH_2 mixed macropore silica gel was obtained from Nano-Micro Co., Ltd. (Suzhou, China) and its physical characteristics were as follows: particle size, 50 μm to 100 μm; pore size, 300 Å. A C_{18} column (150 mm × 2.1 mm i.d., Thermo Fisher Scientific) was used as the analytical column.

ESI–MS was carried out on a TSQ quantum mass spectrometer (Thermo Fisher Scientific) equipped with an ESI source. The instrument was interfaced with a computer running Xcalibur software (Thermo Fisher version). The ESI–MS detection conditions were as follows: spray ion voltage, 3500 V; sheath gas (N_2) pressure, 30 arbitrary units; auxiliary gas (N_2) pressure, 5 arbitrary units; capillary temperature, 350 °C; collision gas (Ar) pressure, 0.2 Pa. Ionisation parameters and collision cell parameters were optimised for the four mycotoxins. The quantification of mycotoxins was achieved in selected reaction monitoring mode. The mass analyzers Q1 and Q3 were operated at unit mass resolution. Parameters, including monitoring ion pairs, corresponding skimmer offset, and the collision energy, are shown in Table 1.

2.3. Preparation of fortified analytical samples

Liquid milk including raw milk (3.8 g fat and 3.3 g protein per 100 mL, pasteurised milk (3.0 g fat and 3.6 g protein per 100 mL), sterilized milk (3.5 g fat and 3.3 g protein per 100 mL), and UHT milk (3.0 g fat and 3.2 g protein per 100 mL), milk powder including skimmed milk powder (0.1 g fat and 3.3 g protein per 100 mL of reconstituted milk), whole milk powder (23.1 g fat and 17.9 g protein per 100 g), and infant milk formulae (3.2 g fat and 2.2 g protein per 100 mL of reconstituted milk) were purchased from local markets.

Fortified liquid milk and milk powder samples were prepared by adding standard stock solutions of each mycotoxin to liquid milk and milk powder samples. The concentrations of mycotoxins in the fortified liquid milk samples ranged from 10.0 ng L^{-1} to 500.0 ng L^{-1} (Table 2). The concentrations of mycotoxins in the fortified milk powder samples ranged from 0.05 μg kg^{-1} to 2.5 μg kg^{-1} (see supplementary material, Table S1). The fortified

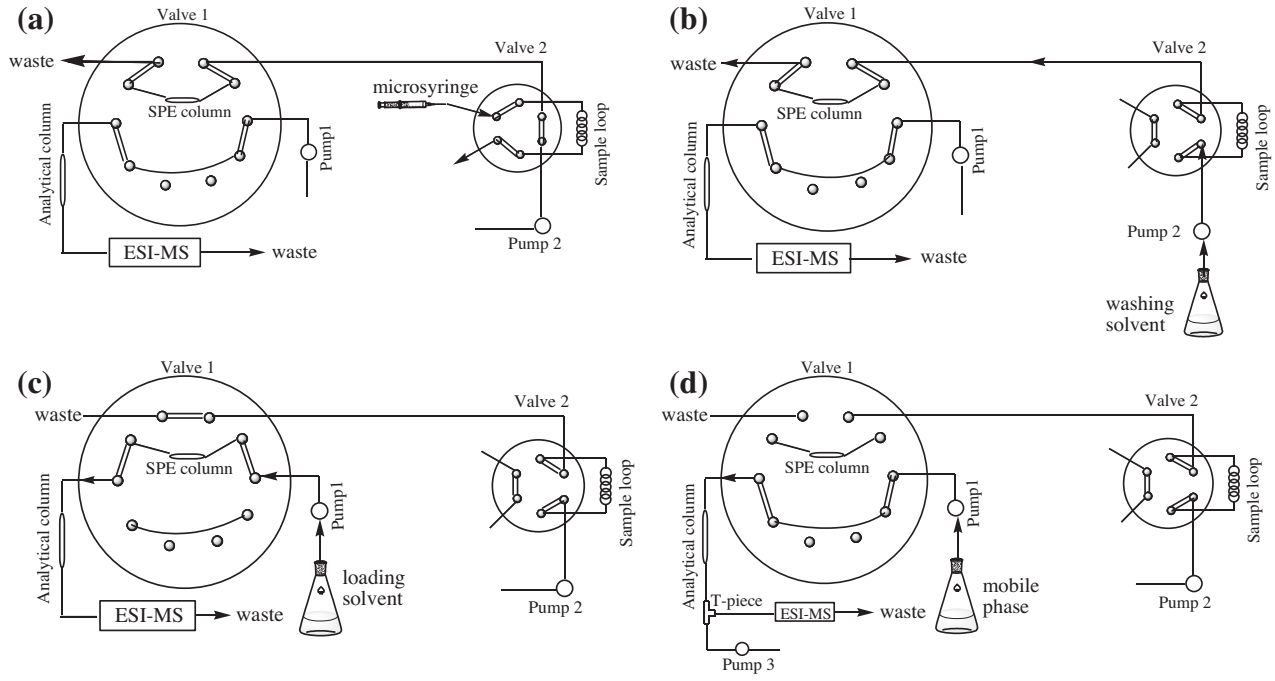

Fig. 1. The manifold diagram of the automated exclusion SPE-UPLC–MS/MS system.

Table 1
Monitored ion-pairs of OTA, ZEN, AFB_1, and AFM_1 in the SRM mode and linear range and detection limit of OTA, ZEN, AFB_1, and AFM_1 in liquid milk and milk powder samples.

Analytes	Scan mode	Ions of qualification	Collision energy (V)	Ions of quantification	Collision energy (V)	Liquid milk		Milk powder	
						Linear range ($ng\ L^{-1}$)	Detection limit ($ng\ L^{-1}$)	Linear range ($ng\ kg^{-1}$)	Detection limit ($ng\ kg^{-1}$)
OTA	+	404/358.1	34	$[M+H]^+$ /$[M+H-H_2O]^+$ (404.0/386.2)	23	2–2000	1.0	10–10000	5.0
ZEN	+	319/283.1	28	$[M+H]^+$ /$[M+H-H_2O]^+$ (319.0/301.2)	31	5–1000	2.0	25–5000	10.0
AFB_1	+	313/241.1	37	$[M+H]^+$ /$[M+H-CO]^+$ (313.1/285.2)	35	0.1–100	0.05	0.5–500	0.25
AFM_1	+	329/259.3	46	$[M+H]^+$ /$[M+H-CO]^+$ (392.1/301.2)	34	1–300	0.3	5–1500	1.5

samples were stored at +4 °C in dry, dark bottles and equilibrated for 48 h. Blank liquid milk and milk powder samples were stored at +4 °C.

2.4. Sample preparation

About 5 g of milk powder was dissolved in 25.0 mL of water. The mixture was placed in a water bath at 40 °C and mixed using magnetic stirring for about 10 min until a homogeneous sample was obtained.

In sequence 1 (Fig. 1a), the liquid milk or milk powder homogeneous samples were first passed through a 0.45 μm filter and then injected into the 1 mL sample loop using a microsyringe.

In sequence 2 (Fig. 1b), valve 2 was in the fill position, pump 2 was activated for 8 min, and the washing solvent (100% water, v/v) was passed through the sample loop at a flow rate of 1.0 mL min^{-1}. The sample in the sample loop was introduced into the SPE column and the objective compounds were pre-concentrated. Other interfering components, such as proteins in the samples, were discarded as waste.

In sequence 3 (Fig. 1c), valve 1 was in the fill position, pump 1 was activated for 10 min, and the loading solvent (ACN/water, 30/70, v/v) was passed through the SPE column at a flow rate of 0.8 mL min^{-1}. Analytes enriched in the SPE column were focused onto the top of the analytical column, which had been pre-equilibrated using the mobile phase.

In sequence 4 (Fig. 1d), valve 1 was in the injection position, pump 1 was activated, the mobile phase was injected into the analytical column at a flow rate of 200 μL min^{-1}, and the column temperature was kept at 30 °C. The mobile phase consisted of ACN (A) and water (B). The gradient conditions were as follows: 0–10 min, 30–100% A and 10–12 min, 100% A. Detection were completed by the ESI–MS system.

2.5. Quantification of matrix effects over the whole chromatographic run time

The quantification of matrix effects was performed over the whole chromatographic run time on the basis of two injections. The pure mobile phase was injected as a reference, after which the extract of an unspiked sample was injected in a second run. During both runs, pump 3 (Fig. 1d) was activated and a mycotoxin mixture was permanently infused to the MS through a T-piece with a flow rate of 20 μL min^{-1}.

The matrix effects at each retention time t_i over the whole chromatographic run time (ME_{t_i}) were determined according to

Table 2
Within- and between-day precisions and recoveries of OTA, ZEN, AFB_1, and AFM_1 in fortified liquid milk samples utilising standard (SC) and matrix-matched calibrations (MMC) ($n = 6$).

Analytes		Calibration	R^2	Within-day recovery ± R.S.D./% (at spiking level)			Between-day recovery ± R.S.D./% (at spiking level)		
				10.0 ng L^{-1}	100.0 ng L^{-1}	500.0 ng L^{-1}	10.0 ng L^{-1}	100.0 ng L^{-1}	500.0 ng L^{-1}
OTA	SC	$Y = 1.36 + 004X + 3.53e + 003$	0.9964	157 ± 5	171 ± 4	132 ± 1	151 ± 8	176 ± 7	129 ± 6
	MMC	$Y = 3.58e + 004X + 9.24e + 002$	0.9935	120 ± 4	113 ± 2	109 ± 3	117 ± 9	117 ± 6	120 ± 4
ZEN	SC	$Y = 7.92e + 003X + 8.75e + 002$	0.9921	142 ± 7	150 ± 3	123 ± 4	139 ± 7	155 ± 8	128 ± 7
	MMC	$Y = 9.34e + 003X + 8.66e + 002$	0.9929	114 ± 6	107 ± 5	97 ± 2	112 ± 8	114 ± 7	101 ± 4
AFB_1	SC	$Y = 3.92e + 004X + 5.78e + 003$	0.9963	140 ± 4	135 ± 6	114 ± 5	137 ± 6	132 ± 6	112 ± 8
	MMC	$Y = 5.54e + 004X + 4.86e + 003$	0.9972	105 ± 6	115 ± 3	89 ± 4	104 ± 9	114 ± 9	93 ± 5
AFM_1	SC	$Y = 5.51e + 004X + 3.17e + 003$	0.9984	136 ± 5	128 ± 2	133 ± 3	136 ± 5	125 ± 8	127 ± 6
	MMC	$Y = 7.18e + 004X + 4.59e + 003$	0.9915	111 ± 4	105 ± 4	118 ± 4	109 ± 8	107 ± 6	110 ± 6

the method reported by Stahnke (Stahnke et al., 2009). ME_{t_i} can be calculated according to following formulations:

$$ME_{t_i}(\%) = \left[\frac{\text{smoothed SI}_i\ (\text{sample extract})}{\text{smoothed SI}_i\ (\text{corresponding reference})} \times 100\%\right] - 100\% \quad (1)$$

$$\text{Smoothed SI}_i = (1-f)\text{SI}_i + f\text{SI}_{i-1} \quad (2)$$

All of the signal intensity values at retention time t_i (SI_i) were transferred from the analytical software to a spreadsheet program. A smoothing factor f of 0.6 was used.

2.6. Matrix-matched calibration

To construct matrix-matched calibration curves, blank samples (i.e., non-spiked liquid milk or milk powder) were spiked with standard solutions of the mycotoxins. MS response values versus spiked concentrations were used to construct the calibration curve. A five-point calibration curve was drawn based on the average of three parallel measurements.

3. Results and discussion

To simultaneously detect the four mycotoxins in liquid milk or milk powder using the established automated exclusion SPE–UPLC–MS/MS system, the chromatographic conditions, MS parameters, and the automated exclusion SPE conditions were optimised.

3.1. Optimisation of the chromatographic conditions

LC separation of the pure standard mycotoxins was carried out in reversed-phase mode on a C_{18} column by MS detection. To optimise chromatographic separation, the effect of the volume ratio of ACN to water on the separation of mycotoxins was studied. Chromatograms of each mycotoxin were obtained after gradient elution (see supplementary material, Fig. S1).

3.2. Optimisation of the MS parameters

The fragmentation process to produce detectable ion–molecule complexes of mycotoxins was achieved via two mechanisms: dehydration and elimination of CO. Both reaction mechanisms resulted in skeletal rearrangements of ion–molecule complexes of mycotoxins, as shown in Fig. 2. Dehydration from protonated OTA lactone (Fig. 2a) was achieved by skeletal or hydrogen rearrangements that move the positive charge site away from the C–OH group. The newly formed carbo-cationic centre then activates protons in 3-positions for transfer onto the basic hydroxyl group. Ion–molecule complexes of ZEN can also undergo such group migrations with elimination, as shown in Fig. 2b. The formation mechanisms and structures of the ions at m/z 386.1 (Fig. 2a) and m/z 301.2 (Fig. 2b) were previously reported by Wang, Wu, and Zhao (2001). Ion–molecule complexes of AFB_1 (Fig. 2c) and AFM_1 (Fig. 2d) easily lose CO from their ketone because the loss of a single ring carbon can only occur by cleavage of its adjacent ring bonds with heteroatom stabilization.

3.3. Optimisation of the automated size-exclusion SPE column

3.3.1. Selection of the extraction sorbent

To achieve simultaneous sample clean-up and concentration by size-exclusion SPE, several sorbents for SPE columns, such as C_{18}, C_8, Florisil, alumina, and silica, were tested. The presence of proteins in the milk samples had a significant effect on the robustness of traditional SPE columns and brought about matrix effects. The C_4 and NH_2 mixed macropore silica gel in the SPE column was built up by bonding hydrophilic polyoxyethylene groups and hydrophobic phenyl groups to thin film of high-purity silica in silicone polymer. When a milk sample was introduced into this column, proteins are not retained because the small pores of the silica gel do not allow their retention in the column. In contrast, low-molecular weight compounds are retained and consequently separated from the bulk of the sample matrix according to the size-exclusion principle. The SPE column was thus selected to minimise the interference of proteins in the milk sample and maximise elimination of matrix effects.

3.3.2. Optimisation of washing solvent

A mixture of ACN–water was used as the washing solvent. The effect of the amount of ACN on the determination of the four mycotoxins in pasteurised milk and skimmed milk powder samples was studied. Because the washing solvent was used to transfer milk samples from the sample loop to the SPE column, the content of the organic phase in the washing solvent system had to be as low as possible to prevent protein precipitation, which would increase column pressure. The amount of ACN used in this study was varied from 0% to 50% (v/v) at the flow rate of 1 mL min^{-1}. The peak areas obtained by the analysis of toxin in difference samples are provided in the supplementary material, Fig. S2. The signal intensity of ZEN in fortified pasteurised milk decreased with increasing ACN concentration because of the hydrophobic property of the mycotoxin (Fig. S2a). In contrast, OTA, AFB_1, and AFM_1 in fortified pasteurised milk were hardly affected by the ACN concentration. A solvent system containing 100% (v/v) water was selected as the washing solvent for liquid milk samples. Although results was similar for four types of toxins in skimmed milk powder (Fig. S2b) and pasteurised milk, system pressure in sequence 3 for pasteurised milk was much higher for skimmed milk powder. So the content of fat in milk seriously affected SPE column life.

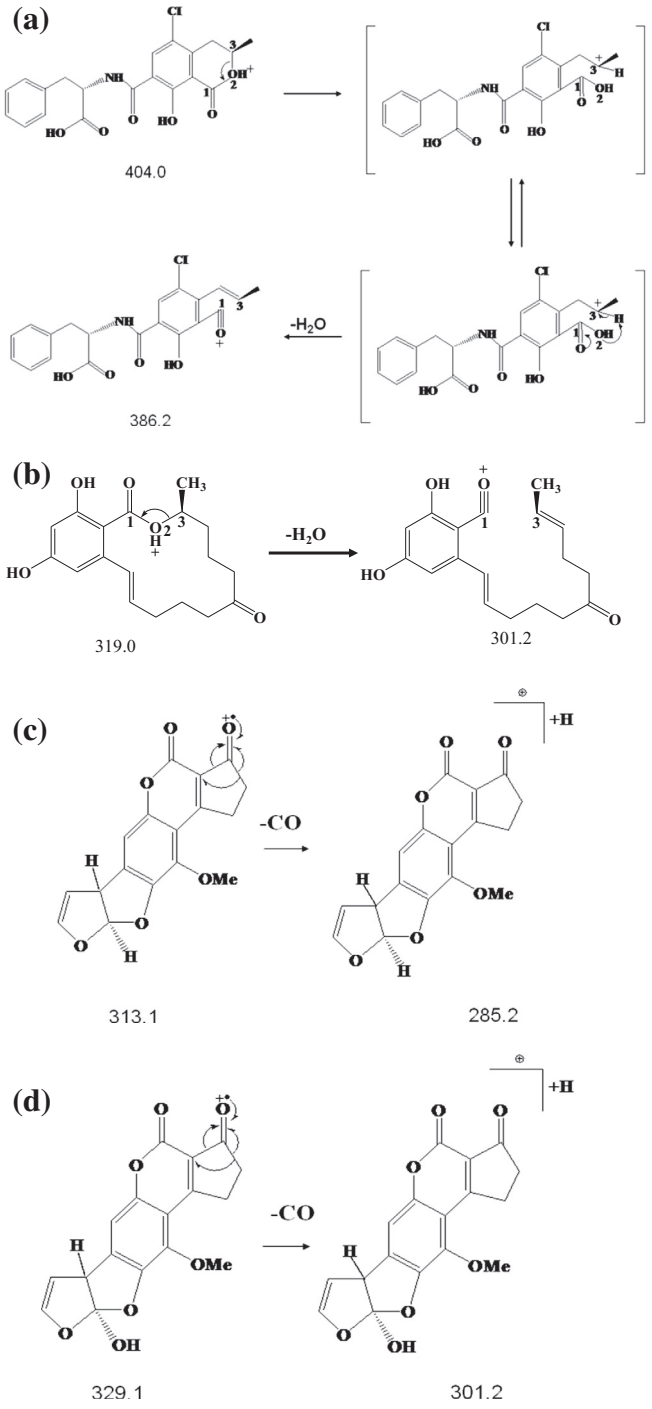

Fig. 2. Fragmentation pathways for OTA (a), ZEN (b), AFB_1 (c), and AFM_1 (d), illustrating the most representative product ions for quantification.

3.3.3. Optimisation of washing time

In this study, the washing time controlled the enrichment degree of the four mycotoxins in the SPE column. The effect of washing time on the efficiency of recovery of the four mycotoxins was studied. The washing times adopted in this study ranged from 2 min to 10 min at a washing solvent flow rate of 1.0 mL min^{-1}. The recoveries of the mycotoxins increased with increasing washing time until 8 min (see supplementary material, Fig. S3). At longer washing times, the recovery of ZEN decreased because it could be eluted from the SPE column. The optimal washing time for maximum recovery was thus determined to be 8 min.

3.3.4. Optimisation of loading solvent

The loading solvent must satisfy two requirements: it must be able to completely elute the four mycotoxins from the SPE column and it must be compatible with the mobile phase. A mixed solvent of ACN/water (30/70, v/v) was chosen as the loading solvent.

The effect of the flow-rate of the loading solvent was also optimised. The recoveries of the mycotoxins decreased with increasing flow-rate of the loading solvent (see supplementary material, Fig. S4). A slow loading process may provide adequate time for mycotoxins in the SPE column to wash off completely and adsorb effectively on the analytical column. To optimise the analytical time, the flow-rate was selected as 0.8 mL min^{-1} with a constant loading time of 10 min.

3.4. Matrix effect

Investigations of matrix effects impact on the electrospray ionisation of four mycotoxins were carried out in liquid milk and milk powder. Results are displayed in Fig. 3. The electrospray ionisation process of mycotoxins was susceptible to eluted matrix components.

For four mycotoxins from the same species (liquid milk or milk powder), the matrix effect profiles obtained were quite similar. The mycotoxins in liquid milk (Fig. 3a–d) showed similarly strong ion enhancement throughout most of the separation time, except the ion suppression section ranged from 1.8 to 2.3 min. Ion enhancement between 40% and 60% was detected in all of the liquid milk samples. Mycotoxins in milk powder (Fig. 3e–h) also showed similarly strong ion enhancement throughout most of the separation time.

In contrast, by comparing Fig. 3a–d (matrix effect from liquid milk) with Fig. 3e–h (matrix effect from milk powder), it was found that the matrix effect profiles for the same mycotoxin from different species (for example, liquid milk and milk powder) were completely different. Furthermore, ion enhancements in milk powder samples were significantly higher (up to 200%) than those in liquid milk before 2 min. Therefore, it was obvious that the differences between matrix effects observed for different analytes in one matrix were much smaller than between the matrix effects observed for different matrixes. The result of matrix effects for four mycotoxins provides a good basis to investigate a suitable quantitative method for four mycotoxins in different matrixes.

3.5. Analytical performance

3.5.1. Matrix-matched calibration and measurement accuracy

Mycotoxins in liquid milk and milk powder showed strong ion enhancement matrix effects, and the slope of the matrix-matched calibration (MMC) for mycotoxins was significantly greater than the standard calibration (SC). Recoveries of the four mycotoxins calculated by MMC were much better than those calculated by SC. These findings indicate that MMC may effectively compensate for the matrix effects of liquid milk and milk powder. The recoveries of mycotoxins in liquid milk samples at low, moderate, and high concentrations are shown in Table 2. As can be seen in the Table 2, the spiked concentrations and concentrations detected subsequently were, generally in good agreement using MMC. MMC could achieve satisfactory results with high recoveries (89–120%) and favourable relative standard deviation (RSD) values (2–9%) in liquid milk samples. The linear ranges and LODs are shown in Table 1. The MMC curves and recoveries of spiked milk powder samples are shown in the Supplementary material.

3.5.2. Measurement precision

Precision was expressed as within-day and between-day RSDs. The within-day precision was evaluated by determining each of

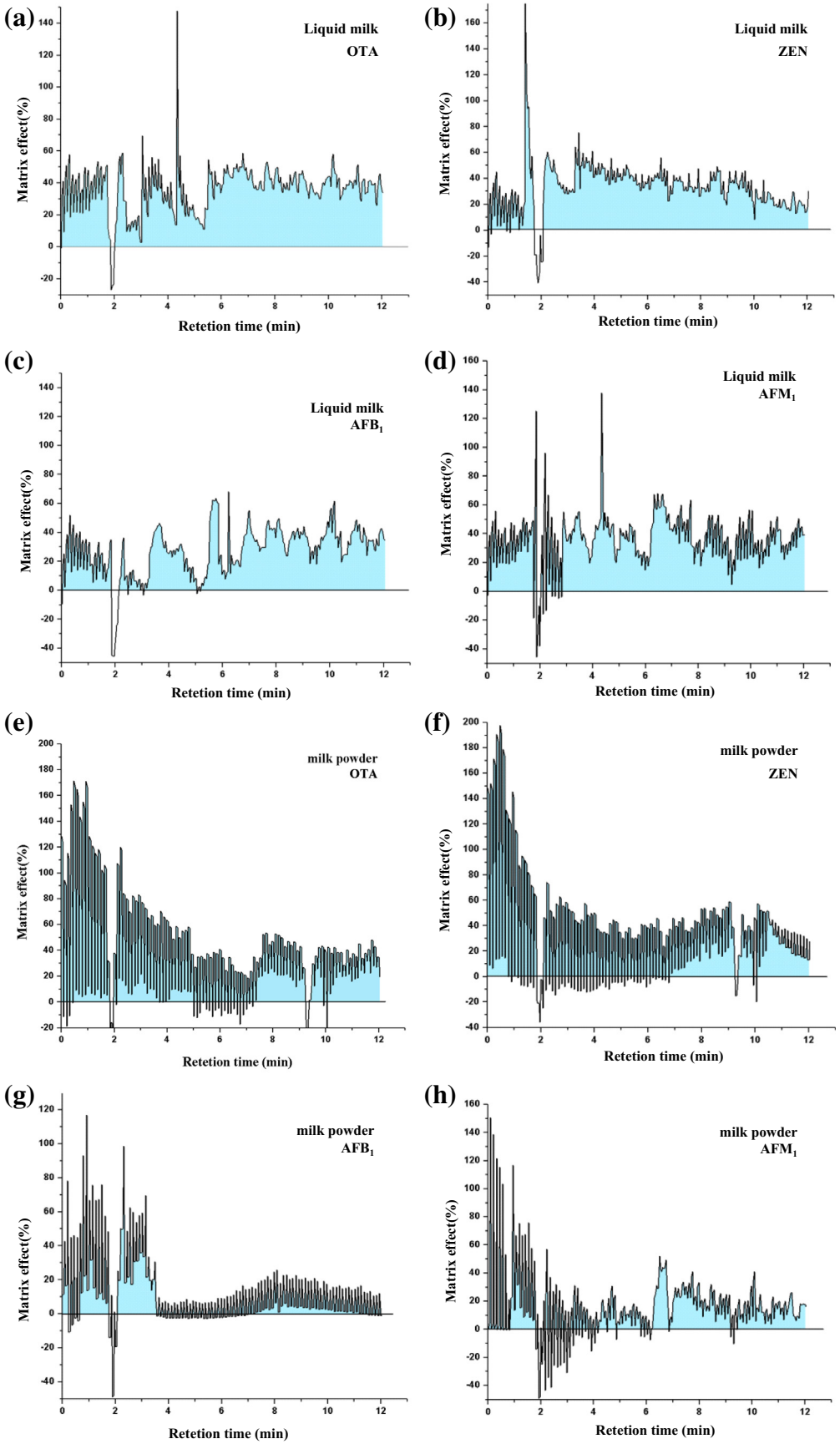

Fig. 3. Matrix effect profiles of OTA (a and e), ZEN (b and f), AFB_1 (c and g) and AFM_1 (d and h) in liquid milk and milk powder.

Table 3
OTA, ZEN, AFB_1, and AFM_1 analysis performed in various liquid milk and milk powder samples. Mean concentrations after $n = 3$ replicate analyses.

Sample		Content of mycotoxins in various samples			
		OTA	ZEN	AFB_1	AFM_1
Liquid milk ($\mu g\ L^{-1}$)	Raw milk 1	1.59	3.25	n.d. [a]	n.d.
	Raw milk 2	n.d.	n.d.	0.52	0.01
	Raw milk 3	n.d.	n.d.	n.d.	n.d.
	Pasteurised milk 1	0.37	n.d.	0.21	n.d.
	Pasteurised milk 2	n.d.	n.d.	n.d.	n.d.
	Pasteurised milk 3	n.d.	n.d.	n.d.	n.d.
	Sterilized milk 1	n.d.	n.d.	n.d.	n.d.
	Sterilized milk 2	n.d.	n.d.	n.d.	n.d.
	Sterilized milk 3	n.d.	n.d.	n.d.	n.d.
	UHT milk 1	n.d.	n.d.	n.d.	n.d.
	UHT milk 2	n.d.	n.d.	n.d.	n.d.
	UHT milk 3	n.d.	n.d.	n.d.	n.d.
Milk powder ($\mu g\ kg^{-1}$)	Skim milk powder 1	n.d.	n.d.	0.03	n.d.
	Skim milk powder 2	n.d.	n.d.	n.d.	n.d.
	Skim milk powder 3	n.d.	n.d.	n.d.	0.04
	Whole milk powder 1	n.d.	n.d.	n.d.	n.d.
	Whole milk powder 2	n.d.	5.64	n.d.	n.d.
	Whole milk powder 3	n.d.	n.d.	n.d.	0.02
	Infant milk powder 1	n.d.	n.d.	n.d.	n.d.
	Infant milk powder 2	n.d.	n.d.	n.d.	n.d.
	Infant milk powder 3	n.d.	n.d.	n.d.	n.d.

[a] n.d.: not detected.

the mycotoxins of each concentration level in fortified samples five times during a single working day. The between-day precision was determined by analysing the same samples (fortified with analytes at different concentration levels) over a period of 5 d. Table 2 lists the recoveries and precision values of the four mycotoxins at three concentration levels in liquid milk samples.

Compared with previously reported methods, detection of one sample using our method can be completed in only 30 min, the volume of organic solvent(acetonitrile) is only 2.4 ml (before chromatography separation), and recoveries are comparable (Sørensen & Elbæk, 2005).

3.5.3. Analysis of real samples

The method described here was applied to quantify four mycotoxins in various liquid milk and milk powder samples. Results are given in Table 3. From 21 samples, 9 liquid milk samples and 5 milk powder samples contained no detectable levels of mycotoxins. Seven samples were found to contain at least one of the target analytes at a quantifiable level.

4. Conclusions

In this study, a novel highly sensitive, accurate, fully automated method for identification and quantification of four mycotoxins (OTA, ZEN, AFB_1, and AFM_1) in liquid milk and milk powder samples was developed. Our method presents two main advantages. (1) A C_4 and NH_2 mixed macropore silica gel size-exclusion SPE column was successfully introduced as an on-line cleanup column. The performance of this column was demonstrated to be excellent, successfully eliminating protein interferences from milk samples and thus realising satisfactory recoveries. (2) Large volume (1 mL) injections improved the sensitivity and LOD of the automated on-line analytical method by achieving a larger concentration factor. This methodology could be classified as a candidate of the green analytical chemistry because it uses less organic solvent compared with other methods (Tobiszewski, Mechlińska, Zygmunt, & Namieśnik, 2009).

The effect of the sample matrix on the four mycotoxins was independent of retention time, as determined from the matrix effect profiles. The main advantage of the matrix effect profiles observed is the direct measurement of signal enhancement or suppression caused the matrix over the whole chromatographic run time. The four mycotoxins in milk showed ion enhancement effects throughout most of the separation time. The matrix-matched standards were demonstrated to be an appropriate approach for preventing problems arising from matrix effects. The assays based on this methodology yielded recoveries ranging from 89% to 120% and precision values ranging from 2% to 9%.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.10.056.

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