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Development, optimization and validation of a multimethod for the determination of 36 mycotoxins in wines by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A fast and efficient multimethod for the determination of 36 mycotoxins in wine, using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS), was developed, optimized, validated and implemented in routine analysis. A simplified, quick extraction was performed with acetonitrile, derived from the QuEChERS (quick, easy, cheap, effective, rugged and safe) approach, which was traditionally developed for pesticides analysis. This study aimed at a single extraction and chromatographic separation for 36 mycotoxins.

Optimization tests were performed to find the proper ratio of wine: water and extraction solvent and the need for an additional buffering step with ammonium formate/formic acid and a dispersive SPE cleanup with various sorbents. The dSPE steps did not show significant improvement in analysis results, therefore, it was not applied in the final method to be validated. The mycotoxins were separated and detected on a UPLC–MS/MS system, used in the ESI positive ionization mode. The various mycotoxins were divided in three different concentration level groups, according to their sensitivity in UPLC–MS/MS. The validation was performed by analyzing recovery samples at three different spike levels with six replicates ($n=6$) at each level. Linearity (r^2) of calibration curves, accuracy (recovery %), instrument limits of detection and method limits of quantification (LOD and LOQ), precision (RSD%) and matrix effects (%) were determined for each individual mycotoxin. From the 36 mycotoxins analyzed by UPLC–MS/MS (ESI+), 35 showed average recoveries in the range 70–120%, and 86% of these with a RSD $\leq$ 20% at the lowest spike level (for Group I, II and III, respectively, 1, 50 and 10 μg kg^{-1}). The higher spike levels showed even better results. Only nivalenol could not be quantified at any concentration level. The method LOQ for 86% of the mycotoxins studied was the lowest spike level tested. The matrix effect observed was low for most mycotoxins analyzed and had no significant influence on the analytical results obtained. The developed procedure was applied successfully in routine analysis in a survey of wine samples originating from different countries.

1. Introduction

Wine is the fermented juice of fresh grapes and usually contains from 10 to 15% of alcohol per volume. Being one of the most consumed beverages around the world, it has a high economic significance and the consumption is still increasing each year. According to FAO, more than 27 million liters of wine were produced in 2009. Italy, France, and Spain produce about 50% of the world's wine and USA, Argentina, Australia, China, Germany, South Africa, Chile and Portugal are also important wine producers [1,2].

According to the *Organisation Internationale de la Vigne et du Vin* (OIV), vineyard area in the world is around eight million hectares and about 80% of the grape production is used in wine-making [1,3]. Even when a very careful treatment is applied in the vineyard and winemaking, contamination by pesticides and other contaminants can occur.

The intentional contamination of food by the use of chemicals, such as pesticides or veterinary drugs, is a worldwide public health concern nowadays. However, food contamination due to natural toxins, as mycotoxins, can also compromise the safety of

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food and feed supplies and adversely affect both human and animal health [4].

Mycotoxins can be defined as toxic secondary metabolites produced under appropriate environmental conditions by filamentous fungi, mainly *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. Their presence can evoke a broad range of toxic properties including carcinogenicity, neurotoxicity, as well as reproductive and developmental toxicity [5]. The impacts of mycotoxins contamination of agricultural commodities on human and animal health as well on domestic and international trade are increasingly recognized in both developed and developing countries [1,6,7].

In general, developed countries have established regulatory limits to protect consumers from exposure to mycotoxins. In many developing countries, however, regulation is insufficient and certain agricultural commodities, including dietary staple foods, can contain unacceptably high levels of mycotoxins [1]. After the first report [8] of the occurrence of ochratoxin A (OTA) in wine, worldwide attention was paid to this contamination, caused by fungal infection of grapes. An extensive overview for European wines was given by Stratakou and Van der Fels-Klerx [9].

Wine analysis may not seem to be difficult, as the matrix looks simple at first sight. During the extraction process the naturally occurring matrix compounds such as fatty acids and their esters, alcohols, sugars and other compounds also get co-extracted in addition to the target analytes, which interferes with the analysis through matrix interference signals and/or matrix-induced signal enhancement/suppression. This can make quantification and identification of the target analytes difficult, erroneous and ambiguous [10]. Also, the presence of ethanol can compromise the results obtained.

Usually, mycotoxins analysis has been carried out by liquid chromatography after immunoaffinity column cleanup or via enzyme-linked immunosorbent assay (ELISA) tests, but all these methods normally involve determination of a single compound only [11]. In general the extraction of mycotoxins from liquid samples such as wine, beer, juices and milk, is based on solid phase extraction (SPE) using immunoaffinity columns or other type of sorbents. However, other extraction techniques such as liquid–liquid extraction (LLE), solid phase microextraction (SPME) and liquid phase microextraction (LPME) have also been applied [12].

Lau et al. [13] were the first to analyze ochratoxin A by HPLC with electrospray mass spectrometry. The application of LC–MS, when compared to the immunoassay cleanup procedures which are specific for the mycotoxin of interest, gave the option to inject crude extracts and thus to discover more mycotoxins then just ochratoxin A in wine. This lead to the finding of the Alternaria toxins alternariol and alternariol monomethyl ether in fruit juices and beverages, including red and white wines [14]. Logrieco et al. first established the presence of fumonisin B_2 in grapes [15] and secondly in Italian red wines [16]. Mogenson et al. [17] investigated a total of 77 wine samples from 13 countries and concluded its occurrence to be widespread. Despite determination of *Aspergillus* and *Penicillium* species on grape surfaces [18] no aflatoxins, citrinin or trichothecenes were found in wines. Measuring wine samples with a multimycotoxin method offers the opportunity to determine the presence of mycotoxins in wine more systematically.

Spanjer et al. [19] were the first to develop a real multimethod for 25 mycotoxins and apply this method successfully in routine analysis for surveys and enforcement purposes. The method is based on an acetonitrile extraction, shaking the sample or an aqueous slurry of a homogenized sample with acetonitrile. The water–acetonitrile extract was injected directly in a LC–MS/MS system, without any partitioning or cleanup step.

Anastassiades et al. [20] described in 2003 a multiresidue method for pesticide residue analysis in fruits and vegetables, based on an acetonitrile extraction, a partitioning with magnesium sulfate and dispersive SPE cleanup step. The QuEChERS (quick, easy, cheap, effective, rugged and safe) approach attracted much attention and became very popular in the last decade. Lehotay et al. [21] proved the wide applicability by validating the method for 229 pesticides, using subsequent, selective GC–MS and LC–MS/MS detection. After a collaborative study of a slightly modified version, the method was accepted as AOAC official method for multiple pesticides in fruits and vegetables.

In 2007, in our laboratories a comprehensive project was started aiming at the integration of the mycotoxins and pesticides sample homogenization, preparation and analysis for relevant matrices where both types of contaminants are likely to be present, such as soya [22,23], rice, coffee [15], cacao, grape juice and wine. Our in-house, standardized slurry-homogenization method was combined with the QuEChERS approach for the development of an integrated mycotoxins-pesticides method. Various results of the method for LC- and GC-amenable pesticides have been published before [22–24]. This paper describes the results for the analysis of 36 mycotoxins in wine, using an acetonitrile-based extraction with magnesium sulfate induced phase separation. Various dispersive SPE cleanup sorbents were evaluated and compared relative to omitting the cleanup step. Especially, the combination of acetonitrile extracts with a LC–MS/MS detection method is very attractive, because an evaporation step can be avoided before direct injection. The validated, optimized method has been applied successfully in the survey of mycotoxins in routinely analyzed wine samples.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (pesticide grade), acetic acid (p.a.) and anhydrous magnesium sulfate (99.9%) were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q Gradient Water System (Millipore, Bedford, MA, USA).

The studied mycotoxins were: Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2, Ochratoxin A (OTA), DeOxyNivalenol (DON), DiAcetoxyScirpenol (DAS), Fumonisin B1, Fumonisin B2, Fumonisin B3, Fusarenone-X, HT2-Toxin, T2-Toxin, 15-Acetyl-DON, 3-Acetyl-DON, Mycophenolic acid, Nivalenol (NIV), Penicillic acid, Citrinin, Cyclopiazonic acid, Roquefortine-C, Sterigmatocystine, Zearalanone (ZAN), α-Zearalanol (α-ZAL), β-Zearalanol (β-ZAL), Zearalenone (ZEN), α-Zearalenol (α-ZEL), Alternariol, Alternariol methyl, Ergocornin, Ergocristin, Ergokryptin, Ergonovin, Ergosin, Ergotamin and Mevinolin. Reference standards were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands), Biopure (Tulln, Austria), Acros (Geel, Belgium), Fermentek Biotechnology (Jerusalem, Israel) and Iris Biotech GmbH (Marktredwitz, Germany).

During optimization tests, formic acid from Merck (Darmstadt, Germany) and ammonium formate from VWR (Amsterdam, The Netherlands) were applied. Dispersive solid phase extractions (dSPE) sorbents, C_{18}, Florisil, Nexus® and Graphitized Carbon Black were purchased from Varian (Middelburg, The Netherlands). Chlorofiltr® was obtained from UCT (Bristol, PA, USA) and Oasis HLB® from Waters (Milford, MA, USA).

2.2. Standard solutions

Mycotoxins standard stock solutions were prepared at concentrations as indicated in Table 1. The mixture stock solutions were subdivided in three different groups of concentration levels, according to their sensitivity in UPLC–MS/MS. Table 1 contains all mycotoxins that were studied and their grouping, the

Table 1
Mycotoxins studied, grouping, solvent and concentration of individual standard stock solutions and mixture stock solutions.

Mycotoxin	Group	Solvent	Concentration in stock solution ($\mu g\ mL^{-1}$)	Concentration in mixture stock solution ($\mu g\ mL^{-1}$)
Aflatoxin B1	1	MeOH	54.0	0.5
Aflatoxin B2	1	MeOH	56.5	0.5
Aflatoxin G1	1	MeOH	100	0.5
Aflatoxin G2	1	MeOH	41.5	0.5
Ochratoxin A	1	Toluene/ 1% HOAc	32.0	0.5
Deoxynivalenol (DON)	2	ACN	1000	25
Fumonisin B1	2	ACN/ water (1:2)	1000	25
Fumonisin B2	2	ACN/ water (1:3)	1000	25
Fumonisin B3	2	ACN	105	25
Nivalenol (NIV)	2	ACN	1000	25
Diacetoxyscirpenol (DAS)	2	ACN	1000	25
T2-Toxin	2	ACN	1000	25
HT2-Toxin	2	ACN	1000	25
3-Acetyl-DON	2	ACN	1000	25
Zearalenone (ZEN)	2	ACN	1000	25
15-Acetyl-DON	2	ACN	1000	25
Penicilic acid	2	ACN	1000	25
Fusarenon-X	2	ACN	1000	25
β-Zearalanol (β-ZAL)	2	ACN	1000	25
α-Zearalanol (α-ZAL)	2	ACN	1000	25
Citrinin	2	ACN	1000	25
Zearalanone (ZAN)	2	ACN	1000	25
Cyclopiazonic acid (CPA)	2	ACN	1000	25
Sterigmatocystine	2	ACN	1000	25
Roquefortine C	2	ACN	100	25
α-Zearalenol (α-ZEL)	2	ACN	1000	25
Mycophenolic acid	2	ACN	1000	25
Altenariol	2	ACN	1000	25
Altenariol-methyl	2	ACN	1000	25
Ergotamin	3	ACN	500	5
Ergonovin	3	ACN	500	5
Ergocornin	3	ACN	500	5
Ergokryptin	3	ACN	500	5
Ergocristin	3	ACN	500	5
Ergosin	3	ACN	500	5
Mevinolin	3	ACN	100	5

concentration levels and the solvent of the individual stock solutions and mixture stock solutions. The stock and mixture stock solutions were stored at $-18\ ^{\circ}C$.

2.3. Samples

A biological red wine (Cabernet Sauvignon) with an alcohol percentage of 13.5 (v/v), originating from South Africa, was purchased from a Dutch wine store. This wine was used as blank sample for recovery studies and matrix-matched calibration standard solutions, after the absence of mycotoxins and pesticides was established. Wine samples intended to be analyzed for a survey were bought during the years 2008 (68 samples), 2009 (62 samples), 2010 (50 samples), 2011 (60 samples) and 2012 (40 samples) from various Dutch supermarkets, wine stores and importers.

2.4. Analytical instrumentation

Chromatographic analyses were performed by a Waters Acquity (Milford, MA, USA) equipped with a quaternary solvent delivery system, degasser, autosampler and column heater. The separation was performed on a BEH C_{18} analytical column (100 mm × 2.1 mm I.D.), with 1.7 μm particles, from Waters. The detection system applied was a Waters Quattro Premier XE tandem mass spectrometer (Manchester, UK), equipped with an electrospray ionization interface (ESI) operating in the positive ion mode, using multiple reaction monitoring (MRM).

2.5. Chromatographic and mass spectrometric conditions

A gradient elution was performed using a mobile phase (flow rate at 0.40 mL min^{-1}) constituted by water (0.1% formic acid) and acetonitrile (0.1% formic acid), eluent A and B, respectively. The program started at 90% eluent A and was directly ramped to 30% at 10 min, kept constant for 0.1 min and decreasing linearly to 10% of eluent A. This condition was kept constant for 2 min. The column was re-equilibrated in the last minute to the initial mobile phase composition, resulting in a 13 min chromatographic run. The injection volume of each sample was 5 μL and the autosampler was flushed with methanol/water solution (1:9, v/v) before sample injection. The optimal column temperature (in order to obtain maximum resolution) was kept at 50 °C.

The mass spectrometer ion source parameters applied were: capillary voltage, 2.0 kV; the sample cone voltage was set at an optimal value for each individual mycotoxin (Table 2); source temperature, 120 °C and desolvation gas temperature, 400 °C. The desolvation gas and cone gas (N_2) flow rates were set at 100 and 700 L h^{-1}, respectively. Collision-induced dissociation was performed using argon as collision gas at 3.5×10^{-3} mbar. Optimization of the collision energy and cone voltage for each individual mycotoxin was done by infusion of the mycotoxin directly into the LC effluent using a syringe pump (Harvard, Kent, UK).

For instrument control, data acquisition and processing, Mass Lynx and QuanLynx software 4.1 (Waters) were used. Precursor and product ions monitored, time windows/functions and optimized collision energies for the ESI positive ionization mode are also shown in Table 2.

2.6. Experimental procedures for optimization studies

Optimization tests were performed to improve selectivity, specificity, accuracy and precision of the method, in order to select the most appropriate method for validation purposes.

2.6.1. Wine/water ratio optimization

In order to reduce interference of matrix compounds and solve problems of phase separation during the extraction process, due the presence of ethanol, different ratios of the mixture of wine and water were tested during the method development. The scheme of the procedure is shown in Fig. 1A.

2.6.2. Cleanup and buffering studies

For the development of an effective cleanup procedure in order to decrease matrix effects and/or interferences during chromatographic analysis of the mycotoxins, various sorbents and sorbent mixtures were studied using dispersive Solid-Phase Extraction (dSPE), such as C_{18}, Oasis HLB (Oasis), Nexus, Graphitized Carbon Black (GCB) and ChloroFiltr (CF).

From in-house experiences, it is known that the extraction efficiency and/or stability of some mycotoxins, especially the fumonisins, are pH dependent. The control of this parameter

Table 2
LC–MS/MS (ESI, positive mode) parameters for mycotoxins analyzed.

Mycotoxins	t_R (min)	Precursor Ion	Cone voltage (V)	MRM transitions			
				Quantification		Confirmation	
				Product ion	Collision energy (eV)	Product ion	Collision energy (eV)
Aflatoxin B1	7.3	313.1	50	241.1	37	285.0	23
Aflatoxin B2	7.0	315.1	50	259.1	30	287.1	26
Aflatoxin G1	7.0	329.1	40	243.1	25	214.3	25
Aflatoxin G2	6.7	331.1	50	245.1	30	275.1	30
Ochratoxin A	8.9	404.1	25	239.1	22	241.1	22
Deoxynivalenol (DON)	1.5	297.2	23	249.1	10	231.2	12
Fumonisin B1	7.3	722.4	58	334.4	42	352.4	37
Fumonisin B2	8.0	706.5	59	336.4	34	318.4	40
Fumonisin B3	7.8	706.5	59	336.4	34	318.4	40
Nivalenol (NIV)	1.0	313.0	13	175.0	20	195.0	8
Diacetoxyscirpenol (DAS)	7.3	367.3	14	307.3	10	289.3	12
T2-Toxin	8.7	467.4	8	305.2	8	245.3	7
HT2-Toxin	7.8	425.3	23	263.2	10	105.1	37
3-Acetyl-DON	5.7	339.3	28	231.2	13	203.2	18
Zearalenone (ZEN)	8.8	319.1	24	187.0	18	184.9	26
15-Acetyl-DON	5.7	339.3	19	137.2	15	261.2	10
Penicilic acid	3.5	171.1	20	125.1	12	153.1	6
Fusarenon-X	2.9	355.0	20	247.0	16	229.0	16
β-Zearalanol (β-ZAL)	7.8	323.3	16	305.3	8	277.1	16
α-Zearalanol (α-ZAL)	8.2	323.3	16	305.3	8	277.1	16
Citrinin	7.9	251.2	30	205.2	27	191.2	23
Zearalanone (ZAN)	8.8	321.3	29	303.2	13	285.2	16
Cyclopiazonic acid (CPA)	9.5	337.3	39	196.2	23	182.1	20
Sterigmatocystine	9.0	325.1	50	281.1	34	253.3	42
Roquefortine C	7.4	390.3	44	193.1	27	322.3	22
α-Zearalenol (α-ZEL)	8.3	321.0	15	303.0	7	285.0	12
Mycophenolic acid	8.3	320.8	22	206.8	23	274.8	16
Altenariol	7.6	258.8	48	184.8	29	213.0	25
Altenariol-methyl	8.9	273.1	50	183.8	36	198.8	29
Ergotamin	7.0	582.3	38	268.2	27	208.2	48
Ergonovin	1.9	325.9	38	222.9	22	283.0	18
Ergocornin	7.2	562.0	36	268.0	26	544.0	17
Ergokryptin	7.5	576.4	32	268.0	26	305.0	25
Ergocristin	7.7	610.0	25	268.0	28	304.9	23
Ergosin	7.0	548.0	35	223.0	35	208.0	45
Mevinolin	10.6	405.1	20	198.9	13	285.0	10

during the extraction procedure can thus prevent lower recoveries and precision. During the cleanup studies, simultaneously a buffering step using formic acid and ammonium formate was tested (Fig. 1 B—Procedure 1). The measured pH values of the wine samples were around 3–4.

The sorbent that showed the best results (ChloroFiltr) for cleanup was also tested without the buffering step (formic acid/ammonium formate), that is just with acetonitrile acidified with acetic acid (1%). In addition, some other sorbents (C_{18}, Florisil, GCB) and their mixtures (GCB+Florisil and GCB+C_{18}) were evaluated in this type of experiment (Fig. 1C—Procedure 2), in order to obtain the best cleanup performance.

2.7. Final extraction procedure

In the final, optimized extraction procedure developed for wine samples (Fig. 2), an amount of 5.0 ± 0.02 g of wine was weighed into a 50 mL PTFE centrifuge tube and 5.0 g of water was added. A volume of 10 mL acetonitrile, containing 1% of acetic acid and the procedure internal standard (P.I.S.), was added to the tube, which was shaken with a mechanical shaking machine (IKA H501), at 300 rpm during 1 min. Then, 3.0 g anhydrous magnesium sulfate was added to each tube and immediate vigorous manual shaking was performed, followed by mechanical shaking for 1 min. Three milliliter aliquot of the acetonitrile extracts (upper layer) were decanted into other tubes, containing 450 mg of anhydrous magnesium sulfate. The tubes were mechanically shaken for 1 min. Thereafter, the tubes were centrifuged (Harrier 18/80) at 4000 rpm for 4 min, at 10 °C, and an aliquot of 0.5 mL extract was diluted with 0.5 mL of methanol with instrument internal standard (I.I.S) into an autosampler vial.

The methanol dilution solvent containing I.I.S., was prepared in a 500 mL volumetric flask by adding 1 mL of a propoxur standard solution (5 μg mL^{-1} in acetonitrile) and making up the volume with methanol.

2.8. Method validation study

Method validation provides evidence that a developed method is fit for the purpose for which it is intended to be used. The validation was performed according to the requirements of the European Union SANCO document [25].

2.8.1. Calibration curves, linearity, LOD and LOQ

To evaluate the linearity of the calibration curves, standard solutions were prepared at seven concentrations in organic solvent (acetonitrile with 1% acetic acid) and in blank wine matrix extract, both diluted with methanol (1:1). They were injected into the LC–MS/MS system. The calibration levels for the three subgroups of mycotoxins are shown in Table 3.

Each calibration solution was injected six times (n=6). Calculations were performed based on the average peak areas by external standard calibration. Relative standard deviations values (RSD) for

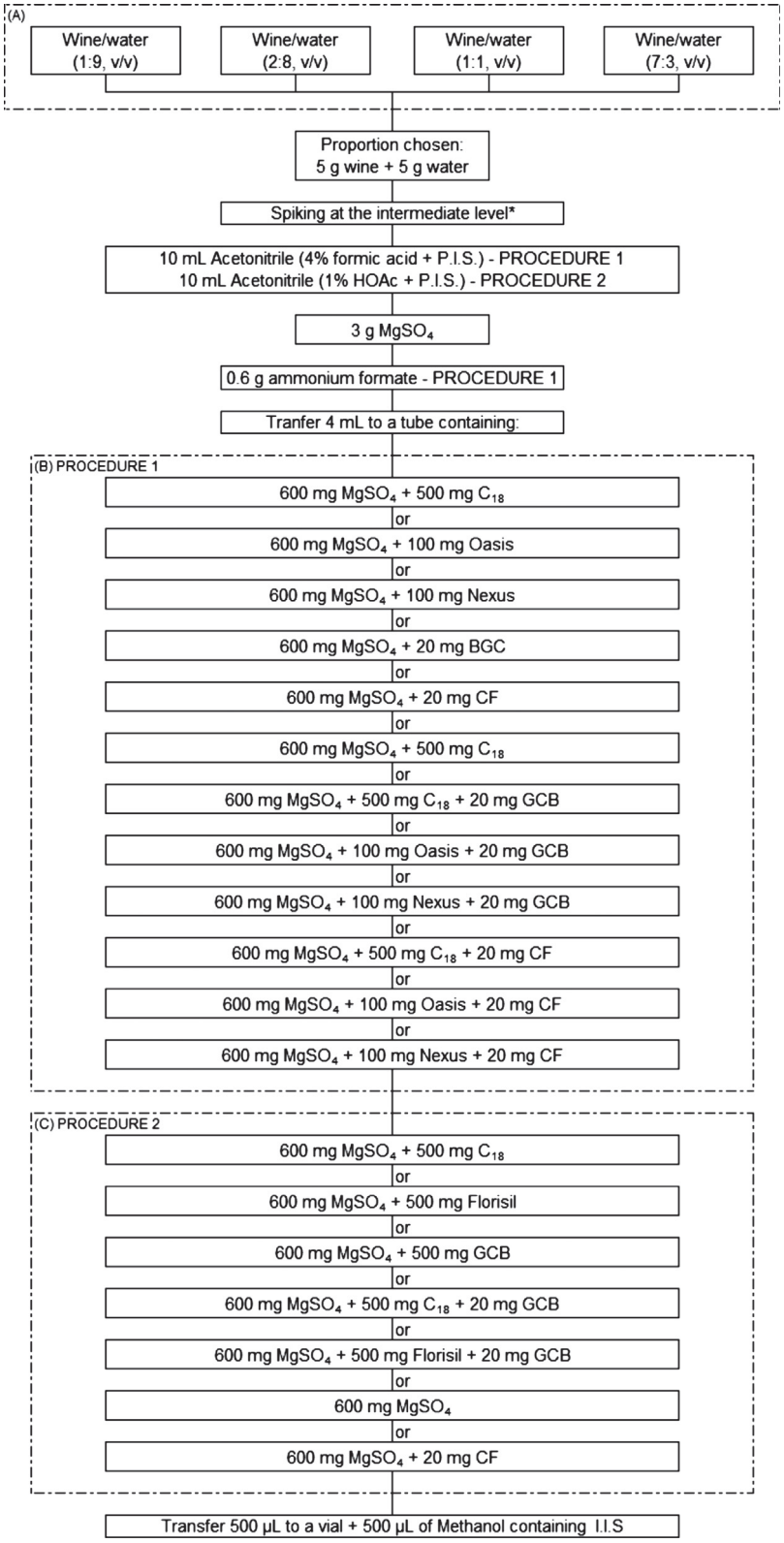
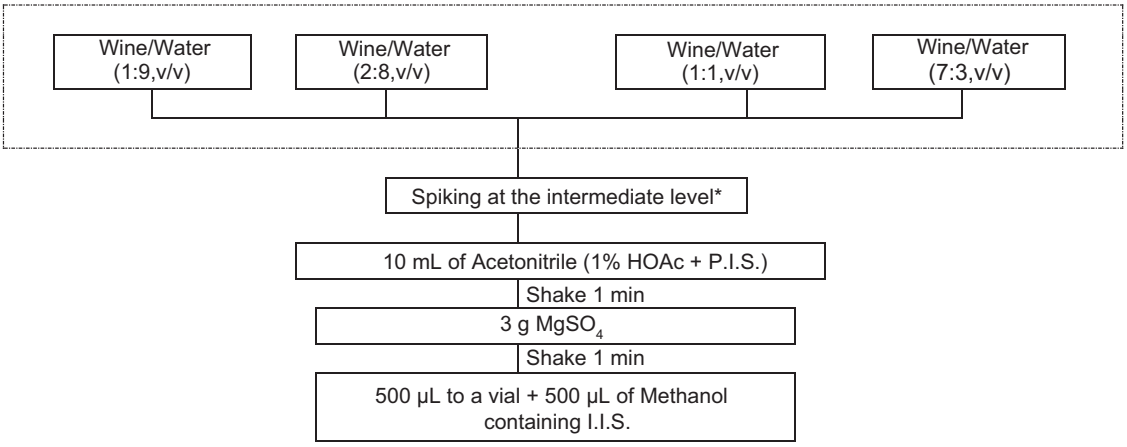


Fig. 1. Experimental procedures schemes for optimization studies. (A) Experiments for wine/water ratio optimization, extraction with acetonitrile +1% HOAc; (B) experiments for testing buffering step with formic acid/ammonium formate and cleanup applying C_{18}, Oasis, Nexus, CF, GCB and their mixtures; (C) experiments for testing with omission of buffering step, only using acetonitrile +1% HOAc, with an extra drying step and dSPE cleanup, applying C_{18}, Florisil, GCB, CF and their mixtures.



* 4 µg kg^{-1} (Group 1) / 200 µg kg^{-1} (Group 2) / 40 µg kg^{-1} (Group 3)

- Procedure Internal Standard (P.I.S.) = Quinalphos (12.5 µg L^{-1})

-Instrument Internal Standard (I.I.S.) = Propoxur (10 µg L^{-1})

Fig. 2. Final extraction procedure for determination of 36 mycotoxins in red wine.

Table 3
Concentrations of calibration solutions for linearity study and selected spike levels for recovery study.

Mycotoxins group	Concentrations of calibration solutions (ng mL^{-1})								Spiking levels (µg kg^{-1})		
	1	2	3	4	5	6	7				
Group 1	0.1		0.2	0.5	1	2	5	10	1	2.5	10
Group 2	5.0		10	25	50	100	250	1000	50	125	500
Group 3	1.0		2	5	10	20	50	100	10	25	100

Table 4
Matrix effect and recovery percentage obtained by applying various dSPE cleanup sorbents and their mixtures, performing the extraction with formic acid/ammonium formate buffer.

Mycotoxin	Matrix effect (%)/recovery (%)										
	CF	C18	Nexus	Oasis	GCB	Nexus+GCB	Nexus+CF	C18+CF	C18+GCB	Oasis+GCB	Oasis+CF
Aflatoxin B1	−26/88	−46/84	−45/104	−25/77	−17/14	2/13	−33/82	−32/80	−15/11	7/16	−39/101
Aflatoxin B2	−25/70	−44/81	−41/74	−17/73	−30/15	−19/17	−37/80	−40/86	−18/9	−26/19	−37/78
Aflatoxin G1	−28/81	−35/77	−29/75	−29/80	−29/49	−18/47	−44/89	−44/94	−31/40	−19/43	−27/73
Aflatoxin G2	−32/104	20/55	10/76	48/69	−9/66	−5/39	−10/74	−26/98	1/33	4/45	10/64
Ochratoxin A	13/76	−1/78	−2/93	12/76	14/30	10/40	−3/69	−1/69	2/34	3/37	−7/71
Deoxynivalenol	−19/79	−23/78	−27/89	−22/80	−28/85	−24/83	−28/81	−27/84	−27/87	−18/86	−32/94
Fumonisin B1	164/90	124/89	102/104	110/87	76/102	135/87	118/107	103/105	147/76	124/77	137/81
Fumonisin B2	117/88	115/88	112/96	116/84	107/88	105/90	101/88	94/89	105/86	106/84	89/88
Fumonisin B3	112/81	139/98	79/160	161/103	168/96	47/106	109/80	47/97	49/99	54/85	78/77
Nivalenol (NIV)	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/ nd	nd/nd
Diacetoxyscirpenol	−1/93	6/71	−1/78	10/76	34/68	36/80	−10/105	15/82	−8/95	14/85	29/63
T2-Toxin	−5/87	−3/87	0/84	1/98	−12/104	9/82	1/78	0/78	29/65	14/78	−12/82
HT2-Toxin	−7/100	1/78	1/85	−10/85	−12/89	7/77	2/78	5/69	2/82	23/82	−10/92
3-Acetyl-DON	−32/86	−22/88	−3/68	−1/70	−33/102	−18/68	−31/75	−30/82	−24/95	−18/83	−34/76
Zearalenone (ZEN)	7/80	−6/78	5/77	−2/68	−13/83	12/74	−13/83	−12/80	4/72	5/67	−17/84
15-Acetyl-DON	−8/64	−18/95	−34/106	−19/110	−20/91	39/53	−44/102	−24/81	−27/101	−20/94	−35/78
Penicilic acid	33/84	23/88	16/86	18/88	11/84	34/86	22/88	4/79	19/82	41/80	1/92
Fusarenon-X	−21/99	−12/84	−17/88	−14/87	−3/73	−12/88	−27/98	−22/87	−4/85	2/80	−22/82
β-Zearalanol (β-ZAL)	6/80	4/83	10/75	8/77	−11/88	7/76	−6/78	−8/80	1/70	1/82	−15/81
α-Zearalanol (α-ZAL)	0/87	1/78	1/80	−3/77	−6/74	4/78	−11/80	−9/80	4/71	−1/75	−23/83
Citrinin	2/66	−3/80	0/80	0/79	−5/38	−2/45	−6/66	−7/66	−3/35	0/35	−8/62
Zearalanone (ZAN)	19/81	13/71	4/79	−3/79	4/81	8/91	−9/84	3/76	26/67	24/70	−9/85
Cyclopiazonic acid	7/78	3/77	1/77	1/74	−4/63	−8/72	−3/70	−5/67	−10/53	−4/68	−13/66
Sterigmatocystine	7/84	3/78	−1/80	−1/80	−5/14	8/16	−3/80	1/76	3/11	9/13	−5/78
Roquefortine C	0/85	−14/83	−25/78	−29/86	−31/76	−6/79	−13/90	−13/84	−6/73	−4/78	−10/82
α-Zearalenol (α-ZEL)	19/80	13/72	3/79	−2/78	4/81	6/93	−8/84	3/76	25/67	25/70	−11/87
Mycophenolic acid	12/88	3/77	3/88	5/78	8/81	−5/83	−5/82	−8/80	15/72	5/74	−6/77
Altenariol	−6/80	15/82	12/69	11/54	18/11	15/16	0/76	7/64	−4/12	12/12	−4/56
Altenariol-methyl	27/76	−2/81	4/85	−1/74	−2/10	23/11	2/73	6/71	16/9	19/9	0/59
Ergotamin	−11/79	−19/86	−11/73	−10/80	−28/52	−24/56	−22/79	−28/91	−16/40	−9/48	−34/91
Ergonovin	−12/82	−26/88	−13/77	−16/84	−9/72	−28/81	−20/82	−26/82	−16/74	−16/76	−25/82
Ergocornin	−12/75	−9/68	−37/99	−25/79	−40/68	−33/77	−35/91	−27/79	−24/57	−26/72	−41/95
Ergokryptin	−18/78	−37/86	−44/87	−32/87	−46/66	−23/71	−36/83	−34/85	−17/63	−17/69	−38/89
Ergocristin	−1/73	0/55	−31/79	−31/83	−34/42	−7/46	−24/84	−25/79	4/27	−22/53	−30/86
Ergosin	−21/86	−20/83	−17/79	−19/85	−19/68	−21/69	−30/84	−31/82	−19/60	−19/68	−34/90
Mevinolin	−4/86	−1/64	−8/83	−14/85	−10/78	−6/79	−14/78	−9/67	1/64	−3/79	−18/82

each individual calibration level, calibration curve regression equations with their determination coefficients (r^2) were calculated and the linear range for each mycotoxin was determined.

The instrument limits of detection (LOD) were calculated from the calibration curves and the repeatability (RSD, $n=6$) data of injected calibration solutions at the lowest detectable levels.

The minimum concentration of an analyte that can be detected by the instrument, at the 95% confidence level, with a response significantly higher than the background is defined as the instrument LOD (LOD_i). This lowest concentration should always have been really injected and detectable repeatedly (with a signal-to-noise ratio >3) all six times at that level, while the RSD should not have exceeded 33% [14]. The minimum concentration of an analyte that can be quantified with 99% confidence is defined as instrument LOQ (LOQ_i). The lowest validated spike level meeting the requirements of a recovery within the range 70–120% and a RSD $\leq 20\%$ [16] is defined as method LOQ (LOQ_m) and the calculation is based on the accuracy and precision data, obtained via the recovery study.

2.8.2. Matrix effect evaluation

The influence of (mostly) undetected components from the matrix on the measurement (detector response) of the analyte concentration is the definition of matrix effect [25]. In wine, the main co-extracted compounds are fatty acids, esters, alcohols and sugars, which can decrease or increase the detector response of the analyte. Sometimes these effects on the ionization process are difficult or not at all to eliminate [26]. The result of matrix effect can be determined by comparing the response obtained from standard solutions ($n=6$) of each mycotoxin in solvent and in blank wine extract. It is possible by this evaluation to observe, to calculate and to compare the positive or negative matrix effect that is an increase or decrease of the detector response, respectively. The matrix effect (in %) was calculated by dividing the average peak response (area) of the mycotoxin in matrix extract minus the average peak response of the mycotoxin in solvent by the average peak response of the mycotoxin in solvent.

When the average matrix effect exceeds around 20%, it can generally be considered to have a significant effect on the quantitative analytical results, but it is also necessary to take the repeatability (expressed as RSD values) of the average peak areas into account [23].

2.8.3. Accuracy and precision (recovery experiments)

The accuracy and precision of the method were evaluated through recovery experiments by spiking mycotoxins to a “blank” aliquot of wine (wine without mycotoxins), at three different concentration levels, with six replicates at each level ($n=6$). Mycotoxins from each group were spiked at different concentration levels, which are shown in Table 3. The “blank” wine extract was also analyzed six times.

The spiking procedure was performed by adding the standard mixture solution containing 36 mycotoxins to the mixture of water and wine, at the beginning of the procedure, before applying the extraction method (Fig. 2). After 1 min manual shaking/mixing, the contact time of the mycotoxins with the wine, before addition

Table 5
Matrix effect and recovery percentage obtained by applying various dSPE cleanup sorbents and their mixtures, performing the extraction only with acetonitrile/1% acetic acid.

Mycotoxin	Matrix Effect (%)/Recovery (%)						
	CF	C18	Florisil	GCB	Florisil + GCB	C18 + GCB	Extra drying step
Aflatoxin B1	−7/95	−15/85	−38/41	−8/0	−28/9	−22/23	−24/98
Aflatoxin B2	−31/96	−22/82	−45/37	−29/0	−41/6	−35/15	−47/89
Aflatoxin G1	−25/90	−15/86	−33/62	−17/1	−40/43	−25/50	−31/87
Aflatoxin G2	−23/83	2/91	−32/70	5/0	−43/39	−5/31	−26/75
Ochratoxin A	−2/86	11/71	−9/72	−120/	−13/31	−6/42	−16/115
Deoxynivalenol (DON)	−32/89	−28/85	−24/95	−19/81	−22/85	−34/40	−37/78
Fumonisin B1	9/77	−41/93	18/0	8/32	−50/1	−28/91	−3/90
Fumonisin B2	2/77	14/90	2/1	−1/31	−3/1	6/85	−14/87
Fumonisin B3	−8/85	6/86	−27/0	−19/40	−3/0	12/81	−9/83
Nivalenol (NIV)	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd
Diacetoxyscirpenol (DAS)	7/109	−27/89	−34/88	−46/87	−6/76	−5/56	−9/89
T2-Toxin	11/79	7/89	3/80	−5/72	20/79	3/93	−3/93
HT2-Toxin	−18/86	−13/79	−13/81	−7/74	−18/95	−17/100	−8/75
3-Acetyl-DON	−37/83	−2/78	−10/126	32/76	−1/84	21/67	−42/75
Zearalenone (ZEN)	4/78	−4/89	−4/85	−12/8	−11/81	−4/71	−5/90
15-Acetyl-DON	−29/104	−38/85	−3/54	0/64	−26/78	−34/91	−14/69
Penicilic acid	−6/85	12/86	6/99	0/84	0/89	−7/92	−22/88
Fusarenon-X	−14/92	−19/86	−24/94	−14/79	−26/78	−29/96	−34/97
β-Zearalanol (β-ZAL)	2/82	1/79	−4/83	−5/6	−9/82	−8/74	−11/94
α-Zearalanol (α-ZAL)	6/83	8/75	−4/87	−4/9	0/92	5/82	−10/93
Citrinin	11/65	7/84	4/90	0/4	0/57	−1/29	9/81
Zearalanone (ZAN)	−1/88	6/91	−6/90	−7/18	−9/87	−1/81	1/83
Cyclopiazonic acid	13/79	−8/71	11/73	−13/1	7/60	−16/48	6/83
Sterigmatocystine	1/85	10/87	11/86	−3/0	−1/9	2/18	−3/89
Roquefortine C	5/88	−25/68	−42/ 75	1/17	−41/54	−35/74	−4/86
α-Zearalenol (α-ZEL)	0/88	8/90	−2/87	−4/18	−6/86	2/82	1/83
Mycophenolic acid	12/84	21/87	6/83	−12/10	−4/76	−9/80	8/82
Altenariol	−2/90	−21/101	0/61	3/0	13/7	−9/23	1/77
Altenariol-methyl	16/78	36/74	24/76	7/1	20/6	14/16	−10/105
Ergotamin	26/80	86/83	45/72	91/0	38/14	100/44	4/91
Ergonovin	−21/87	−13/79	28/58	−2/24	36/53	−9/80	−30/88
Ergocornin	−5/87	47/76	−10/50	36/3	−56/41	30/66	−14/82
Ergokryptin	−3/89	11/83	−49/64	26/3	−60/37	−7/73	−11/93
Ergocristin	15/82	18/63	−39/67	10/0	−66/10	−15/49	3/76
Ergosin	−1/88	29/82	21/58	69/3	6/45	51/63	−15/97
Mevinolin	−30/76	1/72	−6/93	−16/33	−8/91	−5/70	−14/81

of extraction solvent, was kept at 10 min, but not longer than necessary, in order to avoid possible degradation of some mycotoxins.

3. Results and discussion

3.1. Wine sample amount

Different ratios of the mixture of wine and water were tested. Applying too low amounts of wine, like 1 or 2 mL, resulted in loss of sensitivity and increase of detection limits. The best compromise was achieved with a 1:1 mixture of wine and water.

3.2. Cleanup and buffer influence studies

Various sorbents were tested for the dispersive solid phase extraction (dSPE) step.

Nexus is a polymeric sorbent with hydrophilic and lipophilic characteristics, capable to obtain good extraction yields of acidic and basic compounds. Oasis is a similar sorbent with balanced hydrophilic–lipophilic selectivity, capable of being wetted with 100% water if applied in a SPE cartridge, which facilitates elution with a non-polar eluent, without the need for a drying step. These characteristics make both sorbents generally suitable for many SPE applications.

The sorbents C_{18} and Florisil are well known for their efficient removal of fatty matrix components [27]. Graphitized carbon black (GCB) is a very effective sorbent to remove matrix interferences, typically chlorophylls from fruits and vegetables extracts. However, it has a disadvantage that simultaneously also certain target analytes, namely nonpolar, planar aromatic compounds, can be lost due to irreversible adsorption. Clorofiltr (CF) is a polymeric sorbent, which has been developed specifically for use in the QuEChERS extraction method, with the aim to replace the use of GCB. Comparison tests applying CF have shown good results, removing interfering matrix (besides chlorophylls) but with the advantage of lower losses of target analytes than with GCB.

In Table 4 recovery and matrix effect results are shown comparing the various dSPE cleanup variations. CF showed the best results, in terms of offering the highest number of mycotoxins with recoveries within the acceptable range of 70–120%. C_{18}, Oasis and Nexus alone showed good results, with acceptable recoveries for most mycotoxins. The combinations of C_{18}+CF, Oasis+CF and Nexus+CF gave very similar results. GCB showed not to be useful for the cleanup step, because too many mycotoxins were lost in the dSPE.

The results obtained with experiments omitting the buffering step, that is just applying acetonitrile acidified with acetic acid, and an extra drying step with magnesium sulfate, with or without dSPE sorbents (C_{18}, Florisil, GCB, CF and their mixtures), are shown in Table 5.

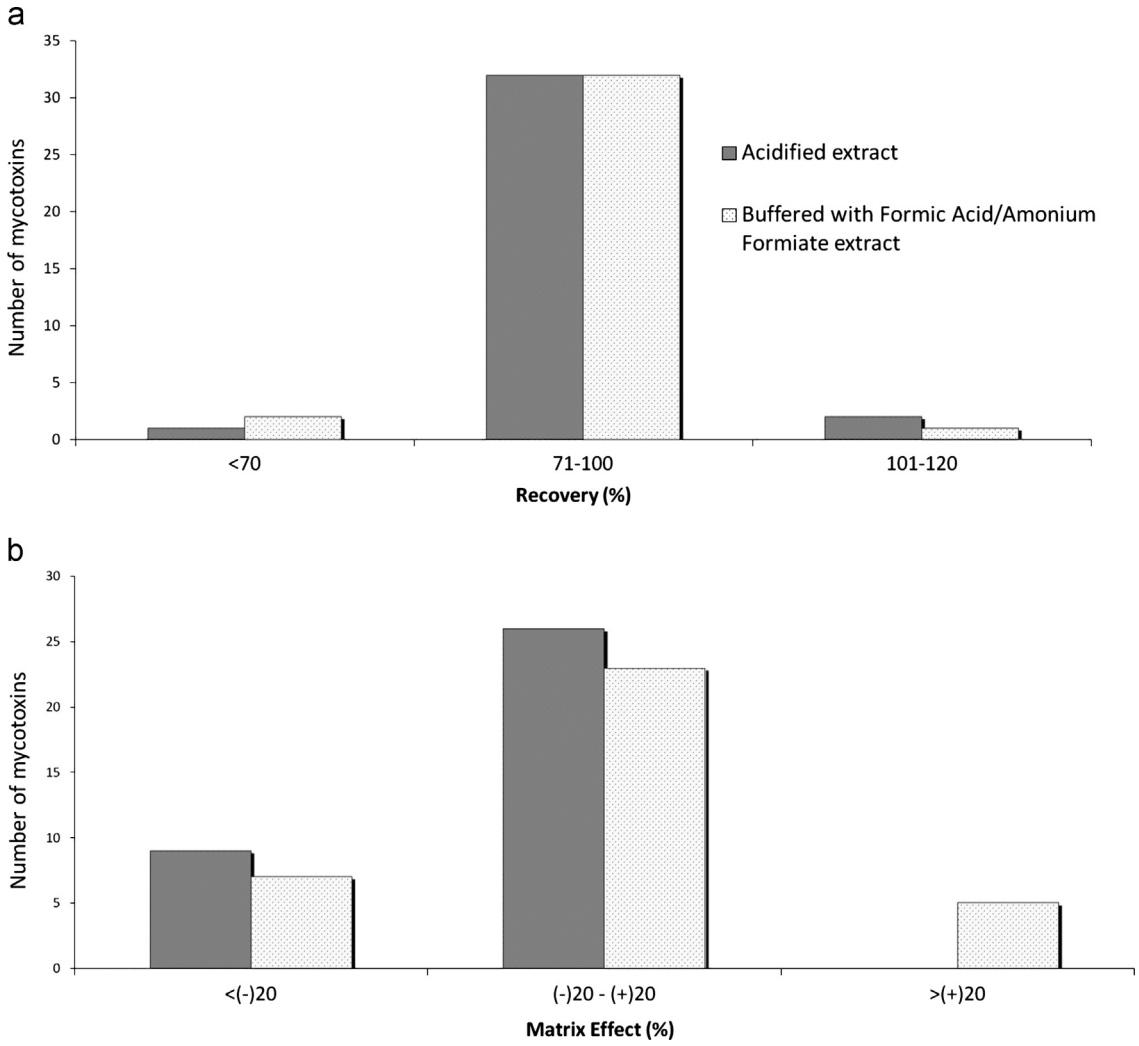


Fig. 3. Comparison between results for (a) recovery and (b) matrix effect, observed in experiments with and without buffering step.

The best results, as to recoveries and minimizing matrix effects, were obtained with CF or even using no sorbent at all, but just applying the extra drying step with magnesium sulfate. The combination of GCB and Florisil results in loss of too many mycotoxins.

In order to compare the influence of buffering in the extraction procedure, the method in which the dSPE is omitted and just the extra drying step is used, was tested with and without using a buffer of formic acid/ammonium formate. The comparison results are summarized in Fig. 3. Wine samples appear to have a low pH, which contributes to the effective extraction yields of mycotoxins. When applying acidified acetonitrile as extraction solvent, the pH is kept low enough for good recoveries, making the buffering step superfluous. The matrix effects are also still within an acceptable range. The optimized method was finally validated.

Table 6
Estimated LOD_i, LOD_m and LOQ_i, for mycotoxin standard solutions prepared in solvent and wine extract.

Mycotoxins group	LOD_i ($\mu g\ L^{-1}$)[a] (LOD_m $\mu g\ kg^{-1}$)[b]	LOQ_i ($\mu g\ L^{-1}$)[c]	Number of mycotoxins	
			Solutions in solvent	Solutions in wine extract
Group 1 (5 mycotoxins)	0.1 (0.4)	0.2	3	1
	0.2 (0.8)	0.5	2	3
	0.5 (2)	2	0	1
Group 2 (24 mycotoxins)	5 (20)	20	18	17
	10 (40)	50	1	2
	25 (100)	100	3	4
	50 (200)	200	1	0
Group 3 (7 mycotoxins)	0.1 (0.4)	5	7	7
	0.2 (0.8)	10	0	0
	0.5 (2)	20	0	0
	10 (40)	50	0	0

[a] $LOD_i = 3 \times RSD \times C$.
[b] $LOD_m = LOD_i \times$ method factor (4).
[c] $LOQ_i = 10 \times RSD \times C$.

3.3. Calibration curves, linearity, LOD and LOQ

The results obtained for mycotoxin standard solutions prepared in solvent and in matrix extract, as to determination coefficients (r^2) and linear range of the calibration curves, were very similar for most mycotoxins studied. For mycotoxin standard solutions prepared in solvent, 11% showed $r^2 > 0.99$, 27% showed $r^2 > 0.98$ and the other 62% had a r^2 in the range 0.95–0.98. For mycotoxin standard solutions prepared in wine extract, 11% showed $r^2 > 0.99$, 41% $r^2 > 0.98$, and the other 48% had r^2 in the range 0.95–0.98. The slightly better results obtained for mycotoxins in wine extracts are

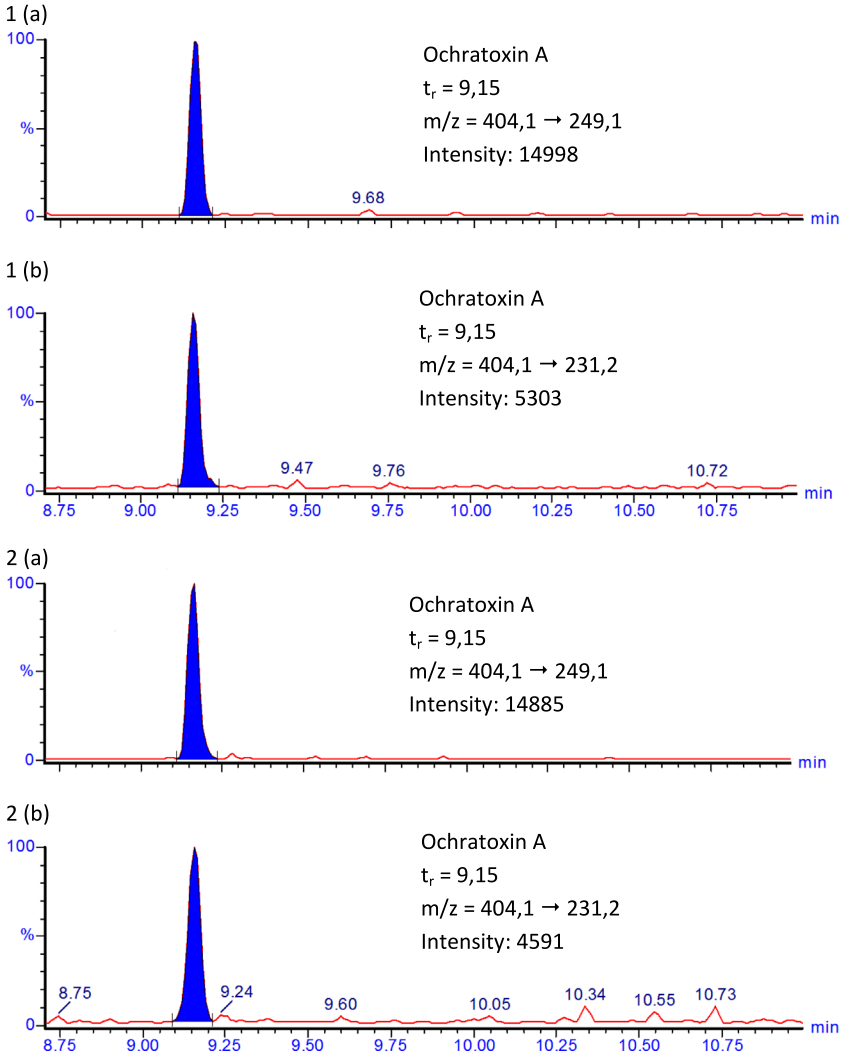


Fig. 4. Reconstructed ion chromatograms obtained by UPLC–MS/MS for (a) the quantification ion and (b) confirmation ion of Ochratoxin A, at 2 ng mL^{-1} concentration, (1) in solvent and (2) in wine extract. Responses are normalized to 100%. Relative intensities of product ions are given in figures of chromatograms.

apparently caused by some co-extracted matrix components, resulting in a more reproducible ionization of the mycotoxins, and thus more repeatable responses and better linearity.

In Table 6, the summarized results are shown for the estimated instrument LOD_i, LOD_m and LOQ_i for each group of mycotoxins from standard solutions in solvent and in wine extract.

3.4. Matrix effect evaluation

The matrix effect usually observed in LC–MS/MS systems is caused by interfering matrix components in the extract, eluting at the same retention time as the analyte and thereby competing during the ionization process in the ion source [23]. In some cases, the number of ions formed can be increased, but more often decreased (due to ion suppression), resulting in a corresponding positive or negative matrix effect, respectively. The significance of the matrix effect at an individual concentration is depending on the percentage difference between the average response of the mycotoxin in matrix and in solvent, and on the RSD values of the replicate injections. Assuming that each of those RSD values is $<10\%$ (or $<15\%$), a matrix effect $>15\%$ (or $>20\%$), has generally to be considered as significant and has to be taken into account when reporting quantitative results. At lower concentrations, approaching the LOD, RSD values of repeated injections tend to be higher and therefore measured matrix effects need to be carefully interpreted.

From the studied mycotoxins, Sterigmatocystin and Fumonisin B1 had the most similar calibration curves that are showing a negligible matrix extract. Fig. 4 shows, as a typical example, the extracted ion chromatograms of standard solutions of Ochratoxin A in solvent and in matrix, at 2 ng mL^{-1}. The signal for the quantification and confirmation product ion of standard solution prepared in matrix extract is slightly lower than the signal for the solution prepared in solvent, indicating that there is a slight suppression of the signal by the presence of the matrix. For the final, optimized method, matrix effects for most mycotoxins were within the acceptable range ($<\pm 20\%$).

3.5. Accuracy, precision, selectivity and method LOQ

The method performance criteria as to accuracy (trueness) and precision in the validation study are typically a recovery within the range of 70–120% and repeatability RSD $\leq 20\%$ [25]. Table 7 shows the summarized data on recoveries and RSD for the 36 mycotoxins, which were spiked at three different levels. From all 36 mycotoxins studied, 35 showed recoveries in the range 70–120%, confirming the high accuracy of the method. Only nivalenol could not be quantified at any concentration studied, which is caused by the almost unretained elution of nivalenol, resulting in peak splitting and also a very low response due to high ion suppression. A specific single mycotoxin method, using optimized mobile phase conditions, could solve this problem. For the 35 mycotoxins, very good repeatability (RSD%) results were obtained for all spike levels studied. For the first and second (lowest) spiking levels, 31 and 32 mycotoxins (out of 36 studied), respectively, showed a RSD below 20%. For the third (highest) level, the RSD obtained for all mycotoxins studied (except nivalenol) was below 20%. Thus, 35 mycotoxins fulfilled the validation criteria at all or at least at one or two of the spike levels. The LOQ_m values, corresponding to the levels where these validation criteria are met, are given also in Table 7.

The pesticides quinalphos (12.5 ng mL^{-1}) and propoxur (10 ng mL^{-1}) were used as the instrument (I.I.S.) and procedure internal standard (P.I.S.), respectively. The P.I.S. was placed into the extraction solvent in order to check the whole analytical procedure. The I.I.S. was added always to the final extracts, at the same concentration in the samples as in the calibration solutions, in order to assess any problem during injection in the UPLC–MS/MS system. As criterion, a maximum response deviation of 20% between an individual injection and the average of all injections of the I.I.S. has been used.

Table 7
Recoveries and RSD data (%) for the 3 spike concentration levels studied and LOQ_m values.

Mycotoxins	Spike levels						LOQ_m[a] (μg kg^{-1})
	1		2		3		
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
Aflatoxin B1	81	13.2	86	8.6	83	8.7	1
Aflatoxin B2	102	13.3	85	12	81	13.4	1
Aflatoxin G1	80	27.9	95	15.4	89	11.8	2
Aflatoxin G2	74	23.2	72	27.5	89	17.6	10
Ochratoxin A	100	18.8	98	12.7	91	11.8	1
Deoxynivalenol (DON)	92	15.4	88	16.2	95	7.4	50
Fumonisin B1	81	16.1	81	7.8	80	7.4	50
Fumonisin B2	82	6.8	81	8.8	82	8.4	50
Fumonisin B3	88	10.1	85	8.6	88	7.8	50
Nivalenol (NIV)	n.q	n.q	n.q	n.q	n.q	n.q	n.q.[b]
Diacetoxyscirpenol (DAS)	79	19.5	82	22.9	85	11.3	50
T2-Toxin	106	11.7	95	12.7	83	6.6	50
HT2-Toxin	83	17.8	88	10.5	84	9	50
3-Acetyl-DON	75	30.9	92	17	88	17.3	125
Zearalenone (ZEN)	88	10.8	90	11.3	90	9.2	50
15-AcetylβDON	104	44.4	113	29.2	110	18.4	500
Penicilic acid	93	4.5	92	8.5	90	8.8	50
Fusarenon-X	89	7.6	86	12.9	84	8.1	50
β- Zearalanol (β-ZAL)	86	5.2	87	6.8	88	9.2	50
α-Zearalanol (α-ZAL)	95	10.7	90	5.4	91	10.1	50
Citrinin	79	4.7	94	6.2	95	4.2	50
Zearalanone (ZAN)	90	10.3	96	17.7	83	9.6	50
Cyclopiazonic Acid (CPA)	70	17	80	16.9	88	9.6	50
Sterigmatocystine	81	13	97	12.2	94	12.2	50
Roquefortine C	71	14.7	92	11.5	100	4.7	50
α-Zearalenol (α-ZEL)	90	10.3	96	17.7	84	9.6	50
Mycophenolic acid	92	10.6	93	8.8	92	10.6	50
Altenariol	72	10.6	86	5.8	93	11.3	50
Altenariol-methyl	82	13.5	91	13.3	93	8.8	50
Ergotamin	78	6.5	82	7.6	82	6.2	10
Ergonovin	80	11.9	86	5.9	90	2.3	10
Ergocornin	94	14.2	87	13.1	92	9.5	10
Ergokryptin	94	10.4	89	9.1	87	7.3	10
Ergocristin	83	9.8	86	9.9	86	6.8	10
Ergosin	93	6.1	90	6.2	87	9.1	10
Mevinolin	90	10.4	89	7.8	89	5	10

[a] LOQ_m=Lowest validated spike level, meeting validation criteria.
[b] n.q.= not quantifiable.

Identification of the mycotoxins is performed according to the newest EU SANCO Document no. SANCO/12571/2013 for pesticides [25], which states the requirement of the ion ratio of the 2 product ions of the 2 MRM transitions to be the same for sample extract and reference (calibration) standard, within a tolerance of 30%.

3.6. Survey of wine samples from the Dutch market

The developed method was applied in routine analysis by analyzing wine samples purchased at various wine stores, supermarkets and importers warehouses. The countries of origin of the samples were both European (France, Italy, Spain and The Netherlands) and non-European (Argentina, Australia, Chili, New Zealand, South Africa and United States). A summary of the results, with the

Table 8
Survey of wine samples from the Dutch market, with different mycotoxins detected their frequency of occurrence and concentration ranges.

Year	Total number of samples	Number of samples without mycotoxins	Number of samples with mycotoxins detected				
			Ochratoxin A	Alternariol	Cyclopiazonicacid	Mycophenolicacid	Zearalenone
2008	68	51	6 (0.2–0.6 μg kg^{-1})	–	8 (1.2–2.7 μg kg^{-1})	1 (6.8 μg kg^{-1})	2 (3.1–3.5 μg kg^{-1})
2009	62	52	–	3 (6.4–12 μg kg^{-1})	–	7 (6.2–35 μg kg^{-1})	–
2010	50	49	–	–	–	1 (54 μg kg^{-1})	–
2011	60	57	–	–	–	3 (11–14 μg kg^{-1})	–
2012	40	40	–	–	–	–	–

different mycotoxins found, their frequency of occurrence and concentration ranges, is shown in Table 8. Results of the years 2008–2010 were still obtained with the original acetonitrile-based extraction of Spanjer et al. [19] and the results of 2011 and 2012 with the new developed method described in this paper. The basic difference between the two methods is that in the original method extraction is achieved by shaking with an acetonitrile–water mixture without partitioning, while the new method is based on acetonitrile extraction with a subsequent partitioning step with $MgSO_4$ and an extra drying step with $MgSO_4$. The main advantages of the new method are a faster sample extraction/preparation step and cleaner extracts. The method LOQ values were similar for both methods.

Measuring wine samples with a multimycotoxin method offers the opportunity to determine the presence of mycotoxins in wine more systematically. In the last 5 years, apart from OTA (0.2–0.6 μg kg^{-1}, $n=6$) and Alternaria toxins (6.4–12 μg kg^{-1}, $n=3$), also cyclopiazonic acid (1.2–2.7 μg kg^{-1}, $n=8$), mycophenolic acid (6.2–54 μg kg^{-1}, $n=12$) and zearalanone (3.1 and 3.5 μg kg^{-1}, $n=2$) were detected in the 280 wine samples analyzed. All concentrations obtained were well below the lowest validated spike levels meeting the validation criteria, that is the LOQ levels (Table 7), but still above the practical LOD concentrations and must thus be considered as semi-quantitative results. From the mycotoxins detected, only for ochratoxin A, an EU maximum limit has been set at 2 μg kg^{-1} and this limit was not exceeded. The concentrations of the other mycotoxins are relatively low, compared with the maximum limits set for other products. Therefore, it may be concluded that the consumer intake of mycotoxins via the consumption of wine does not pose any considerable risk. There appeared to be no clear correlation between the detection of the different mycotoxins in wine and the countries/regions of origin and/or variety of the wine. The mycotoxins detected and their levels correspond nicely with those reported in the literature [8,9,14,28,29]. The developed method applied in our survey affords the simultaneous detection of all mycotoxins present in one analysis and thus improves the efficiency considerably.

4. Conclusion

A fast and simple multimethod was developed and optimized for the analysis of 36 mycotoxins, based on an acetonitrile extraction, followed by a partitioning and a subsequent drying step with magnesium sulfate, and detection using UPLC–MS/MS (ESI positive mode). No cleanup was necessary, because matrix effects were kept at an acceptable level. Compared to most methods described in the literature, which involve determination of a single mycotoxin or a limited group of 10–15 mycotoxins only, this method comprises 35 successfully validated mycotoxins.

The results obtained in the validation procedure showed excellent accuracy and precision for most mycotoxins studied at all three spike levels. Nivalenol was the only mycotoxin that could not be quantified appropriately in this study and needs special LC conditions for analysis. The method presents very good results for Ochratoxin A, the most frequently found mycotoxin in wine samples. The efficiency of the multimethod was confirmed in surveys of wine samples during the last 5 years, revealing, apart from ochratoxin A, also the presence of some less frequently targeted and detected mycotoxins, like alternariol, mycophenolic acid, cyclopiazonic acid and zearalanon. No maximum limits were exceeded and the consumer risk of mycotoxin intake via wine seems negligible.

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