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Simultaneous determination of pesticides, mycotoxins, and metabolites as well as other contaminants in cereals by LC-LC-MS/MS

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A B S T R A C T

A 2D LC-MS/MS method for the simultaneous determination of 350 pesticides, 16 mycotoxins as well as the growth regulators Chlormequat and Mepiquat was developed. The method is applicable to cereals and products thereof. Attention should be paid to the simultaneous analysis of the cereal-relevant mycotoxins aflatoxin B1, B2, G1 and G2, ochratoxin A, deoxynivalenol and zearalenone. Moreover, the tropane alkaloids atropine/scopolamine could be integrated into the final method. The samples were extracted with a mixture of acetonitrile/water (80:20), diluted with acetonitrile and injected into an LC-LC-MS/MS system. There were no further manual clean-up steps. The automatic online clean-up took place during the HILIC-separation in the first dimension (*YMC-Pack Diol*; 2.1 × 100 mm; 5 µm, 120 Å). Here, polar matrix compounds were retained, while the majority of the analyte scope eluted in a fraction at the beginning of the analytical run. This fraction was transferred to the second dimension by a packed loop interface (*Agilent Zorbax* SB-C8; 4.6 × 12.5 mm; 5 µm; 80 Å). On the second column (*Phenomenex Synergi Fusion* RP C18; 2 × 100 mm; 2.5 µm; 100 Å), the majority of the scope was separated by a typical RP-gradient. Only some of the polar pesticides could not be transferred to the second column. They eluted directly after the transfer step from the HILIC-column to the MS/MS. The final method was sensitive enough to meet all the regulated maximum levels for pesticides in cereals according to EU Regulation 396/2005 and those for contaminants according to EU Regulation 1881/2006. Above all, the method was so robust and accurate that nearly 90% of the pesticides and all the tested mycotoxins, growth regulators and tropane alkaloids fulfilled the validation criteria of the *SANTE* guideline document, although the demanding criteria are only applicable to pesticides. For the verification, eight proficiency tests were passed successfully: three for the pesticide analysis, three for the mycotoxin analysis, and two for the analysis of the tropane alkaloids. In addition to the already mentioned contaminants, the six most important ergot alkaloids (e.g. ergotamine/ergotaminine) and two modified mycotoxins (deoxynivalenol-3-glucoside and zearalenone-sulfate, also known as masked mycotoxins) were detected during the routine analysis of rye and corn samples.

1. Introduction

In the European Union (EU), about 13% of the land area is used for crop growing on average. Germany has the third-largest cereal-producing area (6.7 million ha) in the EU, after France and Poland. In 2017, 300 million tons of cereals were produced in all of the EU countries [1]. A Good Manufacturing Practice (GMP) is imperative to be able to guarantee high crop yields; therefore making use of fertilizers and pesticides is essential. Not using pesticides would lead to crop losses for several reasons, one being the growth of fungi resulting in the contamination of the cereals with mycotoxins, particularly under humid climatic conditions. Another possible source of contamination might be due to concurrently harvesting weeds containing toxic tropane alkaloids. For reasons of food safety and consumer protection, it is necessary to screen cereals for both pesticide residues and contaminants such as mycotoxins.

To assure consumer protection, the EU has established the regulation (EC) No. 396/2005 for setting maximum residue levels (MRLs) of pesticides in or on food and feed of plant and animal origin [2]. The regulation (EC) No. 1881/2006 for setting maximum levels for certain contaminants in foodstuffs has also been adopted [3]. During production, processing, storage and transport there are many possibilities for residues or contaminants to get into food and feed. In Germany, food samples were investigated by state laboratories for customer protection and health care. In parallel, many private laboratories were founded to analyze residues and contaminants in food and feed. For pesticide analysis, the majority of both state and private laboratories use QuEChERS-based sample preparation methods [4]. They are usually modified when challenging matrices or alternating analyte scopes have to be analyzed. The numeration of all modifications would go beyond the scope of this introduction. Reviews gave a convenient overview [5–7]. Only a few laboratories use different methods, e.g., the ChemElut-method [8], the SweEt-method [9] or the Luke-method [10]. Irrespective of the chosen pesticide residue method, up to 500 substances were analyzed by using one (or two) extraction approaches and two

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measurement systems. In this regard, GC–MS(/MS) and LC-MS/MS have been established nowadays as state-of-the-art-instruments for the trace analysis of both residues and contaminants.

In the field of mycotoxin analysis, sample preparation techniques are more fragmented. Although the developments in pesticide analysis tend toward a reduction in manual clean-up steps, it is the opposite concerning the mycotoxin analysis. The main reasons are the lower maximum residue levels of the mycotoxins; e.g., the MRL of Aflatoxin B1 in cereals and products thereof is 2 µg/kg. Moreover, the MRL of Aflatoxin B1 in processed cereal-based foods and baby foods for infants and young children is 0,1 µg/kg. These low MRLs demand sensitive and robust analytical methods. To guarantee this, it is common to analyze mycotoxins only as single or group parameter methods, each optimized for the special compounds. For matrix removal and analyte enrichment, solid-phase-extraction (SPE) or the use of immunoaffinity columns (IACs) is widespread. Although, these clean-up tools are selective and robust, they are cost- and time-consuming. However, there is also a tendency toward developing and establishing multimethods for a larger scope of mycotoxins in different matrices [11]. Particularly, the ongoing development of mass spectrometry technology has increased both the selectivity and the sensitivity of the LC-MS/MS-technology. This has enabled a reduction in clean-up steps and, furthermore, the simultaneous determination of more mycotoxins within one analytical run [12–14]. These developments have led to an introduction of many multimethods in several trace analysis fields, not only for pesticides or for mycotoxins. However, the simultaneous determination of different analytes always includes compromises. This is why the conditions are not optimized for each analyte. Lower recoveries, particularly for special analytes, lead to a challenging evaluation of matrix-rich samples.

Nevertheless, three research groups have already tried to summarize the analysis of pesticides and mycotoxins for chosen matrices. The first and most comprehensive approach was described by Mol et al. in 2008. They investigated the simultaneous determination of a wide spectrum of residues and contaminants (pesticides, mycotoxins, plant toxins and veterinary drugs) in different matrices [15]. The sample preparation consists only of a QuEChERS-based extraction step without further clean-up treatment. The whole spectrum was identified in two analytical runs (pos-ESI-LC-MS/MS and neg-ESI-LC-MS/MS). In 2011, Romero-Gonzalez et al. did a similar approach [16]. Their list of investigated substances was clearly shorter. They also used a QuEChERS-based-extraction step and the raw extracts were injected and measured within one run. Lacina et al. studied the application of different extraction options for this comprehensive analytical approach in 2012 [17]. Their spectrum was approximately as large as that by Mol et al.; however, they were able to achieve a lower LOQ for the analytes of interest. They confirmed the general feasibility of the simultaneous determination by reaching very exact results in laboratory comparison trials. In parallel, they achieved very low LOQs, particularly for some mycotoxins (e.g. aflatoxin B1 and ochratoxin A). This is necessary in order to control the low MRLs of these substances in different matrices. In general, all three publications applied a “fast and dirty” extraction step followed by a measurement via LC-MS/MS.

The overview in Table 1 shows a simple comparison between the published methods for the combined analysis of pesticides, mycotoxins and further contaminants.

Considering the above summary, it is obvious that no published method has been able to solve the analytical problem completely, so far. One reason is that the listed multi-contaminants-methods did not concentrate on a single matrix or a matrix category. The demands for the applicability of their methods in all chosen matrices were simply too high.

Therefore, the aim of this study was to summarize the extraction and measurement of a wide spectrum of substances, which are relevant for one matrix group. This had to be carried out in one extraction approach and one analytical run. The spectrum had to include analytes, whose analysis was relevant and mandatory for the matrix cereals. In detail, the list contained pesticides, mycotoxins, growth regulators and tropane alkaloids. Moreover, the possibility of detecting and integrating the metabolites of selected mycotoxins, the so-called masked mycotoxins, was studied. This substance group has been disregarded as yet in the most common mycotoxin analysis methods because of the lack of regulation. However, there are attempts to install a broader monitoring of these plant-generated metabolites for a better risk assessment [18–22].

Table 1
Comparison of the published multi-contaminant-methods.

Substance/substance groups	Method of		
	Mol	*Romero-Gonzalez*	*Lacina*
Pesticides	+	(+)[a]	+
Glyphosate	–	–	–
Mycotoxins	+	(+)[b]	+
Growth regulators	–	–	–
Tropane alkaloids	–	–	–
How many extraction approaches per sample?	1	1	1
How many analytical runs per sample?	2	1	2

[a] Only 85 pesticides included.
[b] Only two mycotoxins included.

Contrary to the existing multi-contaminants-methods, new techniques for both the sample preparation and the analytical run should be used within this study. One way is, to install a multidimensional LC-system. The general advantages of multidimensional chromatography (LC or GC) are an enhanced resolving power expressed as the peak capacity. The theoretical overall peak capacity of a multidimensional system is the product of the peak capacities of each single dimension. The highest resolving power would generate a system consisting of two dimensions, which are orthogonal to each other in their properties [23,24]. Both in gas and in liquid chromatography, many applications have been developed in different analytical fields. These methods can be carried out offline (collecting a fraction of the first dimension and reinjecting it in the second dimension) or online by using an interface. Moreover, there is the opportunity to transfer only one fraction (heart-cut) or all fractions (comprehensive) from the first to the second column. The heart-cut technology is more frequently applied in the field of target analysis, whereas comprehensive chromatography is used in the field or metabolomics, proteomics, and other non-target determinations of very complex, matrix-rich samples [25–32].

The first multidimensional approach in the field of pesticide residue analysis was made in the 1980s by Fogy et al., who coupled two NP (normal phase) columns to separate seven carbamates from co-eluting matrix compounds [33]. Rietveld and Quirijns, Hyötyläinen et al. and Sanchez et al. applied a heart-cut LC-GC-system for the analysis of up to ten pesticides [34–36]. In 2001, Choi et al. studied the matrix effects produced by ESI-MS/MS with the conclusion that multidimensional chromatography is one way to compensate for this problem in LC-MS/MS [37]. In parallel, Pascoe et al. developed the first online heart-cut LC-LC-MS/MS method for three pesticides [38]. Kittlaus et al. and Stahnke et al. also studied matrix effects in LC-MS/MS under different conditions [39] in 2013. To compensate the matrix effects, Kittlaus et al. developed a heart-cut two-dimensional LC-MS/MS method for the analysis of 300 pesticides in various food commodities [40,41]. The special feature was the automatic separation of analytes and polar matrix compounds within the first dimension. To the best of our knowledge, there is no method with a higher number of analytes using the two-dimensional LC-MS/MS-technology.

To reach the aim of this study, the two-dimensional LC-system of Kittlaus et al. was utilized as the basic system. This needs to replace the manual sample preparation after the extraction. Furthermore, the automatic clean-up step integrated in the analytical run must deliver reproducible results and, overall, a robust performance. After the

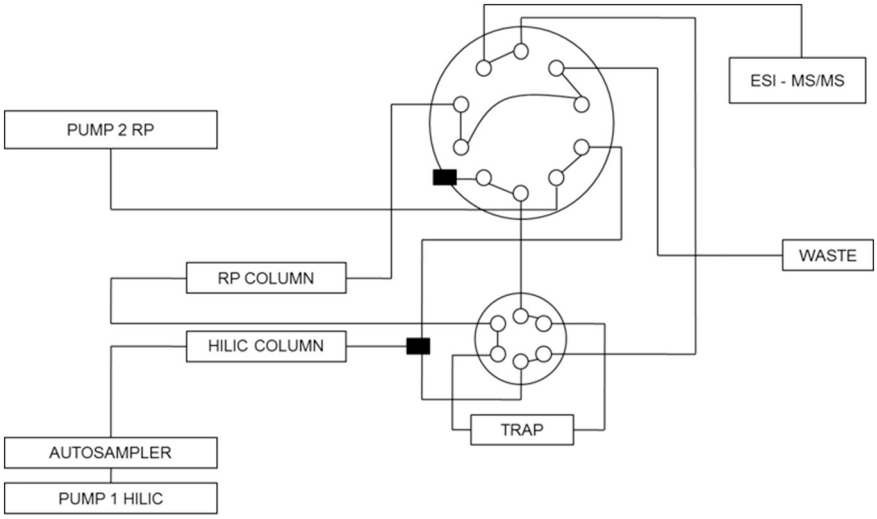

Fig. 1. Set-up of the 2DLC-MS/MS-system with the integrated clean-up feature.

installation of the Kittlaus-system, the LC- and MS/MS-parameters were optimized concerning the scope of interest. The integration of the mycotoxins, growth regulators and tropane alkaloids was checked. For the extraction, a compilation of different solvent mixtures was tested in order to extract the substances completely. The matrix effects were studied by evaluating the recovery rates and by using the post-column infusion approach [41]. With these two options, the best extraction conditions were selected. After the development, the method was validated according to the standards of the German accreditation body (*DAkkS*) and the *SANTE* Document [42]. Several laboratory comparison trials had to prove the feasibility and sensitivity of the system developed. Finally, about 250 samples were tested for the appearance of masked mycotoxins. The integration of these metabolites and further cereal-relevant contaminants was tested.

2. Materials and methods

2.1. Reagents and materials

All pesticide standards including growth regulators and tropane alkaloids were purchased from *Dr. Ehrenstorffer* (Germany) or *Sigma-Aldrich* (Merck, Germany). The standards of nearly all mycotoxins plus DON-glucoside were from *Biopure* (Austria). Only the aflatoxin standards came from *Sigma-Aldrich*. The solvent for the prepared stock solutions was methanol or acetonitrile. The concentration of the stock solution depended on the analytes. For the pesticides, growth regulators and tropane alkaloids, the level of the stock solution was 1 g/L. The pesticide solutions were summarized in six standard mixtures with a concentration of 10 mg/L. The standard solutions of the growth regulators and tropane alkaloids were also diluted to 10 mg/L. In the case of the mycotoxins, it varied due to the different spiking levels. The deuterated substances diazinon-d_{10}, diuron-d_6, imidaclopride-d_4, carbendazim-d_4, propamocarb-d_7, chlormequat-d_4 and mepiquat-d_4 were used as internal standards (ISTD). The ISTD solutions (c = 1 g/L) were prepared with methanol or acetonitrile. The concentration of the ISTD working solution was 10 mg/L. All solutions were stored at −18 °C. Acetonitrile, methanol and water were purchased from *VWR Chemicals* (Germany) in LC-MS-grade. Ammonium formiate and formic acid were from *Sigma-Aldrich*.

2.2. Sample preparation

The focus of the experiments was on cereals. Therefore, wheat and corn samples were analyzed. All the cereal samples were collected from the storerooms of *Eurofins Sofia GmbH*. The materials were ground with a ZM200 *Retsch* ® mill. In case of challenging sample constitutions (corn), cryogenic milling was done. They were tested for pesticide residues and contaminants with established methods before using them as test materials.

For the cereals, 5 g of the samples were weighed in a plastic centrifuge tube. Twenty microliters of the ISTD working solution (c = 10 mg/L) was added. Afterwards, 20 mL of the extraction solvent acetonitrile/water (80:20) was added to the tube. To guarantee equal moistening, the sample tubes were vortexed with a tube vortexer (2800 rpm) from *VWR*. After a soaking time of 20 min, the samples were shaken by an overhead shaker for 30 min. The extracts were centrifuged for 3 min at 3000 rpm. An aliquot of 500 µL of the supernatant was diluted with 500 µL acetonitrile, which was spiked with propamocarb-d_7 (c = 0.01 mg/L). The final extracts were filtered through a 0.2 µm PTFE filter (*Macherey-Nagel*, Germany) in a 1.8 mL vial.

2.3. 2D-LC-MS/MS analysis

For LC-LC-MS/MS-analysis, a 1260 HPLC system (*Agilent*, Germany), containing two degassers (G4225A), two binary pumps (G1312B) and one column oven (G1316) was configured. A PAL-injector (*CTC*, Switzerland) performed the sample injection. The HPLC was coupled to an API 4500 mass spectrometry system (*SCIEX*, Germany) by an Electrospray Ionization-Interface (ESI). For coupling the columns, the 6-port-valve (*Vici*, USA) of the column oven and an additional 10-port-valve (*Vici*, USA) were used. The preinstalled 6-port-valve (*Vici*, USA) of the MS/MS-instrument was configured to lead the analyte-free flow into the waste. All system components were controlled by the *SCIEX Analyst* acquisition software. For data evaluation, the *SCIEX* software *MultiQuant* was applied (Fig. 1).

Separation was performed by using two different columns, which differ in their stationary phases. In the first dimension, the analytes were separated on a *YMC Pack Diol* (2.1 mm × 100 µm; 5 µm; 120 Å). After the first column, the analytes were transferred by a packed loop interface (*Agilent Zorbax* SB-C8 column [4.6 mm × 12.5 mm; 5 µm; 80 Å]) to the second dimension. Here, the separation of the substances was performed by a *Phenomenex Synergi Fusion* RP C18 column (2 mm × 100 mm; 2.5 µm; 100 Å). All three columns were stored in the column oven. The temperature was set to 30 °C. The injection volume was 10 µL.

For the separation, a gradient program was used for both columns. In the first dimension, the mobile phases for the HILIC column were

Table 2
Set-up of the LC-LC-MS/MS method, LC parameters.

Phase	Time 1 [min]	Time 2 [min]	Time 3 [min]	Pump 1 (HILIC)		Pump 2 (RP)		Valve 1 (6-port)	Valve 2 (10-port)
				Flow [µl/min]	B [%]	Flow [µl/min]	B [%]		
1	0.00	0.00	0.00	200	100	200	5	pos. 1	pos. 1
	1.10	1.10	1.10	200	100	200	5		pos.2
2	1.20	1.20	1.20	200	100	2000	0	pos. 2	
	2.00	2.00	2.00	200	100	2000	0	pos. 1	
3	2.05	2.05	2.05	200	100	0	0		
	2.50	2.50	2.50	200	100	0	5		
	4.40	7.40	4.40	200		0	5		
4	4.50	7.50	4.50	200		200	5		pos. 1
	5.00	8.00	5.00	200		200	50		
	6.00	9.00	5.00	200	50	200			
	15.50	18.50	10.50	200	50	200			
	16.50	19.50	12.00	200		200	100		
	18.50	21.50	13.50	200	100	200	100		
	19.50	22.50	14.50	200	100	200	100		
	20.00	23.00	15.00	200	100	200	5		
	22.50	25.50	17.50	200	100	200	5		

Table 3
Set-up of the LC-LC-MS/MS method, MS/MS parameters.

ESI-parameters	Value	Value
Ion source	Turbo spray	Turbo spray
Ionization mode	Positive	Negative
Acquisition mode	sMRM	MRM
Ion spray voltage	5500 V	−4500 V
Interface temperature	400 °C	400 °C
Time	23 min	17 min
Curtain gas (CUR)	42 Psi	42 psi
Collision gas (CAD)	9 psi	9 psi
Ion source gas 1 (GS1)	60 psi	60 psi
Ion source gas 2 (GS 2)	60 psi	60 psi
Entrance potential (EP)	10 V	−10 V
Mass resolution Q1	Unit	Unit
Mass resolution Q3	Unit	Unit
Pause between mass ranges	5 ms	5 ms

water (HILIC A) and acetonitrile/water (90:10; HILIC B). Both contained ammonium formiate (5 mMol/L) and formic acid (0.1%). The flow rate was 200 µL/min. The gradient profile started at 100% B, holding for 2.5 min and afterwards decreasing linearly down to 50% B within 3.5 min. This was kept constant for 9.5 min before being returned to 100% B in 3 min. The starting conditions were held for 4.5 min before the next run.

The mobile phase of the RP-column consisted of water (RP A) and methanol (RP B), also containing 5 mMol/L ammonium formiate and 0.1% formic acid, each. Therefore, the A-eluent of both dimensions was the same. The gradient of the RP column started with 5% B for 1.2 min. Then, the flow rate increased to 2000 µL/min and 0% B (100% A = water). These conditions were constant for 0.8 min and necessary for saving the substances on the trap column. Afterwards, the flow of the RP binary pump stopped (for 4.0 min) in order to measure the direct eluting analytes from the HILIC column. After this step, the flow increased again to 200 µL/min and 5% B and at 5.0 min the amount of the organic solvent (channel B) rose to 50%. To elute all the remaining substances, the gradient changed to 100% B within 11.5 min, linearly. These conditions were constant for 3 min, before being returned to 5% B within 0.5 min. The re-equilibration settings were held for 3 min in the starting composition. Altogether, one analytical run took 23 min. An overview of the gradient, the flow rates and the valves is described in Table 2.

For MS/MS detection, the parameters were as follows: polarity: ESI positive; interface temperature: 400 °C, ion spray voltage: 5500 V, curtain gas: 42 psi, collision gas: 9 psi, ion source gas 1 & 2: 60 psi, entrance potential 10 psi. The mass spectrometer worked in the scheduled multiple reaction monitoring (sMRM) mode. Thus, the two most intensive transitions were only measured when the relevant substance was eluting to the MS/MS. The dwell times were optimized by the *Analyst* Software, automatically. The time window of one MRM transition was 1.2 min and the cycle time was 1.1 s. All the transitions including the declustering potentials (DP), collision energies (CE), collision cell exit potentials (CXP) and the retention times (R_t) of the substances were shown in the supporting information. For the detection of the masked mycotoxins and few other additional analytes, an additional LC-LC-MS/MS-run in ESI-negative mode was essential. The gradient parameters including the flow rates are shown in Table 2; the MS/MS-parameters are described in Table 3.

3. Results and discussion

3.1. Installation and optimization of the 2D-LC-MS/MS system

In a first step, the two-dimensional LC-MS/MS-system by Kittlaus et al. was prepared according to the literature [40]. The method can be divided into four phases. The first phase is defined by the equilibration and the sample injection. After 1 min, the majority of the analytes, especially the unpolar pesticides, eluted from the HILIC-column and were saved on a C8-trap column through an enhanced water flow provided by the pump 2 (see Table 2, phase 2). After a setting point, the valves changed their position again and the next phase began when the still retained polar compounds eluted from the HILIC and went directly to the MS/MS. After the last analyte had left the HILIC-column, the fourth and last phase started. Here, the trapped unpolar substances were rinsed from the C8-column and were separated on a C18-column with a typical RPLC-gradient program. In parallel, the matrix compounds eluted from the HILIC-column into the waste.

This system was optimized for only analyzing pesticides. However, the spectrum of this study contained pesticides, mycotoxins, growth regulators and tropane alkaloids. This was a mixture of polar as well as many unpolar substances. Therefore, the challenge was to find a way to determine all these compounds in one measurement. The analysis of polar substances by RPLC is critical due to a suffering peak shape and an increased matrix effect. Two-dimensional LC is one possible chance for solving the problem. Although the system by Kittlaus et al. was developed to separate matrix components from the analytes, the system also had an enormous capacity to analyze a wide spectrum of substances with a sufficient performance. Particularly, the combination of

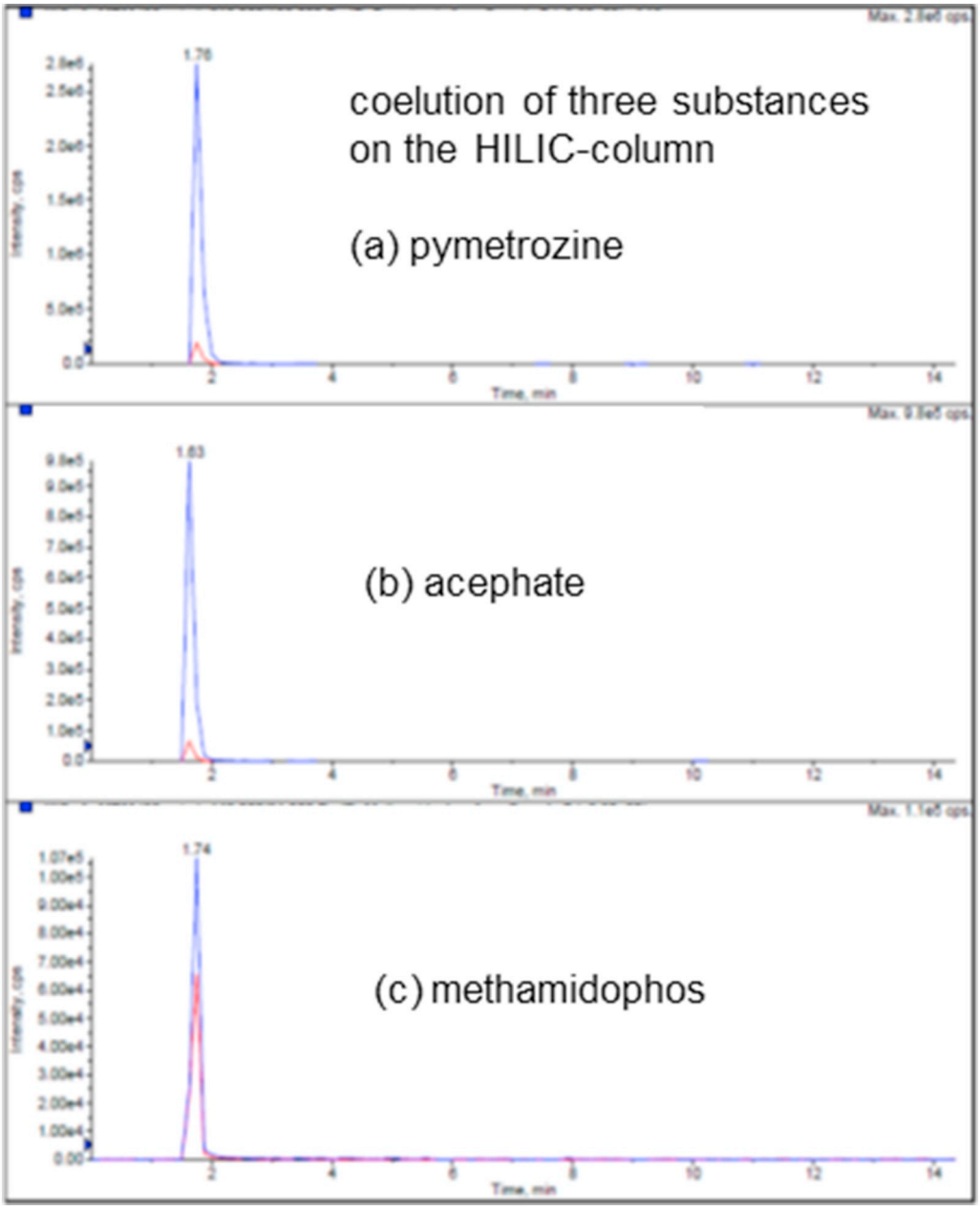

Fig. 2. HILIC-MS/MS-Chromatograms of a standard solution containing (a) pymetrozine, (b) acephate and (c) methamidophos.

the stationary phases with opposed characters was a promising approach.

First, at least two MRM-transitions were determined for each mycotoxin, the growth regulators and the tropane alkaloids. For the pesticides, there was a database available with two or more MRMs for each substance. Then, standard solutions of the analyte mixtures were injected into the system to identify the retention times (R_t). As expected, most of the substances eluted in phase 4 (see Table 2). Only 6% of the analytes eluted in the third phase. A critical point in the set-up of the system was the transition from the second to the third phase. The longer the second phase lasted, the more substances would be transferred to the trap. However, matrix compounds would also enrich on the trap column, simultaneously. In any case, there are substances (pesticides, tropane alkaloids and growth regulators) which are so polar that a separation from the matrix is not possible. Therefore, it was necessary to set a point where the eluent flow from the HILIC is divided. To optimize the transition between the second and the third phase, a standard control mix (c = 0.1 mg/L in ACN/H_2O [90:10]) was prepared, consisting of a few of the critical polar analytes (among others pymetrozin, acephat, methamidophos).

Due to the coelution (Δ R_t = 0.13 min) of these substances on the HILIC, three different settings were tested to find the optimum changing point. The studied transitions were after 1.9, 2.0 and 2.1 min. The transition tested after 1.9 min lead to a split of the pymetrozin peak. It was detected in the third and in the fourth section. Acephat and methamidophos eluted nearly completely in the fourth phase. When the valves moved after 2.1 min, the majority of pymetrozin eluted in the fourth section, while acephat and methamidophos were purged into the waste because of the expanded trapping phase. The best result was to set the transition point after 2.0 min. Pymetrozin eluted completely in the third phase and the majority of acephat and methamidophos were detected in the fourth phase. Only a small, negligible amount of acephat and methamidophos was purged into the waste (Figs. 2 and 3).

Along the way, the dead volume of the system was decreased by installing short connections between the single system components and operating the HPLC-binary pump in the bypass mode.

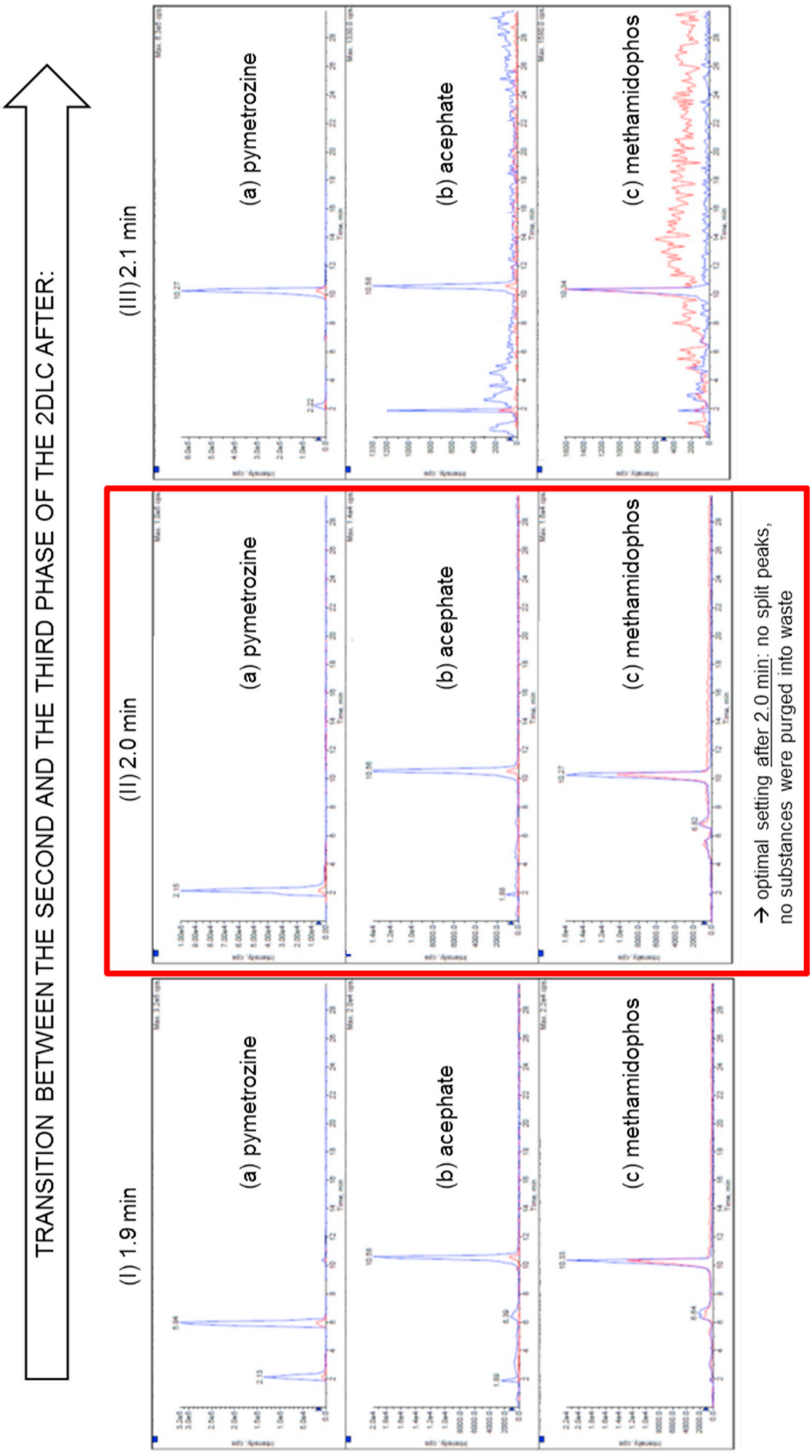

Fig. 3. 2DLC-Chromatograms of a standard solution containing (a) pymetrozine, (b) acephate and (c) methamidophos with different LC set-ups (I) transition between second and third phase after 1.9 min, (II) transition after 2.0 min, (III) transition after 2.1 min.

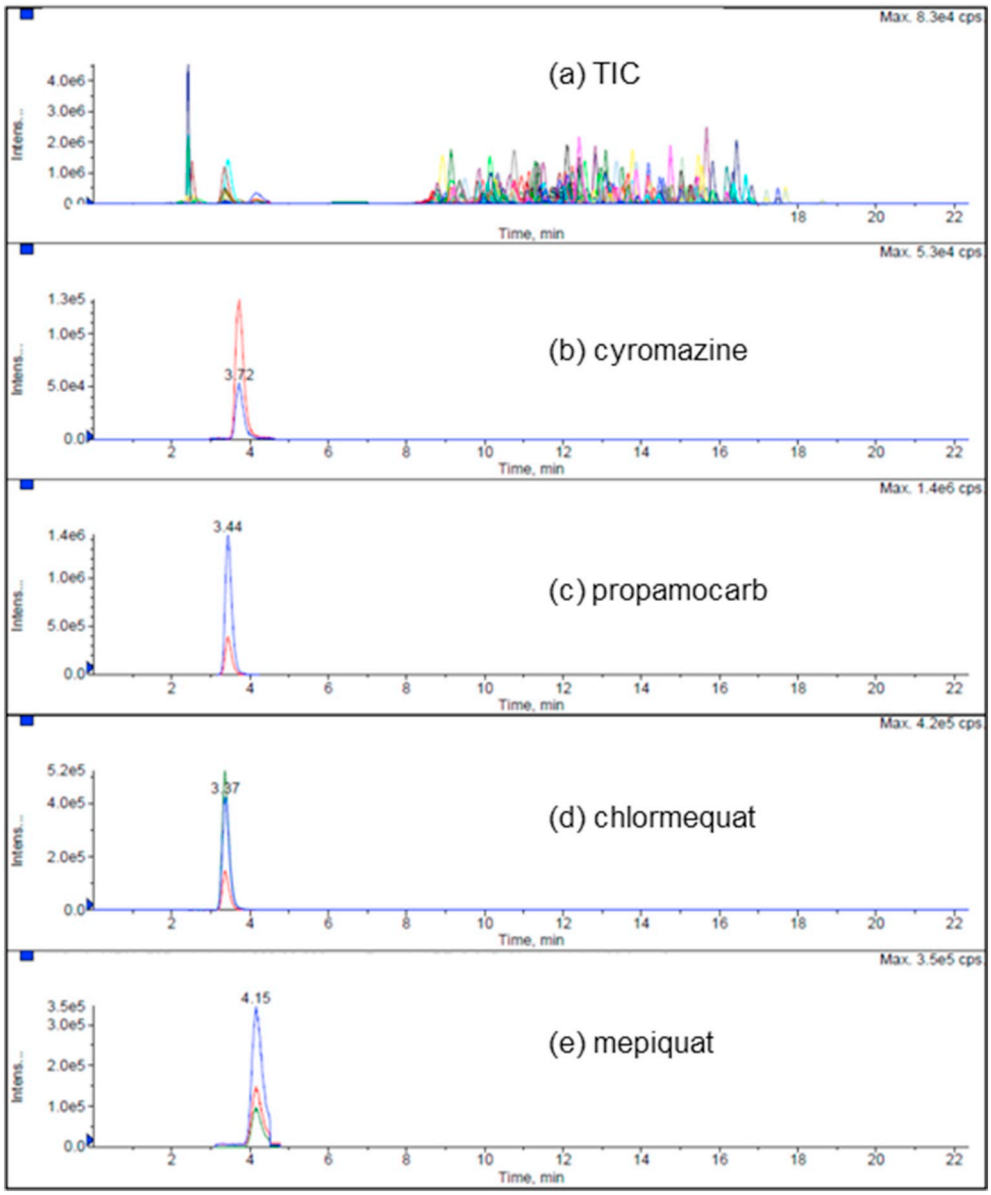

Fig. 4. 2DLC-Chromatogram of a standard solution (c = 0.1 mg/L), single analytes each with two transitions.

After optimizing the valve settings between the second and the third section, the transition between the third and the fourth phase must be optimized, too. In order to measure the very polar growth regulators, the third phase needs to be expanded. Chlormequat and mepiquat eluted after 3.2 and 4.0 min. Therefore, the transition was set after 4.5 min. Tailing effects increased with the increasing polarity of the analytes (Fig. 4). In the case that the fumonisins B1 and B2 also belong to the scope, the third phase must be expanded to 7.5 min (gradient "time 2" in Table 2).

During the fourth phase, 94% of the substances eluted from the RP-column. Early eluting analytes suffered slightly from the viscous fingering effect. It occurs when the solvents utilized for the two dimensions in the LC-system have a different viscosity (methanol $\eta = 0.544$ mPa s [25 °C]; acetonitrile $\eta = 0.316$ mPa s [25 °C]). It leads to turbulences and an unstable flow. In the chromatograms, the effect produces fronting peaks for early eluting substances as acephat or methamidophos (Fig. 3). The effect decreases in the course of the analytical run.

In a next step, different RP-columns (*Phenomenex Synergi Fusion*, 2 × 100 mm, 2.5 µm, 100 Å; *Phenomenex Kinetex*, 2.1 × 100 mm, 2.6 µm, 100 Å; *Agilent Poroshell*, 2.1 × 100 mm, 2.7 µm, 100 Å) were tested to optimize the chromatographic performance on the second dimension. In parallel, the constitution of the mobile phases was optimized with the tested RP-columns. As mobile phases, LC-water and methanol were used with the addition of ammonium formiate (5 mMol) or ammonium acetate (5 mMol). In addition, the varied addition of ammonium formiate (5 mMol, 10 mMol, 20 mMol) was tested, too. Moreover, the effect of the addition of formic acid was studied (0.001%, 0.01%, and 0.1%).

Independent from the selected RP-column, the substances gave an overall higher response adding ammonium formiate to the mobile phases. Among others, this favored the production of ammonium adducts in the MS. The amount of added salt had no significant effect. However, the amount of added formic acid had a great influence. The presence of acid reduces the response of substances like tebufenozide, halofenozide (diacylhydrazine), and the type-A-trichothecenes HT2-/T2-toxine. In contrast, there was an opposite effect for the following substances: ochratoxin A, deoxynivalenol, fumonisine B1/B2 and derivatives of the arylurea herbicides. The more formic acid the mobile phases contained, the higher the response of the analytes in the MS was.

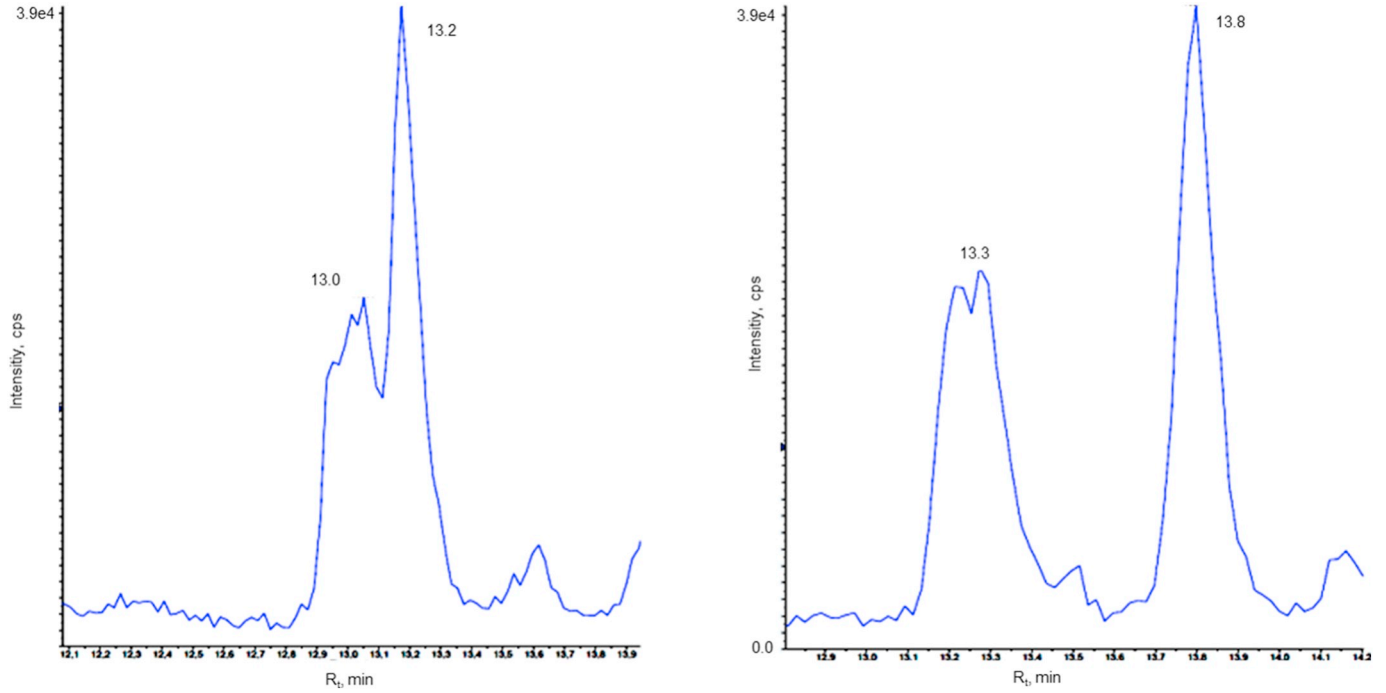

Fig. 5. 2DLC-Chromatogram of the matrix standard (c = 0.1 mg/L); left: Phenomenex Kinetex - coelution of matrix and zearalenone, right: Phenomenex Synergi Fusion - separation of matrix and zearalenone.

Table 4
overview of the solvent mixtures used during the sample preparation tests.

Solvents mixture	1	2	3	4	5	6
	ACN/H_2O 50/50	ACN/H_2O/H^+ 74/25/1	ACN/H_2O 75/25	ACN/H_2O/H^+ 79/20/1	ACN/H_2O 80/20	ACN/H_2O 84/16
vol% acetonitrile	50	74	75	79	80	84
vol% water	50	25	25	20	20	16
vol% formic acid	–	1	–	1	–	–

Even to obtain the best response for the challenging analytes with very low MRLs (OTA, DON), the focus during the method development was on these substances. Therefore, the highest overall response was given by the mobile phase-combination with 5 mMol ammonium formiate and 0.1% formic acid.

To select the best RP-column, standard solutions (analytes in solvent) and matrix standards (analytes in matrix) were measured using all three columns and the optimized mobile phase mentioned (H_2O/MeOH + 5 mMol NH_4^+-formiate + 0.1% HCOOH) for the second dimension. To produce the matrix standards, residue-free cereal samples were prepared according to the sample preparation in Section 2.2. After the treatment, they were spiked so that the concentration of the standards was 0.1 mg/L. For comparison, unspiked matrix solutions were measured, too.

The differences between the three RP-columns were small. Running with the same gradient, the retention times of the substances were slightly smaller in the two columns with partial porous particles (*Kinetex*, *Poroshell*). However, the viscous fingering effect was more visible instead. An expansion of the gradient did not improve the peak performance for this column type. Measuring the matrix standards, the influence of co-eluting matrix components was minimal, too. However, it was possible to separate the zearalenone peak from a co-eluting matrix peak with the *Synergi Fusion*. Due to the fact that corn is often contaminated with zearalenone, this separation is very important. So, for further experiments the *Synergi Fusion* was selected as RP-column in the 2DLC-system (Fig. 5).

The run time was reduced to 23 min. No negative effects were observed. The analytes had enough dwell time in the MS and the starting conditions could be achieved very fast after the run because of the bypass mode in the binary pump.

For the measurement in the ESI-negative mode, another gradient was developed (see Table 2, “time 3”). Thereby, additional substances (nivalenol, 3-actely-deoxynivalenol, DON-3-glucoside, zearalenone-sulfate) with a higher response in the negative mode could be measured with the same configured 2D-LC system.

3.2. Optimization of the sample preparation

Summarizing different single methods in combination with a reduction of manual sample preparation should help to increase the daily sample throughput in the laboratory. In this study, there were two main groups, pesticides and mycotoxins. These two considered groups led to a scope of about 350 substances. In sum, 307 substances were tested in the following sample preparation optimization. In the field of pesticide analysis, the QuEChERS method offers a simple and effective approach to determine the analytes in different matrices. However, the simultaneous determination of the mycotoxins demands another solvent combination for the complete extraction of all analytes. Therefore, six different solvent combinations with acetonitrile (ACN) and water (H_2O), plus an optional addition of formic acid (H^+) were tested. Methanol (MeOH) or acetone was not taken in consideration due to the insolubility of the mycotoxins in MeOH and due to the unselective solvent power of acetone.

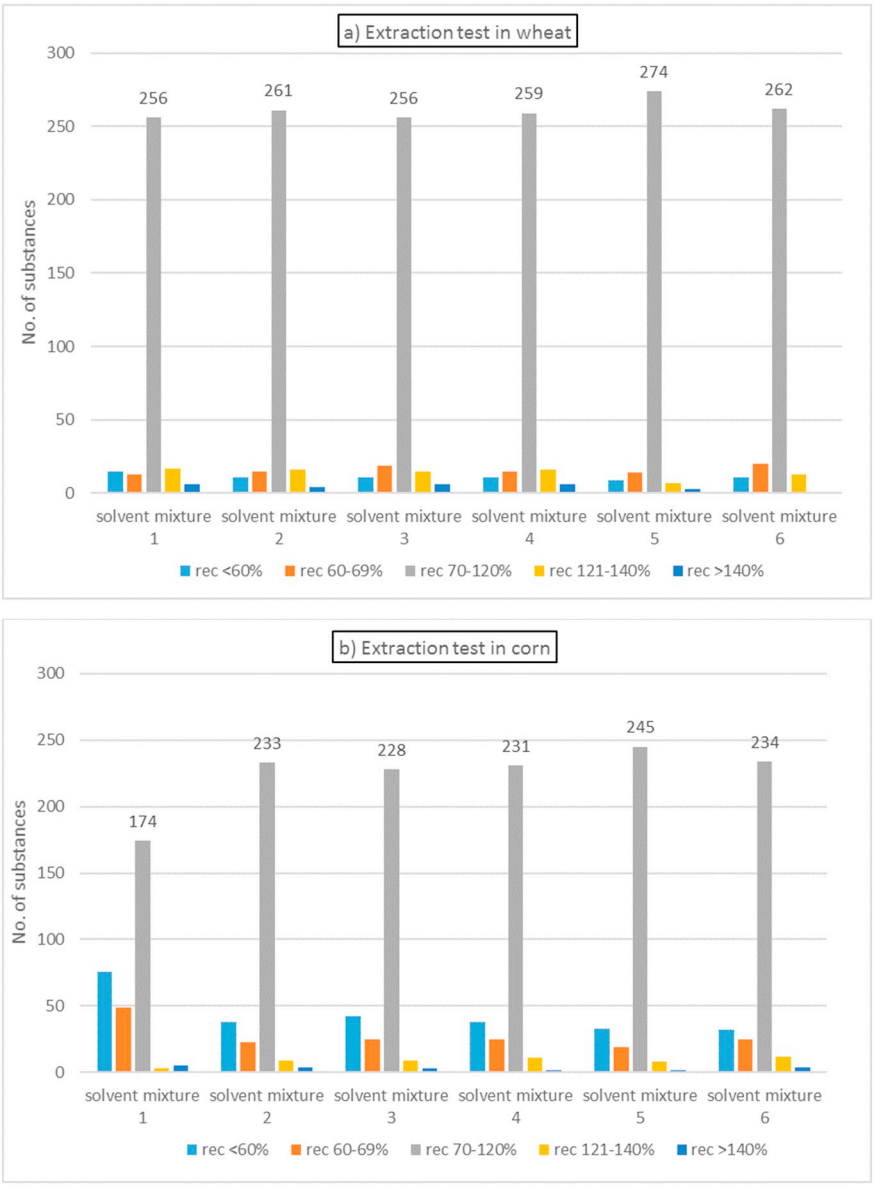

Fig. 6. a, b Distribution of the apparent recovery rates (rec) of the tested substances with six different extraction solvents, in wheat (top, a) and corn (bottom, b).

An overview to the solvent combinations used and their extraction performance is given in Table 4 and Fig. 6. Residue-free samples were spiked with standard solutions so that the resulting concentration in the samples was 0.1 mg/kg. The apparent recovery rates were determined by using the areas of the spiked samples and of the solvent standards. The sample preparation according to Section 2.2 was carried out after the addition of the ISTD-solution and a waiting period of 30 min. The extraction test was made with wheat and corn. The distribution of the apparent recoveries displayed the different resolving power of the solvents tested. Overall, with every solvent, more than 75% of the substances could be resolved out of the matrix. One exception was the solvent acetonitrile/water (50:50) in corn. Here, only 57% of the analytes could be recovered. This solvent combination matched the QuEChERS-solvent. Because of the water ratio, it was not suited as extraction solvent for this approach.

In wheat, the general apparent recovery rate was higher than in corn. The highest number of analytes, which were extracted with a recovery of 70–120%, could be achieved with the un-acidified combination of acetonitrile and water (80:20). The analytes with recoveries smaller than 60% are polar substances, which elute directly to the MS/MS in phase 3 (e.g. cyromazine, pymetrozine, spinosad). Evaluating the recoveries against matrix-matched standards (spiking with 0.1 mg/kg after sample treatment) showed higher recoveries (nearly 100%) in comparison to the evaluation against solvent standards. Obviously, the matrix effect suppression was much higher during phase 3 than during phase 4 and led to the low apparent recovery rates of the polar analytes.

Matrix effect (ME) studies by using the postcolumn-infusion set-up confirmed the enhanced matrix effect during phase 3. Due to the direct measurement of the very polar substances, the signal suppression is very strong (average ME in wheat: −30%; corn: −35%). This led to the low apparent recoveries. The matrix effect profiles and the measurement conditions are shown in the supplementary data.

The corn test samples showed the same tendency, even when some other substances (aflatoxins B2, benzoyl urea class herbicides) showed apparent recoveries smaller than 70%. The addition of acid to the solvent led to better apparent recovery rates for the fumonisins. In contrast, some acid-labile analytes suffered under these conditions (e.g. propamocarb, atropine, carbosulfan). Due to the constant highest apparent recoveries with the acetonitrile/water (80:20) mixture, this solvent was favored to be the optimum extraction solvent. This choice could be confirmed in a test with several reference materials. The reference materials were treated with the two best extraction solvents so

Table 5
Analysis of several reference materials with two selected extraction solvents.

Matrix	Analyte	Reference value [µg/kg]	ACN/H_2O/H^+ (79:20:1) [µg/kg]	Δ (79:20:1)/reference [%]	ACN/H_2O (80:20) [µg/kg]	Δ (80:20)/reference [%]
Corn	AFB1	19	21.5	13	26.5	39
	AFB2	0.9	1.4	56	1.2	33
	AFG1	2.4	4.8	100	3.2	33
	AFG2	4	4.8	20	5.2	29
	OTA	4	4.8	20	5.2	29
	HT2	523	430	−18	430	−18
	ZON	350	295	−16	284	−19
Breakfast cereals	T2	205	150	−27	154	−25
	HT2	167	130	−22	143	−14
Oats	T2	220	163	−26	174	−21
	HT2	89	80	−10	104	17
Animal feed	DON	1375	1512	10	1467	7
Corn	AFB1	6.8	6.5	−4	6.7	−1
	OTA	8.3	14.1	70	12.1	46
Wheat	DON	699	600	−14	567	−19
Wheat	OTA	7	9.5	36	9.3	33
Corn	AFB1	4.8	4.4	−8	4.1	−15
	AFB2	1.9	2.5	32	1.3	−32
	AFG1	1.1	2	90	0.9	−14
	AFG2	0.9	1.2	33	0.9	0
Corn	AFB1	6.8	5	−26	6.4	−6
	OTA	7.5	9	20	5.3	−29

Table 6
Analysis of samples with pesticide residues with the optimized extraction solvent ACN/water (80:20), *LOQDimethoate = 0.005 mg/kg.

	Matrix	Analyte	Reference value [mg/kg]	2DLC-MS/MS [mg/kg]	Δ reference/2DLC [%]
Contaminated samples	Corn	Dichlorvos	0.039	0.025	−36
		Piperonylbutoxide	0.26	0.19	−27
		Trichlorfone	0.019	0.023	21
	Wheat	Dimethoate	0.007	< LOQ*	–
		Flutriafol	0.016	0.014	−13
	Wheat	Imidaclopride	0.026	0.016	−38
		Thiamethoxam	0.027	0.022	−19
	Oats	Piperonylbutoxide	0.039	0.030	−23
FAPAS reference-material 0985	Rice	Carbofurane	0.21	0.20	−5
		Fenamiphos-sulfon	0.19	0.19	0
		Imazalile	0.098	0.095	−3
		Imidaclopride	0.21	0.20	−5
		Pyraclostrobine	0.32	0.36	13
		Thiabendazole	0.34	0.41	21
		Thiamethoxame	0.12	0.12	0
		Thiaclopride	0.28	0.27	−4
		Triadimefone	0.089	0.11	24

far (acetonitrile/water [80:20] and acetonitrile/water/formic acid [79:20:1]).

The test displayed that the differences to the reference value of the acidified extraction solvent are higher than the differences to the un-acidified one. In general, the deviations of the acidified one were higher, too (Table 5). Therefore, the optimum extraction solvent for this approach was the un-acidified acetonitrile/water-mixture (80:20).

Finally, several samples were tested for pesticide residues with the 2DLC-MS/MS method in combination with the optimized solvent mixture for the sample preparation (Table 6). Reference values were determined with the ChemElut-method in combination with the LC-MS/MS analysis. The results obtained with the 2DLC-MS/MS approach were similar to those obtained with the ChemElut-method. The differences were low. The developed method consisting of an extraction step with acetonitrile/water (80:20) and the following analysis by LC-LC-MS/MS was able to determine pesticides and mycotoxins very accurately.

To avoid further analyte loss during the sample preparation, no further clean-up steps were considered. Above all, the integrated online clean-up in the first dimension of the measurement system removed the majority of the polar matrix components and purged it into the waste. However, a single dilution step was added to the sample preparation in order to improve the peak shape of early eluting peaks from the first dimension of the 2DLC. Therefore, an aliquot (0.5 mL) of the extract was diluted with 0.5 mL acetonitrile (spiked with 0.01 mg/L propamocarb-d7 as an internal standard) to have a solvent proportion of nearly 90:10 acetonitrile/water. This corresponded with the starting gradient of the first dimension and resulted in more narrow peaks of substances like cyromazine, pymetrozine and propamocarb (Figs. 7 and 8).

3.3. Validation and verification of the new method

Three matrices (wheat, corn and soybean) were selected as validation material. The starch content of the cereals is comparable (corn, wheat: 60%). Wheat has a high amount of gluten (8%), whereas corn is gluten-free. However, corn (3.8%) has a much higher fat content than wheat (1.8%). Soybean is a protein- (36%) and fat-rich (20%) food with a lower content of carbohydrates (30%). Although it is not a cereal food, the aim was to test which impact a higher fat and protein content would have. All the samples were tested for pesticide residues and mycotoxin contamination before the validation experiments.

Internal standard (20 µL, c = 0.01 g/L) was spiked to 5 g of sample amount. After the addition of the extraction solvent (20 mL ACN/water, [80:20]), the samples were vortexed for 2 min and shaken for 30 min. Afterwards, an aliquot of the centrifuged supernatant (0.5 mL) was diluted with 0.5 mL ACN (spiked with propamocarb-d_7) and injected into the 2DLC-MS/MS system. For each matrix, five concentration levels and five replicates were prepared, at least. Spiking was carried out after the addition of the internal standard mix to the 5 g sample amount. Recovery rates, standard deviations, and linearity were evaluated using matrix-matched calibration standards for each level (Table 7). These matrix-matched calibration standards were blank sample extracts which had been spiked after the dilution step (before the injection).

The validation criteria were set by the SANTÉ Guideline, although the Guideline is only restricted for pesticides. Recovery rates between 70 and 120% were accepted. The relative standard deviations were evaluated through the intra-day ($RSD_R \leq 20\%$) and the inter-day values

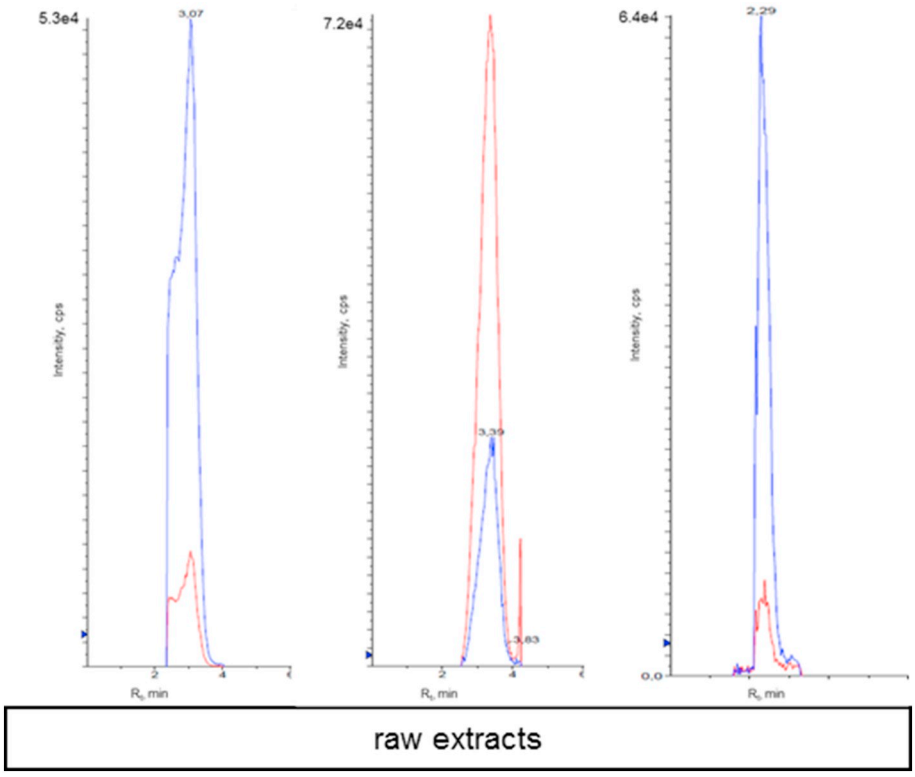

Fig. 7. Peak shapes of the early eluting substances (a) propamocarb, (b) cyromazine and (c) pymetrozine without a dilution step of the sample extract.

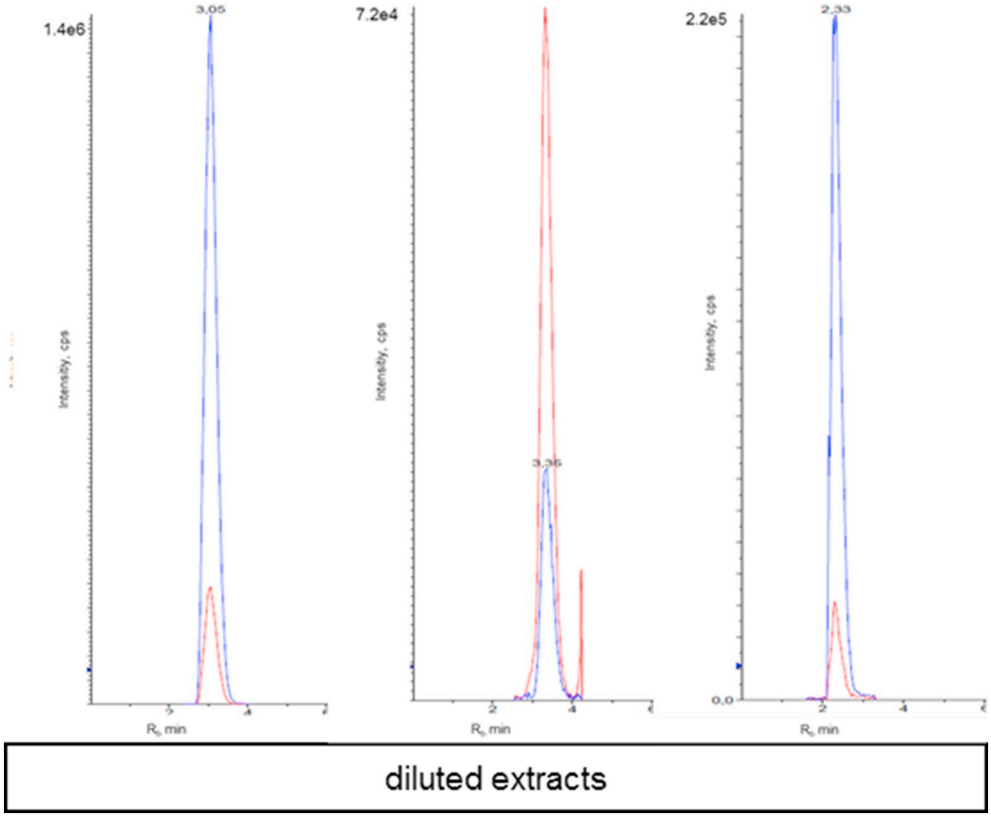

Fig. 8. Peak shapes of the early eluting substances (a) propamocarb, (b) cyromazine and (c) pymetrozine after the dilution of the extract (reduction of the peak width of up to 50%).

Table 7
Overview of the concentration levels for the validation set-up.

Levels	Pesticides, tropane alkaloids [mg/kg]	Aflatoxins, OTA [mg/kg]	DON [mg/kg]	HT2/ T2/ ZON [mg/ kg]	Fumonisins [mg/kg]	CCC/MQ [mg/kg]
1	0.005	0.001	0.2	0.02	0.05	0.01
2	0.01	0.002	0.4	0.05	0.1	0.025
3	0.02	0.005	1.0	0.1	0.2	0.05
4	0.05	0.01	2.0	0.2	0.4	0.1
5	0.1	0.02	4.0	0.4	1.0	0.2
6	0.2			0.5		

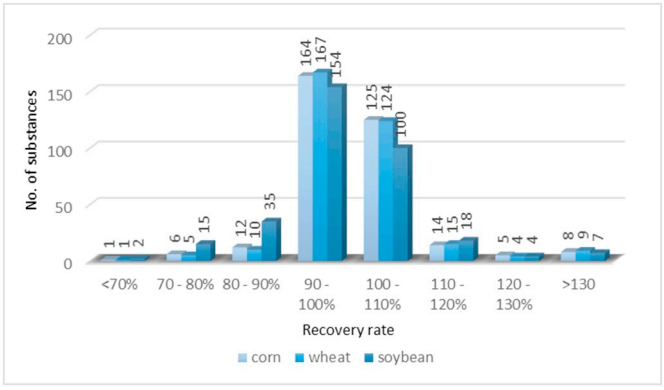

Fig. 9. Recovery rates of the pesticides in three matrices at 0.01 mg/kg.

($RSD_C \leq 25\%$) each at the LOQ of the substances. LOQs of the substance should fulfill the mentioned criteria, should be less than the MRL and the signal-to-noise ratios (S/N) should be equal to 10 at this level, at least.

For the majority of the compounds, pesticides as well as contaminants, the recovery rates were in the tolerable range between 70 and 120% according to the SANTE Guideline document (Fig. 9). For all three matrices, 96% of the pesticide recovery rates were in the tolerable range. Using matrix-spiked calibration levels, the matrix effect could be compensated, particularly for the very polar analytes eluting from the HILIC to the MS/MS, directly. For the routine analysis, the recovery rate should be within the acceptable range (from 60% to 140%). Furthermore, the other analytes of the scope, which were displayed in Table 8, showed recoveries between 70% and 120%, too.

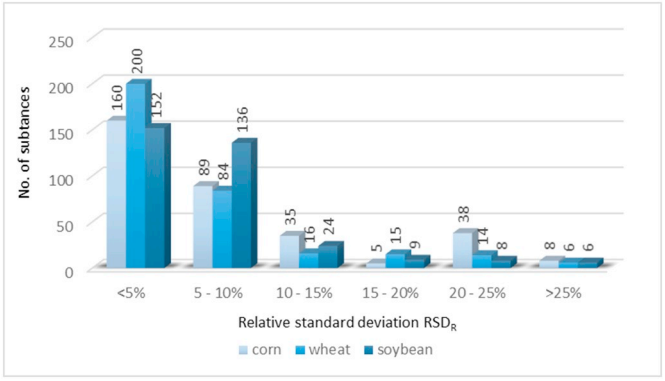

Fig. 10. Relative standard deviation RSD_R (intra-day repeatability) of the pesticides in three matrices at 0.01 mg/kg.

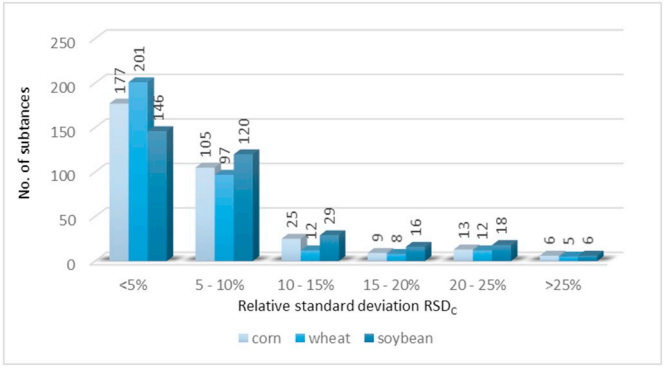

Fig. 11. Relative standard deviation RSDC (inter-day comparability) of the pesticides in three matrices at 0.01 mg/kg.

Two different relative standard deviations were studied at the LOQ of the analytes. The intra-day deviations were evaluated by determining the RSD_R-value. In general, the RSD_R-value of the pesticides was < 20%, according to SANTE. Only for corn, nearly 14% of the pesticides showed higher RSD_R-values than 20%. For wheat and soybean, about 95% of the pesticides showed RSD_R-values < 20%. Considering the inter-day deviations (RSD_C), more than 98% of the pesticides showed RSD_C-values of < 25% in all three matrices, which corresponds to the SANTE-requirements, too (Figs. 10 and 11). For the mycotoxins and further important analytes, which are shown in Table 9, the RSD_R- and RSD_C-values were always below the requirements at the respective LOQ.

Table 8
Recoveries of the other substances of the scope in three matrices at the respective LOQs.

Analyte	LOQs [mg/kg]	Corn		Wheat		Soybean	
		Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
		rec [%]	rec [%]	rec [%]	rec [%]	rec [%]	rec [%]
Aflatoxin B1	0.001	102	104	112	102	89	80
Aflatoxin B2	0.001	95	96	110	98	94	93
Aflatoxin G1	0.001	105	89	105	100	118	101
Aflatoxin G2	0.001	94	103	87	88	84	88
Ochratoxin A	0.001	84	102	86	101	100	82
DON	0.2	107	102	99	104	82	88
Zearalenone	0.05	94	90	76	87	77	89
HT2	0.05	70	80	84	86	72	71
T2	0.02	80	106	78	115	88	95
Fumonisine B1	0.05	97	93	97	109	108	88
Chlormequat	0.01	100	95	93	89	89	90
Mepiquat	0.01	96	104	95	99	103	91
Atropine	0.005	101	89	106	88	97	74
Scopolamine	0.005	85	86	91	91	88	94

Table 9
Relative standard deviations (RSDR and RSDC) of the other substances of the scope in three matrices at the respective LOQs.

Analyte	LOQs [mg/kg]	Corn			Wheat			Soybean		
		Day 1	Day 2	RSD_C [%]	Day 1	Day 2	RSD_C [%]	Day 1	Day 2	RSD_C [%]
		RSD_R [%]	RSD_R [%]		RSD_R [%]	RSD_R [%]		RSD_R [%]	RSD_R [%]	
Aflatoxin B1	0.001	7	5	11	4	6	9	8	6	14
Aflatoxin B2	0.001	4	12	8	5	10	10	11	9	14
Aflatoxin G1	0.001	4	5	6	5	13	9	7	12	11
Aflatoxin G2	0.001	10	19	18	10	7	8	9	14	11
Ochratoxin A	0.001	5	10	7	5	11	8	4	6	5
DON	0.2	6	19	13	9	12	10	18	19	17
Zearalenone	0.05	10	16	14	19	12	16	6	13	10
HT2	0.05	4	2	4	7	7	7	13	5	11
T2	0.02	9	5	8	16	16	15	8	11	10
Fumonisine B1	0.05	5	7	6	3	3	4	3	6	4
Chlormequat	0.01	6	13	10	4	4	4	3	6	4
Mepiquat	0.01	4	3	3	5	6	5	7	5	6
Atropine	0.005	6	4	7	5	11	8	13	5	10
Scopolamine	0.005	9	6	7	6	6	7	8	5	7

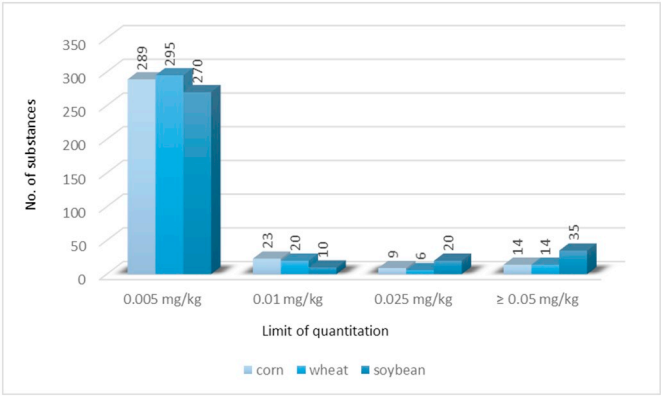

Fig. 12. Limits of quantitation of the pesticides in three matrices.

In Fig. 12, the LOQs of the pesticides are listed. For the majority of the pesticides, an LOQ of 0.005 mg/kg could be observed. For corn, 93% of the pesticides had an LOQ of 0.01 mg/kg or lower (wheat: 94%, soybean: 84%). Therefore, testing pesticide residues in foods from conventional and organic farming is possible with the multimethod developed. The LOQs of the "non-pesticide" analytes are listed in both Tables 8 and 9. The LOQs are lower than the regulated MRLs according to regulation (EC) No. 1881/2006. However, for testing baby food products, it is necessary to apply the reference methods for aflatoxin B1 and the tropane alkaloids. The regulated MRL (aflatoxin B1: 0.1 µg/kg; atropine/scopolamine: 1 µg/kg) are lower than the LOQs achieved with the multimethod developed.

The linearity was studied by the regression coefficients of the calibration curves for the single analytes. However, due to the high amount of substances in the scope, the linearity was evaluated by determining the relative deviation of the standardized signal intensity and the averaged standardized signal intensity. The majority of the substances, pesticides as well as the other analytes, showed a deviation of < 20%. The regression coefficients were higher than 0.99 in, at least, one MRM of the single substances. Both values were in the tolerable range according to SANTE.

3.4. Proficiency tests – inter-laboratory comparison trials

Spiking the samples in the validation approach is a common approach in residue analysis. However, this does not reflect the real-life conditions of samples that had been sprayed with pesticides while they were growing. For the verification of the method trueness, it is mandatory to analyze certified reference materials (see Tables 5 and 6) and to take part in externally organized inter-laboratory comparison trials (proficiency tests, PTs). In sum, seven PTs were studied between 2014 and 2016 with different analyte scopes. The focus was on cereals. The following table give an overview about the respective PTs (Table 10).

Overall, the results determined by the multimethod developed match the assigned values of the PTs. All z-score deviations are within the acceptable range, between −2 and +2. Even at the LOQs of some analytes (OTA, aflatoxins), the determined results are very precise. Again, it was observed that the influence of a higher fat content in the samples was low (e.g. aflatoxins in peanut, FAPAS 04278). Independent from the class (pesticide, growth regulator, mycotoxin, tropane alkaloid), the substances were quantified precisely. There were no false negative or false positive results.

3.5. Analysis of masked mycotoxins

The scope included already the most important mycotoxins, which were to be analyzed in cereals and products thereof. However, plants can metabolize mycotoxins into more water-soluble substances (phase-II-reaction) to reject the toxin. These metabolites are modified mycotoxins, also known as masked mycotoxins. Usually, the mycotoxins are conjugated with sulfate or glucoside. In the gastrointestinal tract of human beings, these conjugates can be cleaved, and the original toxic form of the mycotoxin can be generated. Besides, there exist also derivatives of the most commonly occurring mycotoxins. 3-Acetyldeoxynivalenol (3ADON), 15-acetydeoxynivalenol (15ADON), fusarenone-x (FUS-X), nivalenol (NIV), diacetoxyscirpenol (DAS) are also trichothecenes and structurally relative to DON and HT2/T2, which are already integrated in the scope of the multimethod developed.

In a study investigating more than 200 samples (beer, rye malt and corn), the identification and the occurrence of the fusarium toxins DON and ZON, their derivatives and their conjugated metabolites were considered. For the experiments, the multimethod developed was applied. The sample preparation took place according to Section 2.2 and the measurement was setup according to Section 2.3. To investigate all the substances mentioned, it was necessary to analyze the sample extracts in both an ESI-positive and an ESI-negative MS/MS-run. To identify the transitions of the substances, available standard solutions were used. The LOQs of the additional substances could be determined by a validation study (Table 11).

Analyzing the data of the beer and malt samples, no significant mycotoxin appearance could be observed. Only one sample was contaminated (0.68 mg/kg DON, 0.06 mg/kg 15ADON, 0.21 mg/kg DON3G). In contrast, the analyzed corn samples were largely

Table 10
Overview of the respective proficiency tests (*results are below the LOQ [1 µg/kg]).

Provider	Matrix	Substance	Result [µg/kg]	Assigned value [µg/kg]	z-Score
EU-RL	Rye	Azoxystrobin	90	88	0.1
EUPT-CF 10		Bixafen	79	74	0.3
		Boscalid	477	414	0.6
		Buprofezin	57	48	0.7
		Carbendazim	80	86	−0.3
		Epoxiconazole	196	177	0.4
		Fenpropidin	397	347	0.6
		Fluopyram	267	250	0.3
		Metrafenone	101	88	0.6
		Pencycuron	28	33	−0.6
		Pirimicarb-desmethyl	32	48	−1.3
		Pyraclostrobin	108	98	0.4
		Tebucconazole	97	91	0.3
FAPAS	Brown rice	Carbaryl	122	113	0.4
0997		Malathion	72	61	0.8
		Tebufenozide	131	118	0.5
FAPAS 0990	Oats	Chlormequat	280	282	0.0
		Mepiquat	140	145	−0.1
FAPAS 04269	Corn	Aflatoxin B1	3.20	2.99	0.3
		Deoxynivalenol	1152	1581	−1.8
		Ochratoxin A	1.70	2.15	−0.9
		HT2-toxin	102	111	−0.2
		T2-toxin	100	106	−0.4
		Sum HT2- and T2-toxin	202	205	−0.1
		Zearalenone	199	208	−0.2
FAPAS 04273	Corn	Aflatoxin B1	1.36	1.46	−0.3
		Aflatoxin B2	1.31	1.41	−0.3
		Aflatoxin G1	0.88*	1.00	−0.5
		Aflatoxin G2	0.83*	0.93	−0.5
		Sum	4.38	4.64	−0.3
FAPAS 04278	Peanut	Aflatoxin b1	3.33	3.42	−0.1
		Aflatoxin b2	1.66	1.88	−0.5
		Aflatoxin g1	1.54	1.77	−0.6
		Aflatoxin g2	0.84*	0.96	−0.6
		Sum	7.37	8.29	−0.5
RIKILT (Wageningen)	Sample B	Atropine	85.5	76.0	0.6
		Scopolamine	10.8	10.0	1.5
	Sample C	Atropine	11.3	8.0	0.5
		Scopolamine	59.0	50.0	0.8

contaminated with the fusarium toxins. Both DON, its glycoside-conjugate and its derivatives could be detected. In 61% of the corn samples, DON was detected. The highest amount was 5.2 mg/kg. The MRL for DON in raw corn (1.75 mg/kg) was exceeded in 42% of the positive findings. In 51% of the samples, DON and at least one further form was detected. The second most frequently detected peak was 15ADON (46%). It followed DON3G (39%), NIV (18%) and 3ADON (7%). Fusarenone-X and diacetoxyscirpenol weren't detected. In general, the absolute amounts of the conjugated and derivate forms of DON were less than the DON-amount in the respective sample. There was no positive finding of a conjugated or derivated form of the fusarium toxin without a positive finding of the fusarium toxin.

In case of zearalenone, there was no standard material for the derivatives or the conjugated forms available. Thus, a corn sample with a high amount of zearalenone was prepared to identify related substances occurring simultaneously (Fig. 13). First, the sample extract was measured with an ESI-negative Q1-scan. The studied mass range was set to the area of the theoretical $[M\text{-}H]^-$-ions of the possible conjugates zearalenone-sulfate and- glycoside. Here, only one relevant peak (R_t = 12.1 min) was observed, besides the ZON-peak. The mass corresponded to the $[M\text{-}H]^-$-ion of zearalenone-sulfate. In a following production-scan and with an enhancement of the collision energy, it resulted in the spectrum of zearalenone with the typical ions (*m/z*: 317, 273, 175, 149, 131). Due to this behavior, two MRMs were selected for zearalenone-sulfate. Thus, a qualitative analysis of zearalenone-sulfate could be carried out. Unfortunately, it was not clear whether it was ZON-14- or ZON-16-sulfate. Due to the structure (lower sterically shielding of the hydroxyl group at C14), it was likely to be the zearalenon-14-sulfate. For the potentially occurring glycoside conjugate of ZON, no signals were observed.

Table 11
Validation data of further contaminants.

	LOQ [mg/kg]	Day 1	Day 2	Day 1	Day 2	RSD_C [%]	Day 1	Day 2
		rec [%]	rec [%]	RSD_R [%]	RSD_R [%]		Linearity R^2	Linearity R^2
Diacetoyscirpenol	0.01	111	111	4	2	4	0.999	0.999
Fusarenone-x	0.2	104	88	8	7	7	0.994	0.995
15ADON	0.2	104	97	7	5	6	0.998	0.992
Nivalenol	0.2	51	42	16	16	15	0.998	0.998
3ADON	0.2	92	105	12	11	13	0.999	0.996
DON-3-glucoside (DON3G)	0.02	62	63	15	13	12	0.992	0.991

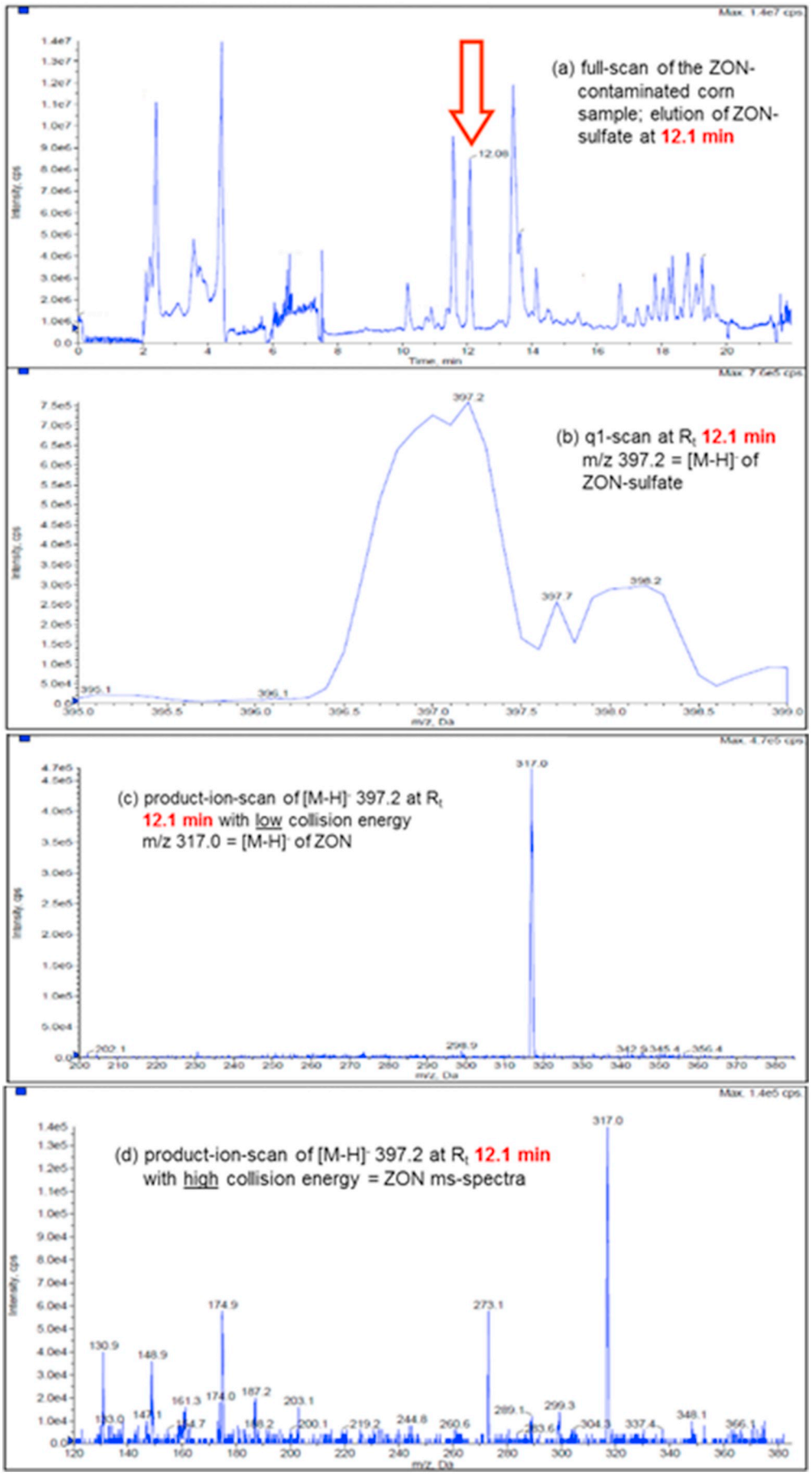

Fig. 13. Identification of zearalenone-sulfate (molar mass = 398.4 g/mol): measurement of a ZON-contaminated corn sample by a q1-scan and a following product-ion-scan with increasing collision energies.

ZON was detected in 54 corn samples from 0.054 mg/kg until 4.1 mg/kg. The MRL for untreated corn of 0.35 mg/kg was exceeded in 25 samples. In 36 of the 54 ZON-positive samples (66%), zearalenone-sulfate was detected, too. Again, there was no positive finding for the sulfate conjugate without a ZON-finding. The ZON-derivatives (zearalenol, zearalanone, zearalanol) were not detected.

4. Conclusion

A total of 20 contaminants and approximately 350 pesticide residues, including several metabolites, can be determined by the multi-method developed. Attention should be paid to the simultaneous analysis of the cereal-relevant mycotoxins aflatoxin B1, B2, G1 and G2, ochratoxin A, deoxynivalenol and zearalenone. Moreover, the growth regulators chlormequat/mepiquat and the tropane alkaloids atropine/scopolamine could be integrated into the final method. In addition to the already mentioned mycotoxins, the six most important ergot alkaloids (e.g. ergotamine/ergotaminine) and two modified fusarium mycotoxins (deoxynivalenol-3-glucoside and zearalenone-14-sulfate, also known as “masked” mycotoxins) were detected during the routine analysis of rye and corn samples.

The final method is sensitive enough to meet all the regulated maximum levels for pesticides in cereals according to EU Regulation 396/2005 [3], and those for contaminants according to EU Regulation 1881/2006 [2]. Above all, the method is so robust and accurate that nearly 90% of the pesticides and all the tested mycotoxins and tropane alkaloids fulfilled the validation criteria of the SANTE guideline document. In detail, the recovery rates were between 70% and 120%, the RSDs were below 25%, at least, and the regression coefficients were above 0.99, which corresponds to a good linearity of the calibration curves. Furthermore, the validation in soybean matrix and a proficiency test with peanut flour as matrix showed that the method is even robust and precise enough despite a higher fat and protein content in the sample matrix. For verification, the analysis of certified reference materials and the participation in proficiency tests was carried out. In sum, eight proficiency tests were passed successfully: three for the pesticide analysis, three for the mycotoxin analysis, and two for the analysis of the tropane alkaloids. The values determined by the two-dimensional LC-LC-MS/MS-method were in accordance with the assigned values. All z-sores were in the range between −2 and +2.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2019.04.013.

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