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Development and validation of an efficient automated method for the analysis of 300 pesticides in foods using two-dimensional liquid chromatography–tandem mass spectrometry

Stefan Kittlaus[a,*], Julia Schimanke[b], Günther Kempe[c], Karl Speer[b]

[a] Joint Analytical Systems GmbH, Carl-Zeiss-Str. 49, D-47445 Moers, Germany
[b] Technical University Dresden, Food Chemistry, Bergstraße 66, D-01069 Dresden, Germany
[c] State Laboratory for Health and Veterinary Affairs (LUA) Saxony, Reichenbachstr. 71-73, D-01217 Dresden, Germany

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ABSTRACT

In this study, a fully automated system was developed for the determination of more than 300 different pesticides from various food commodities. The samples were extracted with acetonitrile prior to the injection into the two-dimensional LC-system. No manual clean-up was needed. The separation of analytes and matrix compounds was carried out by a YMC-Pack Diol (2.1 mm × 100 mm; 5 μm; 120 Å) HILIC column in the first dimension. All analytes eluted within one small fraction at the beginning of the run. With a packed loop interface this fraction was transferred to the analytical reversed phase separation performed on an Agilent Poroshell 120 EC-C18 (2.1 mm × 100 mm; 2.7 μm; 120 Å). Some very polar compounds with a stronger retention on the HILIC column were measured directly. The method was validated for over 300 pesticides in cucumber, lemon, wheat flour, rocket, and black tea. For the large majority of the analytes, the recovery was between 70% and 120% and the relative standard deviation was clearly under 20%. The limits of detection for nearly all the compounds were at least at 0.01 mg/kg. For over 50% of the analytes, good sensitivity was observed even at 0.001 mg/kg. In spite of the injection of a pure sample extract, the method showed robust results even with dirty matrices like hops and tea.

1. Introduction

Pesticides are used intensively in modern agriculture. Due to being hazardous to human health and the environment, it is important to control pesticide residues after their application to food. In 2010, about 80% of the German population estimated the risk of pesticide residues in food as “high” or even “very high”. A majority of 69% of those interviewed even supported more frequent and more intensive control [1].

In the past, various methods were developed to detect the enormous variety of analytes in very different commodities. The simultaneous determination of more than 300 compounds during one chromatographic run is common practice, nowadays [2]. One of the most frequently used methods is the so-called QuEChERS method, an abbreviation which stands for quick, easy, cheap, effective, rugged, and safe [3,4]. In different versions, for fruit and vegetable this is probably the most frequently used method, worldwide [5]. When it was developed, Anastassiades and Lehotay followed the trend of miniaturisation and speeding up for the analytical methods. Due to the needs of modern laboratories, the reduction of solvent use and the decrease in the analysis time are the main intentions of many sample preparation approaches in the pesticide residue analytic procedure, up to now [6,7].

In principal, all important multi-residue methods contain the same sample preparation steps. After homogenisation and the extraction of the sample, a liquid–liquid partitioning separates the polar compounds from the usually more nonpolar pesticides. In the literature, many different ways have been described for this partitioning. In the QuEChERS method this takes place by a salt mix being added to the extract, which is subsequently followed by phase separation [8]. A liquid–liquid partitioning on a solid supporting material (ChemElut) was used by Klein and Alder [9]. Also, classic approaches with highly nonpolar organic solvents (acetone, ethylacetate, cyclohexane) were used [10,11]. Mostly, the partitioning is followed by further clean-up procedures. The aim is to remove the matrix components still remaining in the organic extract. Different procedures were developed e.g. solid phase extraction, dispersive solid phase extraction, and size exclusion chromatography. These are discussed by Zhang et al. [4] and Lambropoulou and Albanis [12]. The impact of the different steps on the reduction of the matrix components varies. In a previous study, we tested the single steps of the QuEChERS method for their reduction of the matrix effects

* Corresponding author. Tel.: +49 176 24874968.
E-mail address: stefan-kittlaus@gmx.de (S. Kittlaus).

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with the permanent postcolumn infusion approach. The result was only a slight separation of the troublesome compounds during the clean-up [13,14].

In the last decade, mass spectrometers, especially triple quadrupole and time of flight, have become the most important detectors for residue analytical applications [15,16]. Typically, the separation of the purified extracts is carried out by liquid and gas chromatography. But, classical one-dimensional approaches do not always provide the resolving power and selectivity needed for the analysis of complex samples. One solution is the use of multi-dimensional chromatography. Different coupling techniques with various interface solutions have been developed. An overview of the application of multi-dimensional chromatography in food analysis is given by Herrero et al. [17]. The published pesticide residue analytical methods typically use two-dimensional gas chromatography for the detection of pesticides in different kinds of food [18–35]. Besides offering better resolving power and peak capacity, multi-dimensional chromatography is also capable of decreasing matrix effects as a result of the advanced separation of analytes and matrix components. Fogy et al. used a two-dimensional LC approach 1980 to analyze 7 carbamates in fruit and vegetables [36]. In later studies, a liquid chromatography in the first dimension was coupled with a gas chromatograph [37–40]. The heart cut technique (collecting all the analytes in one or few fractions) was used to transfer the analytes from the first to the second dimension. Therefore, the number of the analytes was quite small. Cortes et al. and Riedveld and Quirijns analysed individual compounds with the multi-dimensional approach [37,38]. Hyotylainen et al. and Sanchez et al. developed methods for up to ten pesticides [39,40].

In 2001, Choi et al. analysed matrix effects in a pesticide residue analysis with the permanent postcolumn infusion approach [41,42]. Based on their results, they also supported the use of multi-dimensional liquid chromatography for the reduction of matrix effects in the environmental LC–MS/MS analysis. This was carried out by Pascoe et al. [43]. They used a heart cut LC–LC method to analyse three pesticides in complex matrices. Every compound was separated in a single fraction before the analysis in the second dimension. To the best of our knowledge, a two-dimensional LC-multi-method for a higher number of pesticides has not yet been developed.

The aim of this study was to use a HILIC column to replace the classical liquid–liquid extraction step during sample preparation. HILIC is a separation technique using a polar stationary phase as known from normal phase (NP) chromatography. However, here, the mobile phase is much more polar than in the NP-mode. The solvents used in HILIC (water, acetonitrile) are similar to the solvents used in reversed phase chromatography. Yet the elution strength of the solvents and the elution order of the analytes are inverse to the RP mode [44,45]. Four main categories of HILIC columns can be distinguished: unmodified silica gel, neutral phases, charged phases, and zwitterionic phases. All of the materials have highly polar functional groups. It is generally accepted that the water of the mobile phase is concentrated on the polar surface of the stationary phase. The chromatographic separation is based on a partitioning of the analytes between this water phase and the organic mobile phase [44]. Various stationary phases are available from different manufacturers. Partially, they differ significantly in their retention behaviour and selectivity. Good overviews are given by Jandera [46] and Guo and Gaiki [47]. One critical point in the development of the method was to find the best-suited column for the separation of analytes and matrix compounds.

The combination of HILIC and RP chromatography provides a high orthogonality and, hence, a good matrix separation is to be expected. Thus, the two dimensions of the HILIC-RP method need to be coupled by an appropriate interface to create a two-dimensional LC method for the determination of more than 300 pesticides in fruit and vegetables. The fractions of the first dimension (HILIC) usually have a high amount of organic solvent. Due to the high elution strength it is not possible to transfer these fractions directly to the second dimension. The aim was to concentrate all analytes within one fraction and to separate them from the matrix components with the HILIC approach. Afterwards, the analytes need to be trapped on a packed loop interface to change the solvent before the analytical separation on a reversed phase column.

2. Materials and methods

2.1. Chemicals and reagents

Over 300 pesticides of different classes were analysed with the two-dimensional method. All the substances were certified standards at residue analytical grade purchased from Dr. Ehrenstorfer (Germany) or Riedel de Haen (Germany). Individual stock solutions in a concentration of 1 mg/mL were prepared in acetonitrile or methanol, depending on the solubility and stability of the pesticides. Eight standard mixtures were prepared in acetonitrile with a concentration of 10 μg/mL for the pesticides, each. These mixtures were stored at −18 °C. The spiking mixture was always prepared fresh by mixing the eight standard solutions and diluting them with methanol. The final concentration of the mix was 1 μg/mL. For wheat flour, fenbendazol (5 μg/mL in acetonitrile) was used as internal standard. Acetonitrile, methanol, and water were purchased from Merck (Germany) in LC/MS grade. Ammonium formate came from Biosolve (Netherlands). Formic acid was purchased from Sigma–Aldrich (Germany) in analytical grade.

2.2. Matrices and sample preparation

The analysed food commodities were cucumber (high water content), lemon (high acid content), wheat flour (dry crops), and black tea respectively rocket (difficult matrices) [48]. All the fruit and vegetables were taken from our collection of residue-free routine samples. All the samples were homogenized or milled. For cucumber, lemon and rocket 10 g were weighed in plastic tubes. For wheat flour 5 g and for black tea 2 g were used. The samples were spiked with appropriate volumes of the spiking mixtures and afterwards shaken for equal distribution. The validated levels were 0.001 mg/kg, 0.005 mg/kg, 0.01 mg/kg, 0.02 mg/kg and 0.05 mg/kg (only for black tea). Every level was spiked five times. For wheat flour, also 50 μL of the internal standard solution was added. To adjust the amount of water for wheat flour and tea, 10 mL water were added. The soaking time was 20 min. Depending on the volume of the spiking solution, acetonitrile was added to a total volume of 10 mL. All the samples were shaken for 20 min with an overhead shaker. The extracts were centrifuged for 5 min at 3000 rpm (1740 rcf). For the measurement 500 μL of the supernatant were filled into sample vials. No further clean-up steps were carried out. To calculate the recovery, each matrix was also extracted without the addition of the spiking mix. The blank extracts were spiked after the sample preparation. The concentration corresponded to a quantitative extraction of all the analytes.

2.3. 2D-LC–MS/MS analysis

The experiments were performed on a 1200 HPLC system (Agilent, Germany) consisting of a degasser (G1379B), two binary pumps (G1312B), an autosampler (G1367D), and two column oven (G1316B). The system was coupled to an Agilent (USA) triple quadrupole mass spectrometer 6460A with a jet stream ESI ion source (G1958-65138). For the two-dimensional approach, the HILIC and the RP column were connected with a packed loop interface. The pneumatically 6-port valve and 10-port valve were

Table 1
Phases of the two-dimensional method with elution conditions and valve positions.

	Phase		RP pump		HILIC pump		Valves	
	Time (min)		Flow (mL/min)	Composition	Flow (mL/min)	Composition	1	2
I	0–1.2		0.2	5% B	0.2	100% B	Pos. 1	Pos. 1
II	1.2–2.0	(trapping of compounds)	2.0	100% A	0.2	100% B	Pos. 2	Pos. 2
III	2.0–4.0	(direct measurement of polar compounds)	0.0	–	0.2	Gradient	Pos. 1	Pos. 2
IV	4.0–30.0	(separation on RP, flushing of HILIC)	0.2	Gradient	0.2	Gradient	Pos. 1	Pos. 1

from Valco Vici (USA). They were controlled with the Agilent Mass Hunter acquisition software.

For the chromatographic separation of the matrix components and the analytes in the first dimension, a YMC-Pack Diol (2.1 mm × 100 mm; 5 μm; 120 Å) was used. In the second dimension, the separation of the analytes was carried out with an Agilent Poroshell 120 EC-C18 (2.1 mm × 100 mm; 2.7 μm; 120 Å). The trapping column in the packed loop interface was an Agilent ZORBAX SB-C8 (4.6 mm × 12.5 mm; 5 μm; 80 Å). An overview of all the parts of the two-dimensional method and the valve switching times are given in Table 1 and Fig. 1. All the column temperatures were set at 30 °C. The injected volume was 5 μL.

The mobile phase of the first dimension consists of water (A) and acetonitrile/water (90:10) (B) containing 5 mM/L ammonium formate and 0.1% acetic acid, each. The gradient started with 100% B. This was held for the first 2.5 min. Then, the amount of B was decreased to 50% linear within 5 min. This was held for 10 min. Afterwards solvent B was increased again to 100% within 2.5 min and held there for 10 min. The flow was 0.2 mL/min. The mobile phase of the RP separation consisted of water (A) and methanol (B) containing 5 mM/L ammonium formate and 0.1% acetic acid, each. From injection to 1.2 min, the RP column was conditioned with 5% B. From 1.2 min to 2.0 min (2.3 min in the negative method), the RP pump was used to add water in order to retain the analytes on the trap. For this, the flow was increased to 2.0 mL/min, and the water content (channel A) was increased to 100%. After the trapping, the direct measurement of the polar compounds began. Therefore, the flow of the RP pump was set to 0 mL/min. The RP separation started at 4 min with 5% B and increased the amount of B to 50% linear within 0.5 min. Afterwards the amount of B was further increased to 100% within 15.5 min. This was held for 5 min. Then solvent B was decreased to 5% within 1 min and held there for 4 min. This way, the overall length of the methods was 30 min. The flow rate of the RP separation was 0.2 mL/min. All the gradients, flow rates, and switching times are shown in Fig. 1b.

The jet stream ion source parameters were: drying gas temperature: 250 °C; drying gas flow: 7 mL/min, nebulizing pressure: 40 psi; sheath gas temperature: 375 °C; sheath gas flow: 12 L/min; capillary voltage: 2500 V (positive and negative); nozzle voltage: 300 V (positive), 0 V (negative). The mass spectrometer, working in the multiple reaction monitoring mode (MRM), detected the two most intensive transitions of each pesticide. Every analyte was measured in a small time range around its retention time (dynamic MRM, for retention time windows see Table A in the supporting information). All the transitions are summarized in Tables A and B of the supporting information. Positive and negative transitions were measured in separate runs.

The method for the permanent postcolumn infusion and the formulas for the calculation of the matrix effect profiles are described elsewhere [13].

3. Results and discussion

3.1. Optimisation of the HILIC separation

The aim of the study was to replace the classical clean-up by a chromatographic approach. A HILIC separation was used to remove the interfering matrix components in a two-dimensional LC–MS/MS system. A number of vendors offer HILIC columns with manifold chemical modifications. In the first step of the method development we tested different kinds of polar stationary phases for their suitability. Due to the rather nonpolar character of the

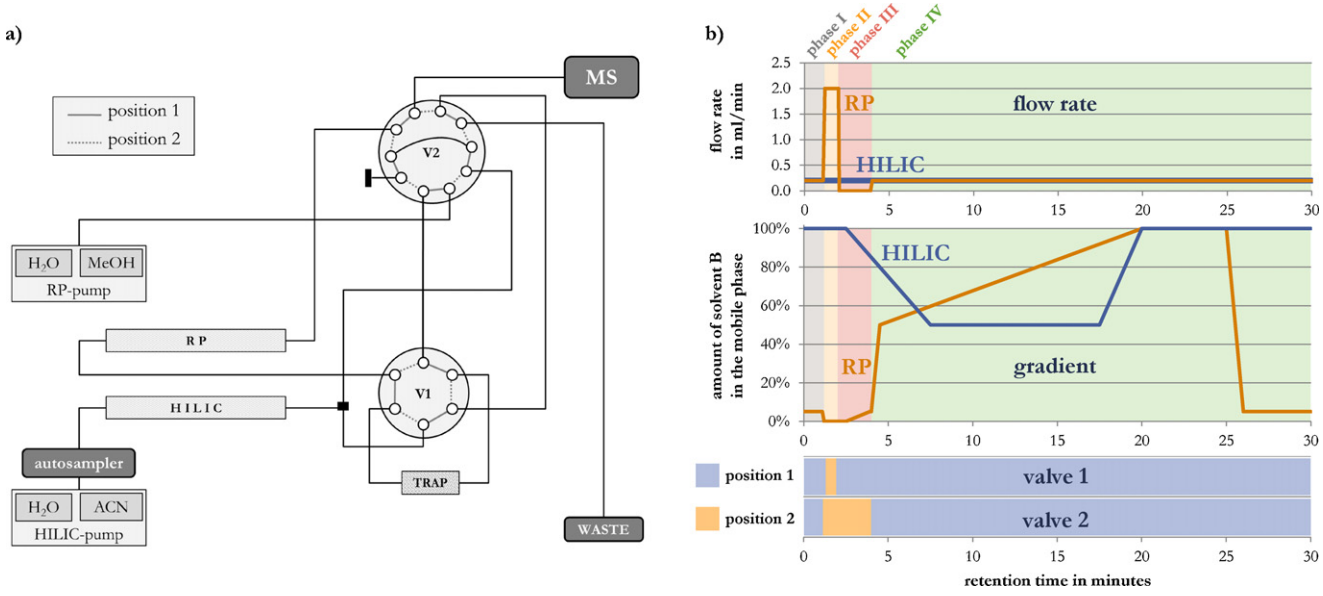

Fig. 1. System configuration (a) and gradients, flow rates, and switching times (b) of the two-dimensional method.

Table 2
Tested HILIC columns for the matrix separation in the first dimension.

Name, character	Dimension, catalogue number
Agilent ZORBAX HILIC Plus	2.1 mm × 100 mm; 3.5 μm; 95 Å
Unmodified silica gel	No.: 959793-901
Phenomenex LUNA 3u HILIC Diol	2.0 mm × 100 mm; 3 μm; 200 Å
Diol (cross-linked hydroxyl-groups bounded on silica gel)	No.: 00D-4449-B0
YMC-Pack Diol	2.1 mm × 100 mm; 5 μm; 120 Å
Diol (1,2-dihydroxypropylgroups bounded on silica gel)	No.: DL12S051002QT
YMC-Pack Polyamine II	2.1 mm × 100 mm; 5 μm; 120 Å
Amine (polymer with secondary and tertiary amino-groups bounded on silica gel)	No.: PB12S051002QT
TOSOH Bioscience TSKgel Amide-80	2.0 mm × 100 mm; 3 μm; 80 Å
Amid (carbamoyl-groups bounded on silica gel)	No.: custom-made product
Nacalai tesque COSMOSIL HILIC	2.0 mm × 100 mm; 5 μm; 120 Å
Triazol (triazol-groups bounded on silica gel)	No.: 08569-11
SeQuant ZIC-HILIC	2.1 mm × 100 mm; 3.5 μm; 100 Å
Sulfoalkylbetain (ammonium sulfonic acid bounded on silica gel)	No.: 150441.0001
Macherey-Nagel NUCLEODUR HILIC	3.0 mm × 125 mm; 3.5 μm; 95 Å
Sulfoalkylbetain (ammonium sulfonic acid bounded on silica gel)	No.: 760531-30

pesticides, the intention of the optimisation was to collect all the analytes in a small fraction at the start of the HILIC separation. At the same time, matrix components were to remain on the column to achieve a maximum cleaning effect.

The tested columns are summarized in Table 2. If available, all columns were ordered in the same dimension. A representative pesticide mix of 100 compounds in acetonitrile (MeCN) was injected into all columns. The mobile phase was water and MeCN. The gradient started with 95% MeCN. This was held for 2.5 min. After that isocratic time, the amount of MeCN was decreased to 50% within 20 min and held there for 10 min. At the end of the test method, the conditions were reconstituted to 95% MeCN within 0.5 min and held there for 4.5 min. The majority of the compounds showed only low retention on the tested columns. About 80% of the pesticides eluted in a small time window between one and three min. As a general rule, compounds with high polarity, small molecular weight, and also tin organic compounds showed the most retention on all HILIC columns. Phenoxyalkan carboxylic acids were problematic. They adsorbed irreversibly on all the stationary phases with amino groups (COSMOSIL HILIC, polyamine II). Generally, these columns showed a different behaviour than the other ones.

To elute all the pesticides within a small time window, formic acid was added to the mobile phase. The tested concentrations were 0.0025% (pH 4), 0.01% (pH 3) and 0.1% (pH 2). The results were different. On Luna HILIC Diol, Nucleodur HILIC, TSKgel Amide, Zic HILIC, COSMOSIL HILIC, and Polyamine II the test compounds showed earlier retention times than without any additive. For YMC-Pack Diol and ZORBAX HILIC, a slightly increased retention was observed with high amounts of acid. In a further experiment, 5 mM/L respectively 10 mM/L ammonium formate was also added to the mobile phase. To dissolve the salt in MeCN, the solvent was premixed with 5% water, and the gradient was adjusted. On most columns, the retention time window was significantly decreased by the addition of the formate. Especially on ZORBAX HILIC, YMC-Pack Diol, TSKgel Amide, and Luna HILIC Diol, the problematic compounds showed less retention. For COSMOSIL HILIC and Polyamine II, no effect was observed. Spiroxamine and propamocarb showed more retention and strong peak tailing on Zic HILIC and Nucleodur HILIC, with high amounts of salt. The differences between 5 mM/L and 10 mM/L on all columns were only small. The results with 5 mM/L were slightly better. In general, the decreased retention times led to narrower peaks and a better peak shape. The smallest retention time window was obtained with 0.1% formic acid and 5 mM/L ammonium formate. The best results were observed for the two tested diol phases (Luna HILIC Diol, YMC-Pack Diol). The detailed results for all columns can be found online in the supporting information. The separation of more than 300 analytes on the YMC-Pack Diol in the final method is presented later in the top of Fig. 3.

In the previous experiments only the analytes were contemplated. However, the aim of the development of the method was to optimize a HILIC based chromatographic method for the separation of analytes from interfering matrix components. Therefore, the behaviour of the matrix components on the chosen columns should also be taken into account. For this purpose, acetonitrile extracts of cucumber, lemon, wheat flour, walnut, and rocket were prepared analogously to the first step of the QuEChERS method [49]. A clean-up did not take place. Onto the two diol columns 5 μL of the crude extracts were injected and analysed with the gradient described above. To obtain a visual impression of the matrix effects caused by coeluting matrix components, the detection was carried out with the postcolumn infusion approach. This technique allowed the visualisation of all interfering compounds in matrix effect profiles [13].

On both columns, a number of matrix components were retained considerably longer than the analytes. Especially for wheat flour and rocket, a good separation was observed. The late-eluting matrix compounds are substances with high polarity. In a classic method such as QuEChERS, they are removed by partitioning between the organic and the water phase. Due to the lack of charged functional groups on the surface of the diol columns, these materials have a separation mechanism very similar to the classic liquid–liquid partitioning between water and acetonitrile. However, the chromatographic approach can lead to a significantly better separation due to the opportunity of an exact adjustment of the switching time.

The results on the tested diol columns were quite similar. The LUNA HILIC showed a smaller retention time window for the analytes. Yet also the matrix components showed less retention than on the YMC-Pack Diol. For the separation of analytes and matrix compound the YMC-Pack Diol was most suitable. The resulting matrix effect profiles for the tested commodities are shown in Fig. 2. Any deviations from 0% indicate matrix effects in that area of the chromatogram. The vertical line indicates the end of the retention time window of the analytes. In all commodities, strong matrix effects were observed at retention times higher than the retention time of the last eluting analyte. With appropriate column-switching, it is possible to separate all the matrix components from the target pesticides eluting at these later retention times.

Also, the retention times of the analytes were tested in the presence of the matrix components. For the tested pesticides, no negative influence of the matrix was observed in the various commodities.

In the last step, the column dimension and the gradient were observed. So far, all the experiments had been carried out with a column of 100 mm length. Now, the 150 mm version of the YMC-Pack Diol was tested to improve the matrix separation. On this column the retention time windows of the analytes was increased to 8 min. A comparison with the resulting matrix effect profiles showed no improvement of the matrix separation. For the final method, the 100 mm column was chosen. Also, modifications of the HILIC gradient were not suitable for increasing the matrix separation. Therefore, the gradient used up to now was also chosen for the final method. The only difference from this was that the water gain to 50% was raised for a better flushing of the column after the transfer of the analytes to the interface.

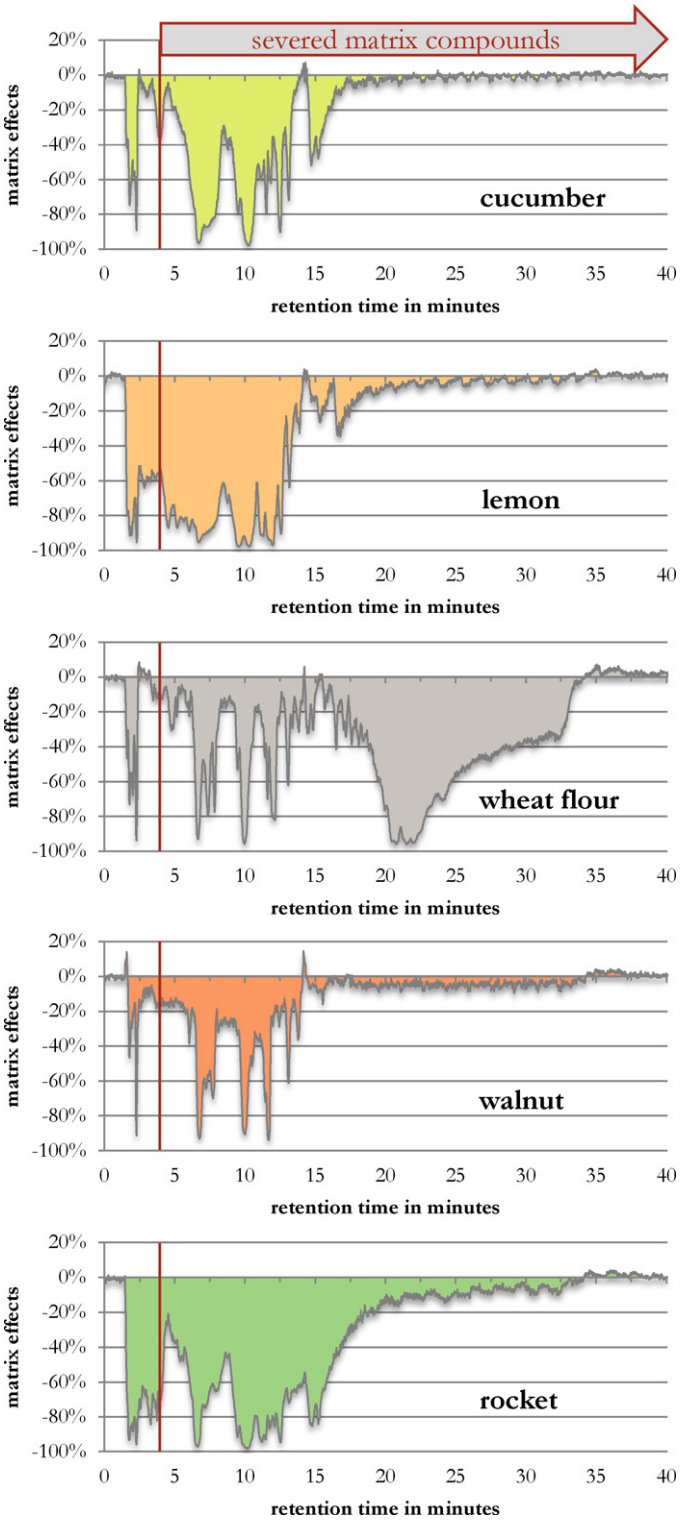

Fig. 2. Matrix effect profiles of different raw extracts after separation on the YMC-Pack Diol HILIC column. Deviations from 0% indicate matrix effects in that area of the chromatogram. The vertical line marks the elution time of the latest eluting analyte. Matrix components on the right side of this line can be removed in the two-dimensional method.

3.2. Development of the packed loop interface and the analytical separation

3.2.1. Interface

To transfer the analytes from the first to the second dimension, an appropriate interface was needed. The aim was to use a packed loop interface to collect all the analytes which eluted in the first small fraction of the HILIC separation. Due to the nonpolar character of the second dimension, reversed phase material should also be used for the interface. The high amount of acetonitrile in the first part of the HILIC separation was problematic for the retention of the analytes on this trap column. To increase the polarity and, thus, to increase the affinity of the nonpolar analytes to the stationary phase of the trap, water must be added to the HILIC eluate. The aim of the optimisation was to trap as many analytes as possible in the interface.

Table 3
Tested columns for the packed loop interface.

Name	Dimension, catalogue number
Agilent ZORBAX SB-C18	4.6 mm × 12.5 mm; 5 μm; 80 Å No.: 820950-920
Agilent ZORBAX SB-C8	4.6 mm × 12.5 mm; 5 μm; 80 Å No.: 820950-915
Agilent ZORBAX SB-C3	4.6 mm × 12.5 mm; 5 μm; 80 Å No.: 820950-922
Agilent ZORBAX SB-Aq	4.6 mm × 12.5 mm; 5 μm; 80 Å No.: 820950-933
Agilent ZORBAX SB-CN	4.6 mm × 12.5 mm; 5 μm; 80 Å No.: 820950-916
Agilent ZORBAX Bonus-RP	4.6 mm × 12.5 mm; 5 μm; 80 Å No.: 820950-928
Agilent ZORBAX XDB-CN	4.6 mm × 12.5 mm; 5 μm; 80 Å No.: 820950-935

Different materials were tested. They are summarized in Table 3. All tested columns were precolumns of the ZORBAX-series from Agilent in the same dimension. To achieve a moderate flow within the trap, an inner diameter of 4.6 mm was chosen. The length was always 12.5 mm. Classic reversed phase materials such as C18 and C8, but also more polar silica gel modifications, were tested. To achieve an appropriate trapping of the polar analytes, most trap columns were not endcapped (SB-series from Agilent). The tested cyanopropyl columns differed in their endcappings (SB without, XDB double). According to the vendor's specifications, the SB-Aq and Bonus-RP should be exceedingly suited for the retention of polar analytes.

The trap columns were directly connected to the end of the selected HILIC column and tested for their ability to retain the analytes. Via a T-piece between the HILIC and the trap column, water with 0.1% HCOOH and 5 mmol/L NH_4HCOO was added.

The cyanopropyl columns (SB-CN, XDB-CN) were unsuitable for the packed loop interface. Only about 70% of the analytes were retained on these materials. Also, the retention of many test compounds on the Bonus-RP column was too low. The alkyl-modified silica columns SB-C18, SB-C8, SB-C3 and SB-Aq showed good performance. About 85% of the analytes showed strong retention on these materials. Especially small and very polar compounds with a stronger HILIC retention such as methamidophos or propamocarb were problematic. These compounds eluted later from the first dimension column than the majority of analytes. Furthermore, it was not possible to collect them on the trap column.

For all trap columns, different amounts of water were tested. The flow rate of the water addition was between 1.0 mL/min and 5.0 mL/min. Together with the water in the mobile phase of the HILIC separation, the concentration was thus between 85% and 96.5%. The results verified the further findings. SB-C18, SB-C8, SB-C3 and SB-Aq showed the best behaviour. At high flow rates, the higher polarity of the mobile phase was connected with an increased flow rate within the trap. So a better retention of the polar analytes was not observed at rates over 2.0 mL/min. At flow rates lower than 1.5 mL/min, the retention of some of the analytes was significantly decreased. The SB-C8 showed the best behaviour and the smallest dependence on the water flow rate. In addition

to these experiments, a 50 mm SB-C8 column was also tested to increase the retention of the polar analytes. This was successful in some cases, but the lack of focussing of these compounds on the trap column and the high column volume led to bad peak shapes and high dead times later in the second dimension separation. Experiments without HCOOH or NH_4HCOO in the added water showed slightly worse results. Finally, the SB-C8 with 12.5 mm length was chosen for the interface.

3.2.2. Design of the 2D-LC system

The previous results showed the possibility to separate a large number of pesticides from the matrix components on a polar HILIC column and to collect the fraction of these analytes on a reversed phase trap column. In the last step of the two-dimensional method the trapped pesticides were to be eluted to an analytical column with subsequent mass spectrometric detection. Problems occurred with small polar compounds. To also detect these analytes, the final method was divided into 4 phases. This principle is shown in Fig. 3.

Within phase I, the starting conditions for both dimensions were adjusted. About 1 min after injection, phase II started. The majority of the compounds eluted from the HILIC. After the addition of water, these compounds were trapped on the packed loop interface. More polar matrix components were still on the HILIC at this time. Due to the separation of the analytes and the matrix components, phase II is one of the most important parts of the method. In phase III, the water addition was stopped. The trapped pesticides remained in the interface column without any flow. The small and polar compounds with more retention on the HILIC were eluted directly to the mass spectrometer without separation in the second dimension. After the elution of the last pesticide from the HILIC, phase IV started. The flow over the trap column was inverted. The trapped pesticides were eluted to the RP column in the second dimension by an increasing methanol gradient. During the entire second dimension separation the mobile phase flowed through the trap column. So the interface was completely purged. The matrix components with a retention on the HILIC were flushed to waste in phase IV by an increased polarity of the mobile phase.

3.2.3. Valve switching times

The aim of the valve switching time optimisation was to achieve a maximized separation of the analytes and the matrix components. Yet also the retention of the polar analytes on the HILIC and the trap column had to be considered. The first pesticides eluted after 1.2 min from the HILIC. This was the starting time for the water addition (phase II). Valve 1 was switched to trap the analytes on the interface. The valve switching time between phase II and phase III was especially important. In phase II, all the analytes with a low retention on the HILIC and a high affinity to the trap column were collected in the interface. Phase III was to determine more polar compounds directly after the HILIC elution. An early switching time would lead to a better separation of the analytes and the matrix components. However, more of the analytes would be measured in phase III without a separation in the second dimension. The aim was to trap as many analytes as possible in the interface. In phase III, only some very polar compounds with no affinity to the trap column were to be measured.

In a first experiment, switching times from 1.8 min to 4.0 min were tested. The majority of compounds were detectable after separation on the RP column with all the switching times. With late switching times, actually all the analytes can be trapped. Yet some polar compounds broke through the trap column when phase II (trapping period) was too long. So the time needed to be shortened until there was no longer any breakthrough to be observed. Early switching times and the following determination of the polar compounds in phase III (direct determination) led to different effects. Due to the low retention on the SB-C8 interface, pymetrozine and spiroxamine showed significantly more intensive signals with direct detection after the HILIC separation. Triclopyr, fenoprop, quizalfop, and 2.4-D retained strongly on the HILIC column, but also on the nonpolar trap. However, significantly better peaks were observed in phase IV after refocusing on the trap. Problems occurred at late switching times with some of the compounds which were unable to bind to the trap column material. Due to the high flow rate within the trap and the long trapping period, e.g. methamidophos and acephat broke through the trap. Instead of the direct connection of the end of the trap column to the mass spectrometer in the final configuration, a sensitive determination of the breakthrough was not possible. Fig. 4 shows the influence of the different switching times for aldicarb-sulfoxid.

A further question was the influence of the matrix on the direct determination of the polar compounds. Cucumber, lemon, rocket, and black tea extracts were spiked with the pesticide test mix in a concentration of 10 ng/mL. The matrix extracts were analysed at different switching times. The peaks after the direct measurement in phase III were compared with the peaks after the RP separation and the peaks in pure solvent, respectively. The majority of the critical compounds showed good signals in the direct determination even in the presence of the matrix. Even in black tea, adequate results were obtained at this low concentration level. But, whenever possible, a transfer of the analytes to the second dimension was to be preferred.

For the final determination of the optimal switching times, all 300 compounds were spiked in acetonitrile/water (v/v 50:50) to consider the influence of the water in the raw sample extracts. The optimal switching time between phase II and III was found at 2.0 min (2.3 min for the negative method).

Acephat, methamidophos, and pymetrozine were defined as marker compounds. These pesticides eluted from the HILIC column close to the switching time. Acephat and methamidophos showed only a low retention on the trap column. Hence, the switching time was set close to their retention times to transfer them to the second dimension. Pymetrozine was the first compound to be measured directly in phase III.

3.2.4. Second dimension separation

At last, also the second dimension was optimized. Different reversed phase columns were tested. They are summarized in Table 4. Due to the mass spectrometric detection, small flow rates and, thus, small inner column diameters (2.1 mm) were used. For the comparison, all the columns were tested with 100 mm length. The pump pressure was limited to 600 bar. Therefore, only particles larger than 2 μm could be used. Besides, three classic C18 modified silica gel columns as well two fused core materials were tested.

For the majority of the compounds, no significant differences were observed between the columns. Variations were found for the retention times but not the peak shape or the signal intensity. The

Table 4
Tested reversed phase columns for the analytical separation in the second dimension.

Name	Dimension, catalogue number
Agilent ZORBAX Eclipse Plus-C18	2.1 mm × 100 mm; 3.5 μm; 95 Å No.: 959793-902
Phenomenex LUNA 3u C18(2)	2.0 mm × 100 mm; 3 μm; 100 Å No.: 00D-4251-B0
Restek Ultra Aqueous C18	2.1 mm × 100 mm; 3 μm; 100 Å No.: 9178312
Agilent Poroshell 120 EC-C18[a]	2.1 mm × 100 mm; 2.7 μm; 120 Å No.: 695775-902
Phenomenex Kinetex 2u C18[a]	2.1 mm × 100 mm; 2.6 μm; 100 Å No.: 00D-4462-AN

[a] Columns with fused core particles.

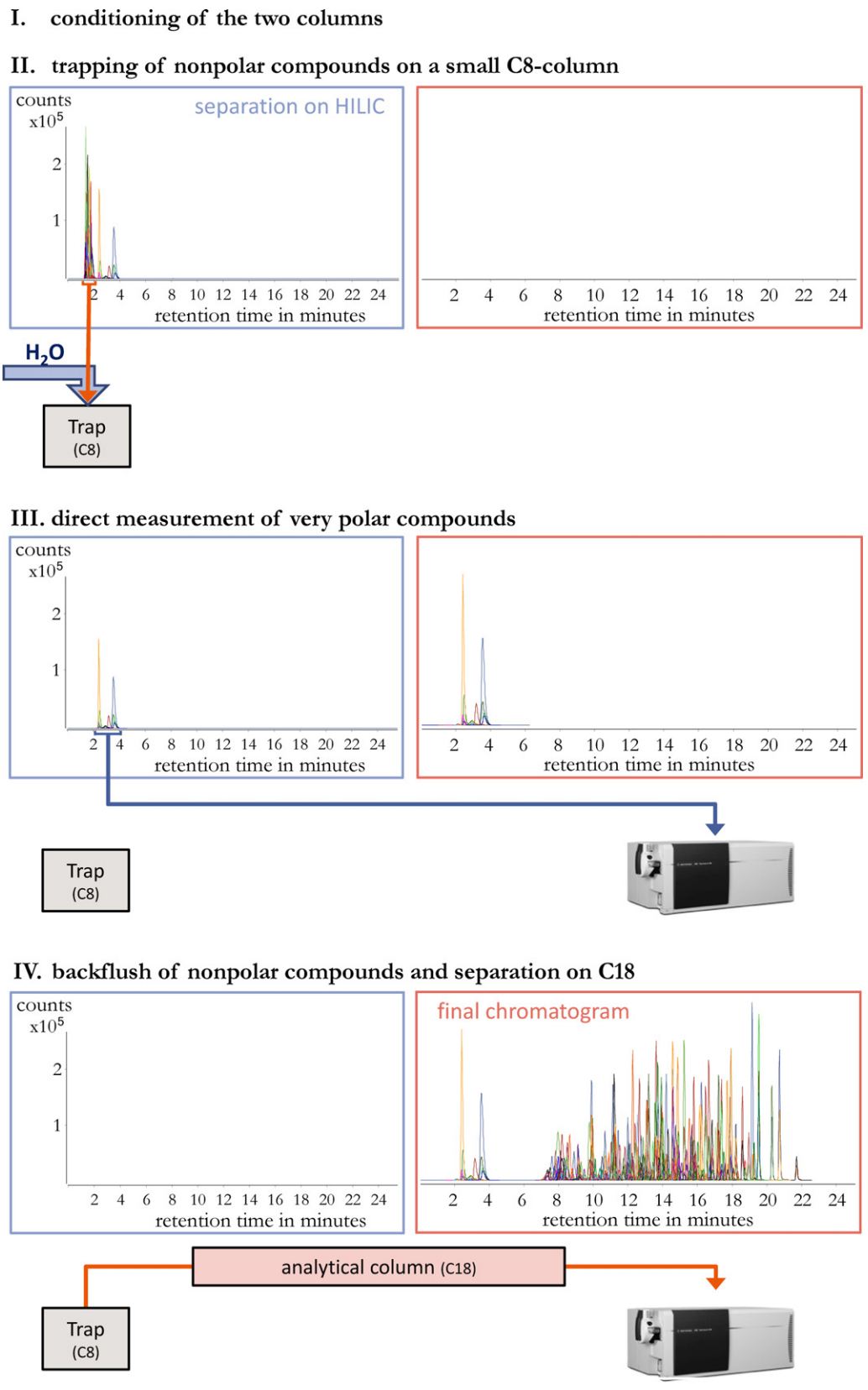

Fig. 3. Principle of the two-dimensional method.

fused core materials showed the best performance regarding peak shape and separation. Especially the Agilent Poroshell showed narrow and well separated peaks and, thus, even the best sensitivity. Even fenbutatin oxide and cyhexatin did not tail like on the other tested columns. Therefore, the Agilent Poroshell was chosen for the second dimension separation.

At last, also the mobile phase of the reversed phase separation was improved. Different combinations of water with methanol and

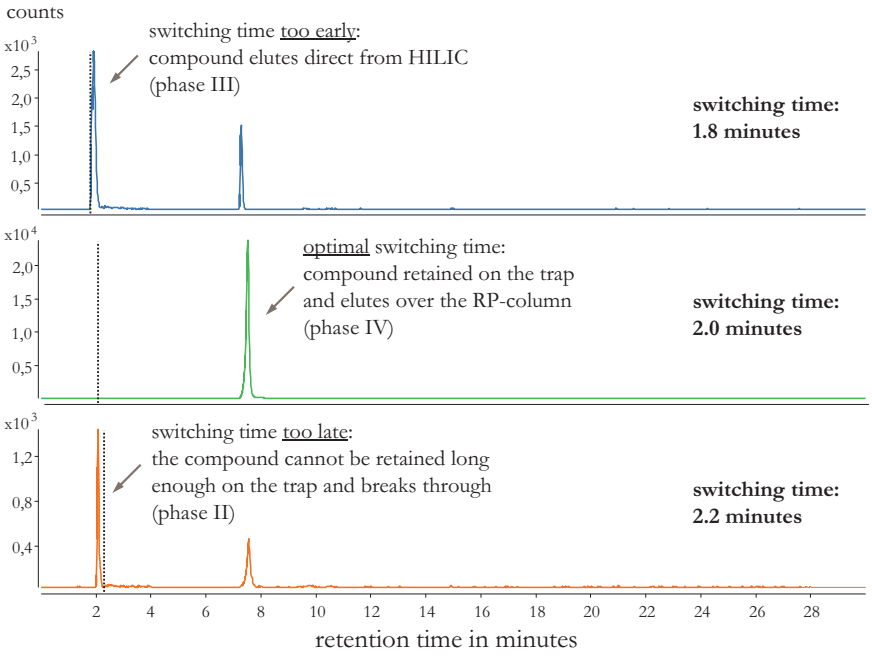

Fig. 4. Influence of the valve switching time on the elution of aldicarb-sulfoxid. The vertical line marks the tested switching time.

acetonitrile with different concentrations of formic acid and ammonium formate were tested. The best results were obtained for water and methanol with 0.1% HCOOH and 5 mM/L NH_4HCOO, each. In the final two-dimensional method the water channel of the second dimension pump was also used for the addition of water during the trapping of the analytes in the interface. Here, the addition of 0.1% HCOOH and 5 mM/L NH_4HCOO to the water also showed the best results. For an optimal separation and distribution of the analytes, the gradient was also optimized.

3.3. Selection of an internal standard for matrices with high amounts of carbohydrates

The aim of the method development was to inject the raw sample extracts without any clean-up. For matrices with high amounts of carbohydrates (e.g. raisins) a phase separation between water and acetonitrile was observed after the extraction. Also, wheat flour showed a slight partitioning, especially at low temperatures. For nearly all the compounds, spiking experiments prior to the validation showed in wheat flour 20–30% higher recoveries than in the other tested matrices. For these matrices with high amounts of carbohydrates an internal standard was needed to compensate the enrichment of the nonpolar analytes in the organic layer. Ideally, an internal standard shows the same behaviour as the analytes. Seven veterinary drugs and drug metabolites were tested. Just as pesticides, these drugs are small and nonpolar organic molecules. The tested compounds are summarized in Table 5.

Table 5
Tested veterinary drugs for use as internal standard.

Compound	Sum formula	Molecular weight (g/mol)
Brillant green	$C_{27}H_{34}N_2O_4S$	482.64
Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	277.19
Dimetridazol	$C_5H_7N_3O_2$	141.13
Fenbendazol	$C_{15}H_{13}N_3O_2S$	299.35
Furazolidon	$C_8H_7N_3O_5$	225.16
Ipronidazol	$C_7H_{11}N_3O_2$	169.18
Leucomalachit green	$C_{23}H_{26}N_2$	330.47

The best results for the recovery compensation were obtained with fenbendazol, ipronidazol, and furazolidon. After the correction of the recoveries with clenbuterol, the amount of the pesticides with recoveries under 70% was slightly increased. With dimetridazol and brilliant green, some pesticides still showed recoveries higher than 120%. Leucomalachit green showed a completely different partitioning behaviour than the analytes. This metabolite remained nearly completely in the water phase and was not suited for use as an internal standard.

For a final decision, fenbendazol, ipronidazol, and furazolidon were spiked again to wheat flour. As in the method validation, the analytes were spiked with 0.001 mg/kg, 0.005 mg/kg, 0.010 mg/kg, and 0.020 mg/kg. In all the concentrations, the correction with furazolidon showed the best recoveries for the analytes. Due to its good peak shape and sensitivity, furazolidon was selected as internal standard for matrices with high amounts of carbohydrates.

3.4. Method validation for over 300 compounds

To test the performance of the two-dimensional method, a validation was carried out for over 300 compounds. Fig. 3 shows the chromatogram of these compounds measured with the final method in the bottom. Recovery rate, relative standard deviation, sensitivity, and linearity were determined according to the SANCO document [48]. Five different matrices were spiked to check the ability for different kinds of food. Cucumber (high water content), lemon (high acid content), wheat flour (high amounts of starch), rocket, and black tea (both difficult to analyze) were tested. All matrices were obtained from the local market and were checked for pesticide residues before the validation experiments. The spiking was carried out at four concentration levels (0.001 mg/kg, 0.005 mg/kg, 0.010 mg/kg, 0.020 mg/kg). Due to the low sample weight, black tea was additionally spiked with 0.050 mg/kg. To determine the relative standard deviations, all the levels were prepared five times. Spiking was always carried out before the addition of water and the extraction of the samples with 10 mL acetonitrile. After shaking for 30 min in an overhead shaker, the samples were centrifuged for 5 min at 3000 rpm (1740 rcf). An aliquot of the supernatant was filled into a sample vial and injected into the 2DLC

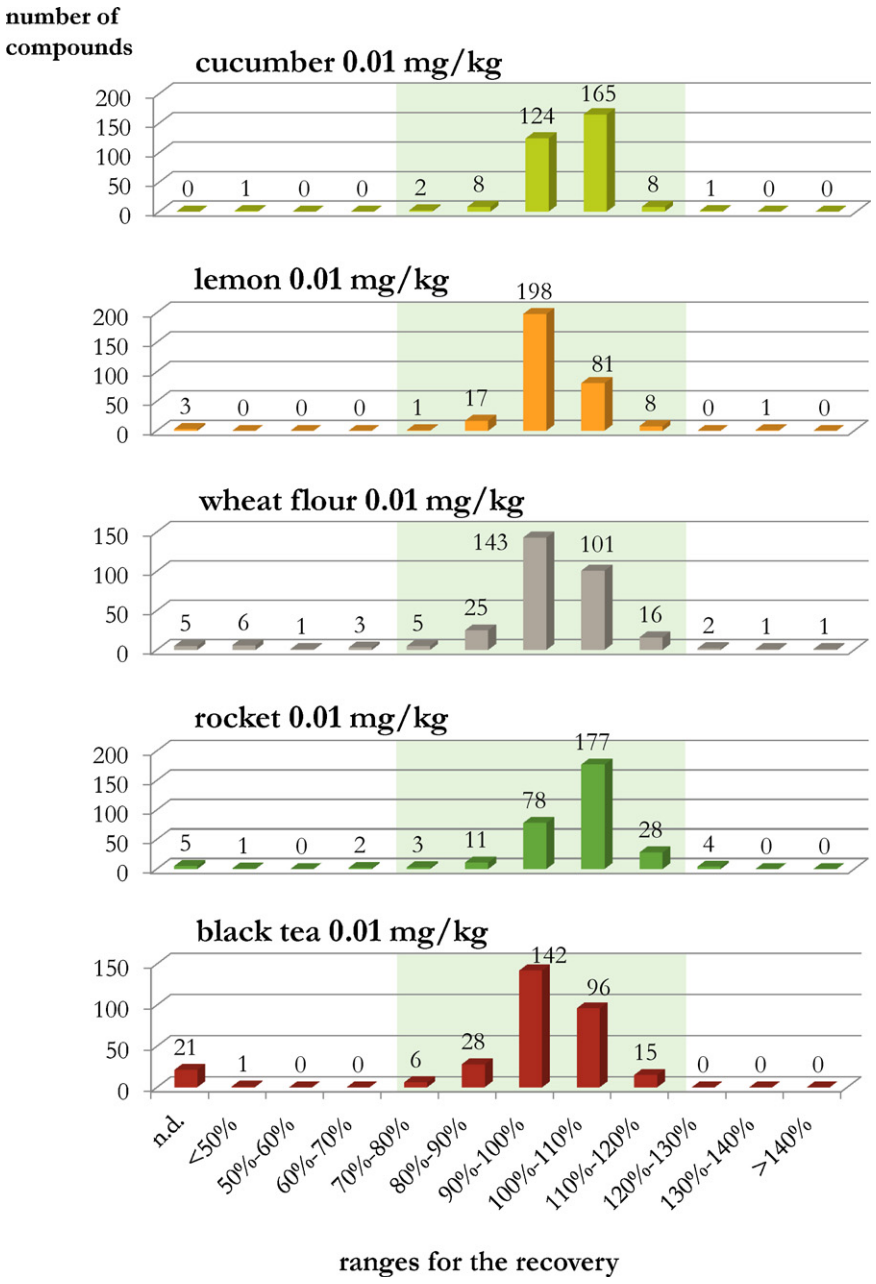

Fig. 5. Recoveries of more than 300 compounds in different matrices at 0.01 mg/kg. The box marks the accepted range from 70% to 120% [48].

system. For the determination of the recovery, blank extracts were spiked after sample preparation.

The detailed results for all the analytes are shown in the supporting information. Tables C and D show the ability of the developed method to analyze more than 300 pesticides in various matrices. For the majority of the compounds, recoveries in the acceptable range between 70% and 120% were observed [48]. In cucumber, 99% of the analytes showed recoveries in this range. For lemon 98%, rocket 96%, wheat flour 94%, and black tea 93% of the compounds showed acceptable recoveries. Fig. 5 summarizes the results for the 0.01 mg/kg level. The majority of the analytes showed recoveries between 90% and 110%. Therefore, only slight deviations from the optimum of 100% were observed.

In lemon and rocket, low recoveries were detected for benfuracarb and carbofuran. Only small amounts of fenbutatin oxide were recovered in all the sample extracts, except lemon. Further experiments showed that a quantitative extraction of fenbutatin oxide was only possible at low pH-values. In wheat flour, some compounds showed recoveries below 70%. For bifenazat and pyridat, recoveries below 60% were detected. Values over 130% were only observed for fenthion-sulfoxid in wheat flour and for 2,4,5-T in wheat flour and lemon.

The relative standard deviations were calculated for every compound on the basis of 5 replicates at each calibration level. According to the SANCO document, values up to 20% are acceptable. Fig. 6 and Tables E and F in the supporting information show the results for all the matrices. Similar to the recovery results, the relative standard deviations were in the acceptable range for the majority of the compounds. For cucumber 99% of the analytes showed values below 20%. For lemon 98%, for wheat flour and rocket 95%, and for black tea 88% of the compounds were in the acceptable range. The best precision was observed for cucumber and wheat flour. About 90% of the analytes showed relative standard deviations below 10% at the 0.01 mg/kg level in these

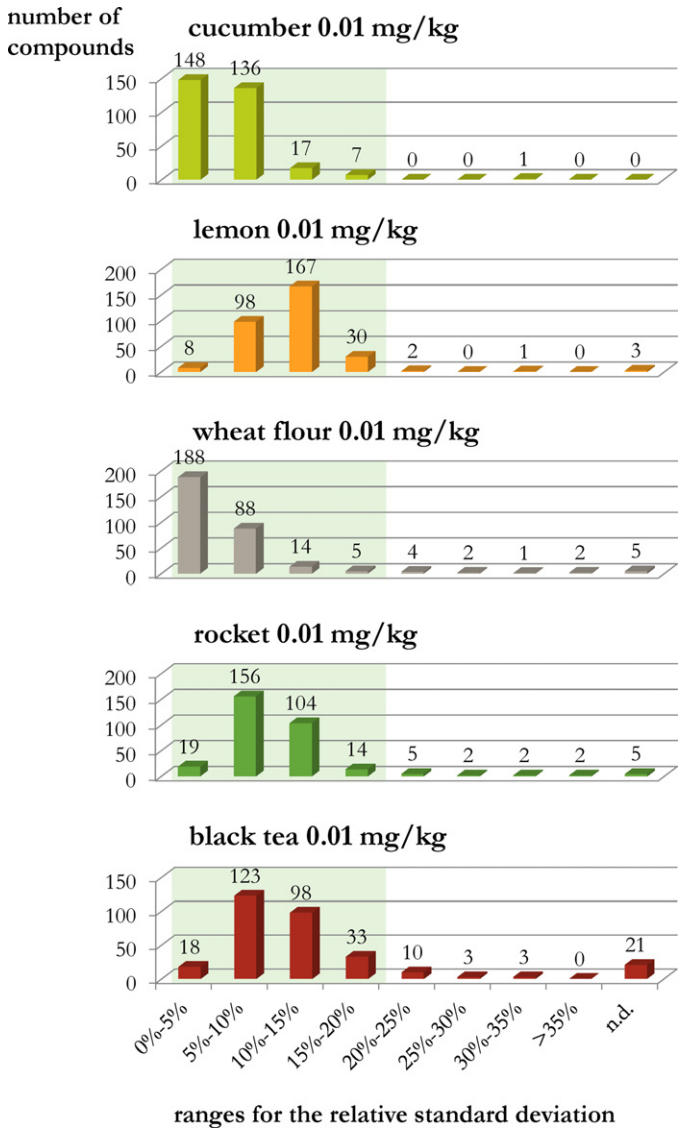

Fig. 6. Relative standard deviations of more than 300 compounds in different matrices at 0.01 mg/kg. The box marks the accepted range up to maximally 20% [48].

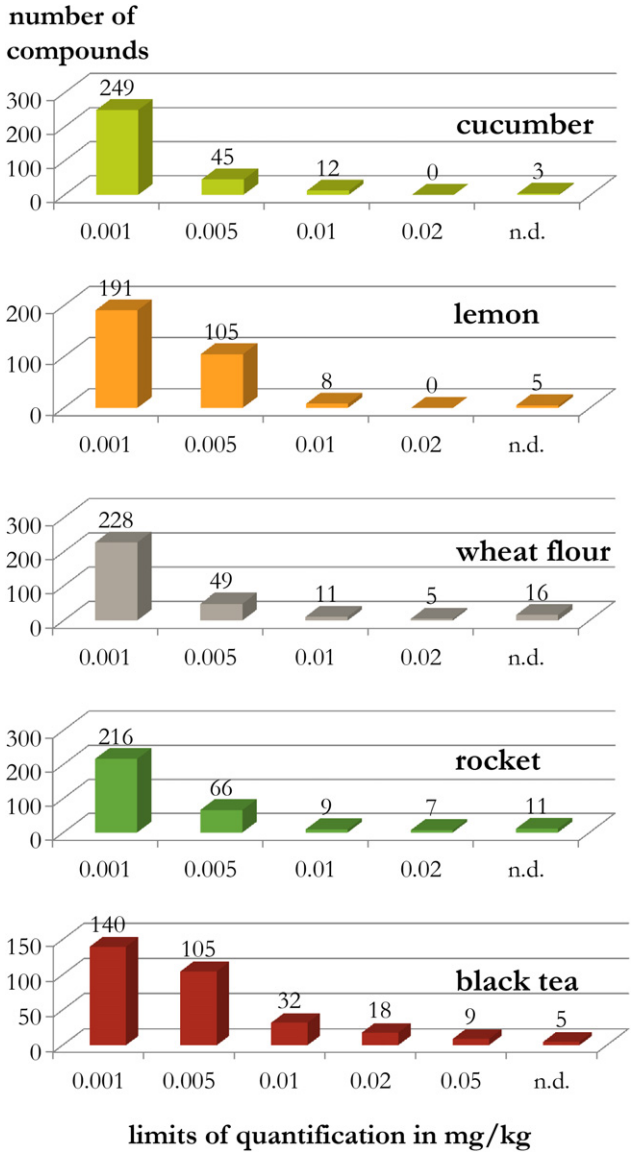

Fig. 7. Limits of quantification for more than 300 compounds in different matrices.

commodities. For cucumber, this was caused by the low number of matrix components in the sample extract. The low standard deviations for wheat flour were observed due to using furazolidon as an internal standard.

According to the SANCO document, the limit of quantification is defined as the lowest validated level meeting the performance criteria for recovery (70–120%) and relative standard deviation (≤20%) [48]. The results for the tested matrices are shown in Fig. 7 and Tables G and H of the supporting information. For the majority of the compounds, the LOQ was the lowest validated level 0.001 mg/kg. For cucumber, all the analytes showed an LOQ of at least 0.01 mg/kg. For black tea, higher LOQs were expected because of its five times lower initial weight. Yet also in this difficult matrix, 89% of the analytes showed an LOQ of 0.01 mg/kg, or even lower.

The linearity was tested by the calculation of the residuals [48]. To have enough data points for the calibration curve, this was only carried out for compounds with at least four detectable concentration levels. Detailed results are shown in the Tables I and J of the supporting information. For all the contemplated analytes, good linearity was observed. Residuals above the accepted 20% were only detected for carbofuran-3-hydroxy and fenhexamid.

3.5. Robustness of the method

inally we wanted to test the robustness of the developed method during routine analysis. Due to the missing clean-up and, thus, the high matrix load, one important question concerned the long-time performance of the HILIC column. To check this, raw acetonitrile extracts of 10 g rocket, 2 g black tea, and 2 g hops were prepared and spiked with all the analytes in a concentration of 10 ng/ml. Five microliters of each of these extracts were injected 100 times into the two-dimensional system. The signal intensities of the spiked compounds were measured. The overall runtime was about one week. The results for some representative compounds are shown in Fig. 8. Instead of the high matrix load and the long time period, no significant changes regarding signal intensity were observed. The method showed high robustness and reproducibility. Deviations in signal intensity were only detected between the different commodities caused by the different matrix effects. Moreover, also the peak shape and the separation of the polar analytes on the HILIC column were checked before and after the high matrix load. No deviations in the retention times were detected for the analytes or the matrix components, respectively. In conclusion, no negative

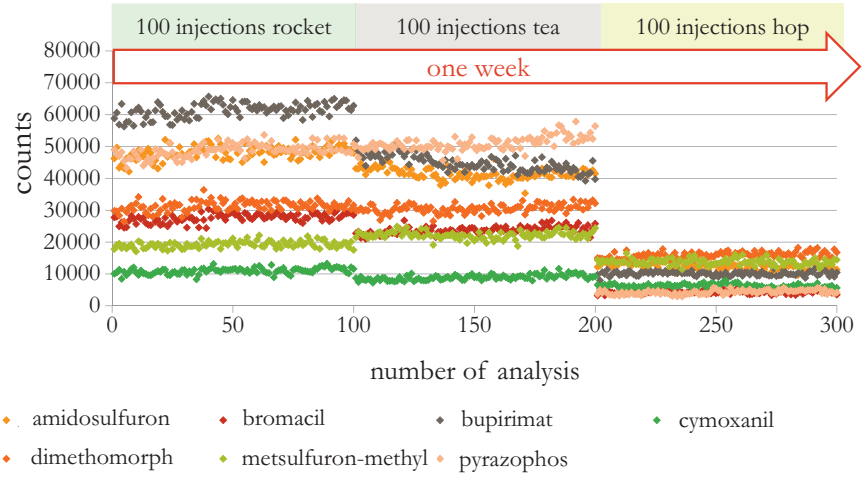

Fig. 8. Long time study with difficult matrices to check the robustness of the method. The figure shows the stability of the signal intensities for representative compounds (0.01 mg/kg). In each case, 100 runs were performed with rocket, black tea, and hop. The total run time was about one week.

effects regarding the robustness of the two-dimensional method were detected during this long-time experiment. Even during the whole method development no change of the HILIC column was needed.

To also ensure the functionality of the method after a change of the first dimension column, six new columns from two different batches were ordered and tested for their separation behaviour. In the experiments, all the columns showed similar behaviour regarding the analytes and the matrix components. For routine analysis, a test mix of five pesticides was composed for the adjustment of the switching times and the method check-up. The chosen compounds and their functions are shown in Table 6.

Finally, we wanted to test the method for routine purposes. For this we analysed the spiked mandarin homogenate of the EURL-PROFICIENCY TEST-FV-13 from 2011. Raw extracts with 10 mL acetonitrile were prepared and injected after centrifugation without any further clean-up. In a first step the spiked pesticides were identified and roughly quantified by external matrix-matched calibration. In the second step the exact quantification was done by standard addition. The whole analysis time was less than one day.

All LC-amenable compounds in the spiked mandarin homogenate were found after the first injection. No false negative results were observed. All pesticides showed good z-scores. For 14 pesticides we detected *z*-scores between −2 and +2. For 2 compounds values between +2 and +3 were observed. Most compounds showed slightly higher *z*-scores than the participating laboratories. For the LC-results the overall classification in case of a real participation would be the highest category ("good").

Table 6
Test compounds for method check-up.

Compound	Function in the test mix
Etofenprox	First compound in HILIC separation; very late retention time in RP separation
Methamidophos, acephat	Last compounds to trap on the interface; switching of valve 1 directly after the elution of these compounds from the HILIC; early retention time in RP separation
Pymetrozine	First compound for direct measurement of HILIC eluate (phase III); switching of valve 1 directly before elution of pymetrozine
Cyromazine	Last compound for direct measurement (phase III); start of second dimension separation (phase IV) after the elution of cyromazine from HILIC

4. Conclusion

For the first time a two-dimensional LC–MS/MS method was developed for the determination of more than 300 pesticides in fruit and vegetables. The homogenized samples are extracted with 10 mL acetonitrile. In contrast to the established methods, no further clean-up is necessary. Matrix components and analytes are separated in the first dimension by a YMC-Pack Diol HILIC column. The majority of the analytes elutes within a small fraction at the beginning of the HILIC separation. At this time, most of the matrix compounds are still retained on the HILIC column. The analytes are trapped on a small reversed phase column to change the solvent prior to the RP separation in the second dimension. Very polar analytes with a high retention on the HILIC can be determined directly without any trapping or RP separation. In the end, the trapped compounds are flushed back to the second dimension RP column. The analytes are separated by gradient elution and detected with a triple quadrupole mass spectrometer working in the MRM mode.

Over 300 compounds can be measured with the developed two-dimensional approach. The method is validated in representative fruit and vegetables. The large majority of the compounds shows recoveries and relative standard deviations within the accepted ranges of the SANCO document [48]. In cucumber and lemon, all the detectable compounds have limits of quantification of 0.01 mg/kg, or even below. For wheat flour, rocket, and black tea, only a few of the compounds showed an LOQ above this value. In all the matrices, the majority of the analytes can be quantified at 0.001 mg/kg. The method showed true, accurate and robust results even with difficult matrices. With these findings we can conclude that our method fits the purpose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.01.106.

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