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Towards a generic extraction method for simultaneous determination of pesticides, mycotoxins, plant toxins and veterinary drugs in feed and food matrices

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ABSTRACT

A fast and straightforward generic procedure for the simultaneous extraction of various classes of pesticides, mycotoxins, plant toxins and veterinary drugs in various matrices has been developed, for subsequent analysis by liquid chromatography with mass spectrometric detection. As a first step, four existing multi-analyte procedures and three newly proposed methods were compared for a test set of 172 pesticides, mycotoxins and plant toxins spiked to a feed matrix. The new procedures, which basically involved extraction/dilution of the sample with water and an acidified organic solvent (methanol, acetonitrile or acetone), were most promising. The three new generic extraction methods were further tested for applicability to other matrices (maize, honey, milk, egg, meat). Overall, the best recoveries were obtained for acetone, followed by acetonitrile. With respect to matrix effects, acetonitrile was the most favorable solvent and methanol the worst. The occurrence of matrix effects decreased for the matrices in the order: feed > maize > meat > milk > egg > honey. The extraction method selected as default procedure (water/acetonitrile/1% formic acid) was also evaluated for applicability to multiple classes of veterinary drugs in all six matrices, with satisfactory results. Finally, the generic extraction procedure was validated for 136 pesticides, 36 natural toxins and 86 veterinary drugs in compound feed and honey at three levels (0.01, 0.02 and 0.05 mg/kg) using UPLC-MS/MS for analysis of the extracts. For over 80% of the analytes, recoveries were between 70-120% and precision (expressed as relative standard deviation) was mostly in the range 5-10% (except for feed at 0.01 mg/kg; adequate recoveries for 62% of the analytes). The limits of detection were < 0.01 to 0.05 mg/kg for most analytes which is usually sufficient to verify compliance of products with legal tolerances. The results clearly demonstrate the feasibility of the generic approach proposed. Application of the method in routine monitoring programs would imply a drastic reduction of both effort and time.

INTRODUCTION

During production, processing, storage and transport of food and feed a variety of residues and contaminants may enter the food chain. Crops and animals are treated with pesticides and veterinary drugs against pests and animal diseases and may leave residues in products of plant and animal origin. Besides anthropogenic introduction of chemicals, natural contaminants like mycotoxins and plant toxins may also appear in feed and food [1-3].

The presence of residues and contaminants can endanger both human and animal health and welfare. Therefore, legislation has been established in which maximum limits of residues and contaminants have been set. This includes legislation on pesticides in food [4,5] and feed [4-6], mycotoxins in food [7] and feed [8] and veterinary drugs in animal products [5,9]. Although plant toxins have hardly been regulated (only to a certain extend in feed [6]), there are concerns, for example regarding the presence of pyrrolizidine alkaloids in feed [10].

To control and monitor the occurrence of undesirable substances in our food and feed, samples are taken at various stages in the food chain and analyzed. The entire scope of residues and contaminants comprises thousands of target analytes. To cover the whole spectrum of residues and contaminants numerous methods are being used. For many food and feed products, residues and contaminants from different classes need to be determined. Consequently, the same samples from these products are often analysed multiple times to cover all relevant analytes. For example, honey may be analysed for residues of acaricides (a subclass of pesticides), sulfonamides (a subclass of veterinary drugs), macrolides (another subclass of veterinary drugs) and pyrrolizidine alkaloids (a subclass of plant toxins).

Within each field of residues and contaminants, a clear trend towards to the use of multi-analyte methods can be seen. Such methods involving MS detection are an efficient way of assessment of occurrence of undesirable substances and can provide both qualitative and quantitative information at the same time. In the field of pesticide residue analysis this has been most well established. Multi-residue methods based on gas chromatography were already developed in the 1980s. During the last five years the scope has been dramatically expanded by complementary methods based on liquid chromatography [e.g. 11-13]. Today samples are being routinely analysed for many hundreds of pesticides by one extraction procedure followed by chromatographic analysis with mass spectrometric detection. More recently, similar developments are going on in the field of mycotoxins [e.g. 14,15] and veterinary drugs [16-18].

Within each residue/contaminant domain it has been shown that combined determination of compounds with a wide variety of physical chemical properties is feasible. The obvious next step forward would then be to combine these methods into an even more generic method to cover residues and contaminants beyond their respective domains. The purpose of the current work is to develop such a generic extraction method which should not only cover a vast number of target analytes, but should also be applicable to different types of food and feed matrices. To achieve this goal, three new extraction/”dilute-and-shoot” type methods are proposed and compared with existing multi-analyte methods from the field of pesticides and mycotoxins. The aim of the current work was to develop one generic sample preparation method, capable of extracting a wide variety of residues and contaminants from various food and feed matrices.

EXPERIMENTAL SECTION

Chemicals and reagents

Reference standards
A list of all analytes used in this work can be found in the Supporting Information (included in Table S-2a). The majority of the reference standards were purchased from commercial suppliers. In other cases, the materials were a gift from other scientific institutes or synthesized in-house. For details the reader is referred to the Supporting Information.

Chemicals
Acetonitrile, acetone, methanol, ethyl acetate, cyclohexane (all HPLC grade or better) and HPLC grade water were purchased from Biosolve (Valkenswaard, The Netherlands). Acetic acid, sodium sulfate and magnesium sulfate were obtained from Merck (Darmstadt, Germany) and formic acid and ammonium formate from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Samples

Six different commodities were used in this work. The feed matrix was a commercially available compound feed sold as horse feed. Compound feeds are feedstuffs that are blended from various raw materials and additives. Maize flour and whole raw milk were samples from the Dutch official monitoring programs. Meat was a mixture of lean pork and beef which was minced in the laboratory. Eggs (chicken) were purchased in a local shop. Honey was obtained from a local bee-keeper. The composition characteristics of the six commodities are given in Table S-1 of the Supporting Information.

Extraction experiments

In all cases the samples were spiked with a mix solution of the test analytes in methanol such that the concentration in the sample was 0.25 mg/kg. The spiked samples were allowed to stand for 30 min before extraction. In all protocols described below, the final concentration of matrix equivalent in the extract was 0.125 g/ml.

Pesticide multi-residue method 1, “Modified Luke method” [19]:
To 2.5 g of feed sample 7.5 ml of water was added and mixed using a vortex. The mixture was allowed to soak for 2 hours. Then 20 ml of acetone and 3.5 g of sodium chloride was added. The mixture was shaken end-over-end for 1 hour. Next 10 ml of a mixture of ethyl acetate/cyclohexane (1/1 v/v) was added to the same tube and shaken by hand for half a minute. The tube was centrifuged (10 min, 2000 rcf). A 1.5 ml aliquot of the clear extract was transferred into a disposable tube and evaporated until near dryness. The residue was reconstituted in 0.5 ml of methanol (vortex, ultrasonication) and then diluted with 0.5 ml water containing 1% acetic acid. The extract was mixed and transferred into an autosampler vial.

Pesticide multi-residue method 2, “Ethyl acetate method” [20]:
To 2.5 g of feed sample 7.5 ml of water was added and mixed using a vortex. The mixture was allowed to soak for 2 hours. Then 20 ml of ethyl acetate was added and the mixture was shaken end-over-end for 1 hour. Next, 10 g of Na_2SO_4 was added to the same tube and shaken by hand. The tube was centrifuged (10 min, 2000 rcf). A 1 ml aliquot of the clear extract was transferred into a disposable tube and evaporated until near dryness. The residue was reconstituted in 0.5 ml of methanol (vortex, ultrasonication) and then diluted with 0.5 ml

water containing 1% acetic acid. The extract was mixed and transferred into an autosampler vial.

Pesticide multi-residue method 3 "QuEChERS method" [21]:
To 2.5 g of feed sample 7.5 ml of water was added and mixed using a vortex. The mixture was allowed to soak for 2 hours. Then 10 ml of acetonitrile containing 1% of acetic acid, 4 g of $MgSO_4$, and 1 g of sodium acetate were added. The mixture was shaken end-over-end for 1 hour. The tube was centrifuged (10 min, 2000 rcf) and a 0.25 ml aliquot was transferred into an autosampler vial and diluted with 0.25 ml of water containing 1% of acetic acid.

Note: the dispersive-SPE clean up step with PSA, normally employed with this method, was omitted.

"Mycotoxin multi-analyte method" [15,22]
To 2.5 g of sample, 10 ml of a mixture of acetonitrile/water 84/16 (v/v) was added and mixed by hand. The mixture was shaken end-over-end for 1 hour. The tube was centrifuged (10 min, 2000 rcf) and a 0.25 ml aliquot was transferred into an autosampler vial and diluted with 0.25 ml of water containing 1% of acetic acid.

Proposed method A: water/acetonitrile/formic acid (MeCN)
To 2.5 g of sample 5 ml of water was added and mixed using a vortex. In case of dry matrices the mixture was allowed to soak for 2 hours. Then 15 ml of acetonitrile containing 1% of formic acid was added and the sample was extracted by end-over-end shaking for 1 hour. The tube was centrifuged (10 min, 2000 rcf) and 0.5 ml of extract was transferred into an autosampler vial.

Proposed method B: water/methanol/formic acid (MeOH)
As proposed method A but with methanol instead of acetonitrile.

Proposed method C: water/acetone/formic acid (ACE)
As proposed method A but with acetone instead of acetonitrile.

Autosampler vials with a build-in syringeless filter device (Mini-UniPrep, 0.45 µm, Whatman, Forham Park, NJ, USA) were used to remove solid materials from the final extract, if any.

LC-MS/MS analysis
For LC-MS/MS analysis an Acquity UPLC system (Waters, Etten-leur, The Netherlands) and a triple quadrupole mass spectrometer (Waters Quatro Premier XE) equipped with an electrospray source were used for determination of analytes measured as positive ions. Separation was performed on a 100 mm x 2.1 mm ID, 1.7 µm BEH-C18 column (Waters) using a flow rate of 0.4 ml/min. The column temperature was maintained at 40°C. Eluent A was 100% water containing 1 mM ammonium formate and 20 µl/l formic acid. Eluent B was water/methanol 5/95 (v/v) containing 1 mM ammonium formate and 20 µl/l formic acid. The LC gradient started with 100% of A for one minute, and was linearly increased to 45% of B over 1.5 min. Then the gradient was increased to 100% of B over 6 min and kept at this phase up to 19.5 min. Finally, the gradient was switched to 100% of A again over 0.5 min and equilibrated for 2 min before the next injection took place. The injection volume was 5 µl.

The LC conditions applied were optimized to have the target analytes distributed over the chromatographic space as much as possible. Despite the small injection volume used and the high initial water content of the eluent, broad peaks were obtained for some of the very polar and basic compounds with the column used.

MS/MS conditions Quattro Premier XE
The following settings were used:
Capillary voltage 3 kV, extractor lens 4 V, RF lens 0 V, source temperature 120 °C, desolvation temperature 450 °C, cone Gas 50 l/hr., desolvation gas 450 lL/hr., LM1 and LM2 resolution 15, ion energy1 0.5, entrance -1, exit 0, HM1 and HM2 resolution 13, ion energy2 1, multiplier 750 V. Argon was used as collision gas at a flow rate of 0.17 ml/min (corresponding to a gas pressure of $3.6x10^{-3}$ mbar). Separate acquisition methods were used for the pesticides/natural toxins and the veterinary drugs (one transition per analyte). Transitions were acquired in up to 17 partially overlapping time windows, containing up to 13 analytes each. The dwell time was 20 ms for each analyte. The inter channel delay and inter-scan delay were both 5 ms. The number of data points across the peaks was at least eight.

For analytes that could only be measured as negative ions, a separate system was used for pragmatic reasons. Here, an Agilent 1100 HPLC system (Agilent, Amstelveen, The Netherlands) with a Waters Quatro Micro triple quadrupole mass spectrometer was used. Separation was performed on a 150 mm x 3 mm ID, 5 µm Symmetry C18 column (Waters) using a flow rate of 0.4 ml/min. The same eluents A and B as described above were used. The LC gradient started at 20% of B (1 min), then linearly increased to 100% of B over 4 min and was kept at this phase for 9 min. Finally, the gradient was switched to 20% of B again over 0.5 min and equilibrated for 6.5 min before the next injection took place. The injection volume was 5 µl.

MS/MS conditions Quattro Micro
The following settings were used:
Capillary voltage 3 kV, extractor lens 2 V, RF lens 0 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas 50 l/hr., desolvation gas 350 l/hr., LM1 and LM2 resolution 15, ion energy1 1, entrance -1, exit 1, HM1and HM2 resolution 13, ion energy2 1, multiplier 750 V. Argon was used as collision gas at a pressure of $3.2x10^{-3}$ mbar
The dwell time for this analysis was between 50 and 100 ms. The inter-channel delay and interscan delay were both 100 ms.

The retention times and analyte specific MS settings (cone voltage, collision energy, precursor and product ion) can be found in the Supporting Information (included in Table S-2a).

LC-TOF-MS analysis
For LC-TOF-MS analysis, an Acquity UPLC system and a time-of-flight mass spectrometer (Waters LCT Premier) were used. The chromatographic conditions were the same as for the UPLC-Quatro Premier XE system described above. The TOF-MS was used with a dual electrospray source. A continuous flow of the calibrant (leucine/enkephaline) was measured every 10 scans. The mass spectrometer was used in W-mode with a resolution of 10,000 (FWHM). Acquisition of m/z 100-1,000 was done with dynamic range enhancement (DRE) on, and a scan time of 0.2 seconds. The source parameters were as follow: capillary voltage 2.8 kV, cone 25 V, desolvation temperature 350°C, source temperature 120°C, cone gas 50 l/hr. and desolvation gas 500 l/hr.

Verification of recovery and matrix effects
For verification of recovery for the different extraction methods, each matrix was fortified in four replicates at the level of 0.25 mg/kg. In addition, one non-fortified sample was included in the test set. The extract of non-fortified sample was also used for preparation of a matrix-matched calibration standard at a level of 31.25 ng/ml (corresponding to 0.25 mg/kg in the samples). In the LC-MS/MS sequence, for each matrix, the 5 sample extracts were bracketed by the matrix-matched calibration standard and a solvent standard at the same concentration. Average recoveries and relative standard deviations (RSDs) were calculated for the fortified samples against matrix-matched standards. Recoveries obtained therefore reflect the recovery from the extraction procedure. Matrix effects were calculated by comparison of the response obtained for each compound in the matrix-matched standard with that of the solvent standard.

Validation
For two matrices, a compound feed and honey, sub samples of 2.5 g were fortified with a mixture of more than 250 pesticides, mycotoxins, plant toxins and veterinary drugs. This was done in five-fold at three levels (0.01, 0.02 and 0.05 mg/kg). Together with two control samples, the fortified samples were processed using proposed method A (MeCN). Aliquots of the control extracts were used to prepare matrix-matched standards at 1.25, 2.5, 6.25, 12.5 and 25 ng/ml (corresponding with 0.01, 0.02, 0.05, 0.10 and 0.20 mg/kg sample). These standards were analysed for verification of linearity of response *vs* concentration. In the LC-MS/MS sequence, the 6.25 ng/ml matrix-matched standard was repetitively analyzed every 5-8 injections. Recoveries were calculated based on one-point matrix-matched calibration, using the average of the 6.25 ng/ml standard preceding and following the sample (bracketing).

RESULTS AND DISCUSSION

The aim of the current work was to develop one generic sample preparation method, suited for extraction of pesticides, veterinary drugs, natural toxins and other contaminants from various food and feed matrices. The extracts obtained should be analysed with a method suited for simultaneous detection of multiple analytes. Chromatography with mass spectrometric detection is very useful for this purpose. At this point there were two possibilities, a method based on LC-MS and a method based on GC-MS. With the target analytes in mind, being pesticides, mycotoxins, plant toxins and veterinary drugs, a method based on LC-MS was considered to cover a wider scope than one based on GC-MS. Therefore, the current work anticipated on a determinative step by LC-MS analysis. With respect to comprehensive screening for residues and contaminants, full scan techniques such as TOF-MS are more appropriate than techniques using targeted acquisition like MS/MS detection [13]. However, since in this work emphasis was on development of a generic extraction procedure, MS/MS detection was used during method development and validation because of its better quantitative performance characteristics (sensitivity, dynamic range). Since speed of analysis and high throughput are important issues in generic residue/contaminant analysis, UPLC was used as separation technique. At the conditions used, all analytes eluted within 9 minutes.

Selection of different extraction procedures and initial evaluation
In the literature, several sample preparation procedures for multi-analyte analysis have been described. Most of these are dedicated to pesticide multi-residue analysis in vegetables, fruits and, to a lesser extend, cereals. The three most frequently used procedures involve an

extraction with acetone followed by partitioning in ethyl acetate/cyclohexane [19], extraction/partitioning with ethyl acetate [11, 20] and extraction/partitioning with acetonitrile [21,23]. Extracts are analyzed as such or cleaned using gel permeation chromatography and/or solid phase partitioning with carbon or amino-based phases. Pesticide methods without involving a partitioning step have been described for example by Granby et al. [24] who used an extraction with methanol, but so far this approach has hardly been pursued by others.

In the field of mycotoxins, many multi-analyte methods described use an extraction with a mixture of acetonitrile/water 84/16 [22] or very similar [15] without any further clean up. For enlarging the scope with respect to mycotoxin analysis, Sulyok et al. [14] investigated other mixtures of water with acetonitrile (with and without acidification) and methanol.

In the field of veterinary drugs, generic methods covering multiple subclasses have been described for milk [16] and meat [18]. In both cases, an extraction using acetonitrile followed by a SPE clean up procedure was used. Lopez et al. [17] described a multi-analyte method for honey which was diluted with water and then further processed using SPE. For the same matrix, a similar approach was followed for combined determination of nine antibiotics and 3 pesticides [25].

Based on the literature cited above, four existing procedures were selected (see Experimental section). Clean up steps based on solid phase extraction were omitted in this work because it was known from literature [23] that certain compounds (e.g. acids) would be removed that way, thereby limiting the scope of the method. In addition to the existing procedures, three alternative extraction methods are proposed here. The proposed methods involved wetting (or dilution) with water (5 ml per 2.5 g of sample) followed by extraction with a water miscible organic solvent, i.e. acetonitrile, methanol or acetone (15 ml). The organic solvent was acidified with 1% of formic acid. This was done because Sulyok et al. [14] showed that acidification was required for adequate extraction of fumonisins. In case of acetonitrile, acidification was also beneficial to prevent degradation of certain analytes [22]. The alternative approaches did not involve a partitioning step, nor any other clean up or concentration steps.

The initial comparison of all seven extraction procedures was done for 172 analytes which included pesticides (basic, neutral and acidic in nature), mycotoxins and plant toxins, spiked to a feed matrix at 0.25 mg/kg (for the list of analytes, see Table S-2 in the Supporting Information). The average recoveries (n=4) and repeatabilities were calculated and evaluated. Recoveries were considered acceptable in the range 70-120% [26]. For each of the seven methods the recoveries for the majority of the analytes were acceptable. This was to be expected since all methods had once been developed to include multiple analytes. However, when evaluating the data in more detail, substantial differences in recoveries were observed for a number of analytes when comparing the seven methods. In Table 1 the average recoveries and repeatability values (expressed as relative standard deviations, RSD) are given for selected analytes for which such differences were observed.

From Table 1 it can be seen that the methods from the field of pesticide residue analysis are more limited with respect to scope than the other methods investigated. This is mainly due to the partitioning step between an aqueous and an organic phase which has always been a typical part of these methods. The reason for this is that at the time of development of pesticide multi-residue methods, pesticides were typically determined by GC which can not handle direct injection of aqueous extracts. Inherent to a partitioning step, the pesticide

methods discriminate against very polar and, depending on the pH, against basic or acidic analytes. With respect to the latter, attempts have been made to solve this by dedicated buffering [11,22] which extended the scope but does not really solve the limitation. For truly generic extraction, we should abandon the concept of partitioning that has always been the core of pesticide multi-methods. Indeed, the number of analytes with low recoveries decreases for the mycotoxin multi-analyte method, which, like the "QuEChERS" method uses acetonitrile as organic solvent but in this case without partitioning. Still, even without losses through a partitioning step, recoveries are low for several analytes, most notably the acids and the ergot alkaloids (which are slightly basic [27]). Apparently, these compounds interact with the matrix. This could be avoided by extracting at low pH. A possible explanation for this could be that at neutral conditions acidic analytes are in their deprotonated state and can interact with (protonated) amino-functionalities on matrix material. Vice versa, basic analytes can interact with deprotonated acidic functionalities on the matrix material. At low pH the acidic groups are protonated and in their neutral state, while basic functionalities are either neutral or protonated (cationic). As a consequence, no or less interactions take place, the analytes do not bind to matrix and remain dissolved in the extract solution. Without partitioning and with acidification, all three organic solvents investigated (acetonitrile, methanol and acetone) showed adequate recoveries for virtually all analytes included in the test. Therefore, it was decided to continue investigations with these three options.

Multi-matrix applicability of selected extraction procedures

A generic method should not only be capable of extracting a wide variety of target compounds from the matrix, but should also be applicable to a variety of matrices. To study this, the three most promising methods from the preceding section were evaluated for extraction of the target analytes from five other commodities (maize flour, honey, milk, meat, egg) with very different compositions (see Table S-1 in the Supporting Information for composition). For all three methods selected, fortifications were done and recoveries determined.

Given the large set of data generated, a detailed discussion of individual analyte/matrix/solvent recoveries was not considered appropriate. Therefore, the discussion is limited to some noteworthy observations and overall results.

For three matrices, consistent low recoveries (<30%) for certain analytes were obtained, irrespective the extraction solvent used. This was case with egg (fluazinam, furathiocarb, methiocarb-sulfoxide and methiocarb-sulfone, pyridate, tolylfluanide), meat (fluazinam, furathiocarb, pyridate, tolylfluanide) and maize (tolylfluanide). Egg, and to a lesser extend meat, differ from the other matrices with respect to their relatively high content of phospholipids, but this could not be related to the low recoveries. Furthermore, from an analytes point of view, no clear common functionality could be found that would allow clarification.

Table 2 gives an overall summary of the recoveries obtained. For maize flour, similar as for feed, acceptable recoveries were obtained for over 90% of the analytes, irrespective the method used. When extracting honey with acetonitrile, an unintended phase partitioning occurred (aqueous syrup:acetonitrile, 5 ml:15 ml). Since the acetonitrile phase was used for further analysis, recoveries were adjusted for the concentration effect (factor 1.33). For meat and egg, methanol was clearly a less favorable extraction solvent than acetonitrile and acetone. For milk, lower recoveries were obtained with acetonitrile. Although better recoveries were obtained with methanol, this was a less practical solvent because turbid

extracts were obtained, most likely due to incomplete precipitation of proteins. Acetone was the most suitable extraction solvent for milk.

From an extraction point of view, acetone was the most favorable solvent. It resulted in favorable recoveries for the highest number of analytes across the matrices investigated, and no undesirable side-effects like phase partitioning or turbid extracts were obtained.

Co-extraction of matrix and signal suppression/enhancement effects in LC-MS/MS
While a wide range of analytes need to be extracted as efficiently as possible, the co-extraction of bulk matrix constituents like fat, proteins and carbohydrates is undesirable. They may reduce the life-time of the (UP)LC column and affect the ionization process in LC-MS analysis causing a suppression or enhancement of analyte response [28]. The latter complicates accurate quantification in quantitative analysis, especially since matrix effects may be sample dependent. In qualitative analysis, suppression results in higher detection limits. Therefore, besides recoveries, such effects were also taken in consideration during evaluation of the generic extraction procedures.

To examine the occurrence of matrix-induced suppression or enhancement in LC-MS/MS analysis, the response of analytes spiked to control extracts was compared with that in solvent standards. Table 3 shows an overview of matrix effects observed for the six matrices and the three extraction procedures found to be most suitable from the recovery experiments. Despite the relatively low amount of matrix equivalent introduced in the LC-MS/MS system (i.e. 5 µl of a 0.125 g/ml extract = 0.625 mg), extensive matrix effects were observed. The worst case was feed when extracted with methanol. Here matrix effects were insignificant for only 14% of the analytes. The response was suppressed by more than a factor of two for over 50% of the analytes and more than a factor of five for 8% of the analytes. The best case was egg when extracted with acetonitrile, here matrix effects were insignificant for 84% of the analytes. Overall, the occurrence of matrix effects decreased for the matrices in the order: feed > maize > meat > milk > egg > honey. For the three extraction solvents, acetonitrile consistently was the most favorable with respect to matrix effects for all six matrices. Comparing methanol and acetone, the results were matrix dependent to some extent, but overall matrix effects were less abundant for acetone.

For two matrices, feed and meat, the amount of co-extracted material was determined gravimetrically by taking a 10 ml aliquot of the clear extract and evaporating the solvent. For feed, the remaining co-extracted material was 107, 237 and 203 mg for acetonitrile, methanol and acetone, respectively. For meat these values were 50, 60 and 58 mg. This corresponded with the trend observed for the matrix effect shown in Table 3.

For selection of the final method, a compromise had to be made between optimum recovery and matrix effect. Acetone was favorable with respect to recovery, but the higher recovery was partly offset by higher suppression of the response which in the end may result in a lower detectability. Therefore, especially for the matrices with extensive matrix effects (feed, maize, meat), acetonitrile, overall, was considered the preferable solvent. For milk and honey, acetone was clearly preferable over acetonitrile in terms of recovery while matrix effects were considered acceptable for both solvents. Therefore, for these two matrices, acetone would be the method of choice. Out of the three solvents considered, methanol was the worst, both from recovery and matrix effect point of view.

If one wants to pursue the approach to have one generic method for all analyte/matrix combinations, the acetonitrile-based method would be the best overall compromise.

Inclusion of veterinary drugs

As pointed out in the introduction, veterinary drugs are a parameter of interest in compound feed (cross-contamination during production of compound feed) and animal products and it would be highly beneficial if these analytes could also be determined using the same method. Based on the wide variety of analytes used in the previous experiments, it was expected that the general observations regarding recovery and matrix effects would also apply for veterinary drugs. To verify this, using the finally selected acetonitrile-based method, recoveries and matrix effects were determined for 86 veterinary drugs from different sub-classes (i.e. benzimidazoles, ionophores, macrolides, nitroimidazoles, NSAIDs, quinolones, sulfonamides, tetracyclines, tranquilizers). Individual recoveries and RSDs are provided in Table S-2a in the Supporting Information, a summary is included in Table 4. In general, the percentage of analytes for which adequate recoveries (70-120%) were obtained was in the same range as with the pesticides/natural toxins. In feed and honey results were slightly worse, in maize and milk slightly better.

A similar trend was observed with respect to matrix effects (Table 4). Feed and maize again were the matrices for which signal suppression was most severe. For meat suppression was less abundant, but a fair comparison with the results for pesticides/natural toxins could not be made because a different meat sample was used.

Validation

During the method comparison, experiments were done at a relatively high level of 0.25 mg/kg. To examine the feasibility of the generic extraction procedure at lower levels, the method was validated for the most complex matrix (compound feed) and one of the 'easier' matrices (honey). Samples were fortified with over 250 analytes (136 pesticides, 36 natural toxins and 86 veterinary drugs) at levels of 0.01, 0.02 and 0.05 mg/kg, in five-fold and analysed by LC-MS/MS. Although it has been demonstrated in many papers before that with MS/MS detection a linear relationship between response and concentration is obtained, this was verified prior to analysis of the extracts. Linearity was examined over the range 1.25-25 ng/ml (corresponding to 0.01-0.20 mg/kg) and confirmed: the regression coefficients were $\geq$ 0.995 and the deviation between actual and calculated concentrations was <20% for most analytes. Since in practice matrix-matched calibration will be required, one-point calibration is more practical than multi-level calibration when dealing with a wide variety of matrices. Therefore, the analytes were quantified using bracketing injections at one concentration level (6.25 ng/ml, corresponding to 0.05 mg/kg) for all three fortification levels. The recoveries and RSDs obtained this way for all individual analytes are included in Table S-2b in the Supporting Information.

To evaluate the suitability for quantitative analysis, different criteria with respect to recovery and repeatability have been established in the different fields of residues and contaminants. Here, the EU criteria from the field of pesticide residue analysis were taken which demands an average recovery (n=5) between 70-120% and a repeatability (RSD) of 20% or less [26]. The number of analytes for which the recovery criterion was met is summarized in Table 5. In this table, the data for all matrices at the 0.25 mg/kg level are also included. Despite the low levels of absolute amount of analyte introduced and the high complexity of the extracts, acceptable recoveries were obtained in the majority of the cases. In honey, this was the case for 77% of all analytes even at the 0.01 mg/kg level. This percentage increased at higher

levels but leveled off to around 86% (caused by the unintended phase partitioning with this matrix when using acetonitrile). For feed, limitations were encountered at the 0.01 mg/kg level, good recoveries were obtained for only 62% of the analytes. This was mainly due to reduced sensitivity (signal suppression) and higher chemical noise (interferences from co-extractants). Results rapidly improved with increasing concentrations. To illustrate differences in analyte response and signal-to-noise for different analyte/matrix combinations, three examples are shown in Figure 1.

The percentage of compounds for which the RSD criterion was met, irrespective the recovery, is presented in Table 6. In most cases the repeatabilities were better than 10%. Not surprisingly, the highest incidence of repeatabilities worse than 20% occurred for the lowest levels in the most complex matrix (feed). Again, this improved rapidly with increasing the concentration levels.

These results demonstrate that the straightforward and generic extraction method, combined with one set of UPLC conditions, is suitable for accurate quantitative analysis of a vast number of analytes from different contaminant classes in various types of products, provided that calibration is performed using standards in the appropriate corresponding matrix.

Covering such a wide scope, the method is highly suited for screening purposes. For this, reliable detectability is the most important aspect (rather then achieving a recovery within a certain range). In a screening approach, one would typically aim for a 95% detection rate. In Figure 2 the number of analytes detected in honey and feed *vs* the concentration level is shown. From the figure it can be derived that the 95% detection rate for honey is reached between 0.02 and 0.05 mg/kg. With the most complex sample (compound feed) this is reached between 0.05 and 0.25 mg/kg. For the analytes evaluated, it can also be seen that there is little difference between the detectability of pesticides and veterinary drugs, but that natural toxins are more difficult to detect.

Outlook to full scan analysis using LC-TOF-MS

The potential of the method is such that targeted data acquisition, as is done with MS/MS detection, will become a limiting factor in the number of analytes that can be determined in one run. Therefore, a screening method would typically involve analysis by full scan mass spectrometric detection techniques such as TOF-MS, as has been pointed out also by others [13]. After analysis, diagnostic accurate mass signals of an unlimited number of analytes of interest can be extracted from the raw data. In Figure 3, as a first example, extracted ion chromatograms of selected residues and contaminants in spiked honey samples are shown. At the 0.05 mg/kg level, good signal-to-noise ratios were obtained and the mass accuracy was in the range 1.2-4.2 ppm. This demonstrates that the generic extraction method proposed in this work is compatible with UPLC-TOF-MS and that this combination has high potential as a generic screening tool in residue and contaminant analysis.

CONCLUSIONS

The simultaneous determination of pesticides, mycotoxins, plant toxins and veterinary drugs in a variety of matrices using one extraction procedure combined with liquid chromatography with mass spectrometric detection has been demonstrated for the first time. Three new extraction procedures proposed were shown to be more generic than those commonly used in the field of pesticides and mycotoxins. Avoiding phase separation and the use of acidic conditions were the key factors for high extraction efficiencies for a wide variety of analytes. The sample preparation procedure is very straightforward and does not involve any clean up.

As a consequence, matrix effects can be abundant, especially for complex samples like compound feeds. This was minimized by injecting small volumes of extracts containing low amounts of matrix equivalent. Overall, extraction with water/acetonitrile/1% formic acid was found to be the default method of choice. For milk and honey, however, water/acetone/1% formic acid was more suited. Despite the simplicity of the procedure and the inherent complex extracts obtained, good quantitative results could be generated for the vast majority of the analyte/matrix combinations using UPLC-MS/MS analysis. The limits of detection were between < 0.01 and 0.05 mg/kg and in most cases low enough to verify compliance of products with the legal tolerances.

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SUPPORTING INFORMATION AVAILABLE
Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org

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Figure legends

Figure 1. Extracted ion chromatograms obtained after UPLC-MS/MS analysis of honey (H) and compound feed (F) samples spiked at 0.01 mg/kg (acetonitrile-based method. Concentration in extract is 1.25 ng/ml; 6.25 pg on-column. (a) azoxystrobin, (b) ethiofencarb, (c) fenhexamide.

Figure 2: The number of analytes that can be detected using the proposed acetonitrile-based method with UPLC-MS/MS analysis. The 100% bar is representing the total validation set: 136 pesticides, 36 natural toxins, 86 veterinary drugs

Figure 3. Example extracted ion chromatograms obtained after UPLC-TOF-MS analysis of a honey sample spiked at 0.05 mg/kg (acetonitrile-based method). Mass window used: accurate mass ± 0.025 Da. VD = veterinary drug, PES = pesticide, PT = plant toxin.

Table 1. Recoveries of selected target analytes in feed matrix using 7 extraction methods

		Generic method:	pesticide multi-methods[1]			mycotoxin multi-method	proposed alternatives [2]		
			Luke	EtOAc	Quechers[3]	MeCN/H_2O	MeCN	MeOH	ACE
	type	Analyte (pKa)	Average recovery (%) (RSD%) (n = 4)						
acids	P	Asulam (4.8)	71 (4)	**64** (4)	73 (1)	85 (2)	81 (3)	85 (4)	90 (2)
	P	2,4-D (2.7)	80 (6)	**67** (6)	**56** (5)	70 (5)	89 (5)	82 (7)	96 (5)
	P	Fluroxypyr (2.9)	86 (12)	74 (12)	**57 (22)**	**55** (20)	79 (19)	84 (8)	99 (23)
	MT	Fumonisin B1 (?)	**< 5**	**< 5**	**< 5**	**10 (46)**	90 (6)	89 **(22)**	**57** (14)
	MT	Fumonisin B2 (?)	**< 5**	**20** (14)	**< 5**	**15** (18)	95 (4)	100 (5)	89 (6)
	MT	Fumonisin B3 (?)	**< 5**	**13** (7)	**< 5**	**15** (9)	103 (3)	92 (1)	96 (6)
	MT	Ochratoxin A (4.4; 7.3)	93 (3)	75 (5)	**31** (6)	77 (3)	91 (4)	82 (3)	96 (3)
	P	Quinmerac (4.3)	**26** (4)	**25** (11)	**36** (3)	**49** (4)	87 (3)	88 (2)	95 (5)
	P	Sulcotrione (3.1)	70 (2)	**67** (8)	**67** (5)	77 (5)	90 (6)	91 (2)	99 (4)
neutrals	P	Acephate	**62** (5)	83 (7)	84 (2)	83 (4)	91 (8)	92 (2)	85 (2)
	MT	Aflatoxin B1	110 (3)	91 (4)	**67** (3)	94 (2)	85 (4)	75 (4)	96 (4)
	P	Aldicarb-sulfoxide	**69** (5)	89 (4)	88 (1)	93 (1)	88 (3)	96 (4)	94 (1)
	P	Clofentezine	**61** (14)	**43** (15)	**63 (25)**	102 (8)	86 (15)	**69** (12)	75 (13)
	P	Dodine	90 (17)	**32 (40)**	**61** (16)	97 (10)	93 (11)	115 (7)	99)12)
	P	Ethiofencarb	**136 (34)**	**37** (18)	**56** (7)	87 (14)	72 (14)	69 (4)	140 (15)
	P	Methamidophos	**56** (15)	70 (7)	86 (5)	90 (5)	79 (9)	107 (6)	97 (15)
	P	Oxamyl	71 (5)	86 (5)	73 (15)	103 (12)	79 (14)	94 (8)	**47 (44)**
	PT	Retrorsine-N-oxide	**5** (18)	**17** (8)	**57** (3)	83 (8)	82 (5)	88 (3)	93 (3)
	PT	Senecionin-N-oxide	**20** (4)	**48** (10)	73 (4)	83 (5)	81 (6)	84 (3)	90 (10)
	PT	Seneciphylline-N-oxide	**18** (9)	**43** (7)	77 (4)	81 (2)	78 (2)	88 (3)	88 (4)
	P	Thiofanate-methyl	98 **(41)**	**13 (97)**	**24** (14)	**68** (16)	**55 (22)**	**46** (10)	94 (19)
bases	PT	Chaconine-alpha (6.7)	**6** (24)	**< 5**	**54** (7)	**65** (7)	80 (7)	104 (10)	111 (6)
	MT	Ergocornine (5.5-6)	74 (5)	**61** (5)	**22** (3)	**47** (6)	75 (4)	72 (5)	92 (3)
	MT	Ergocristine (5.5)	77 (8)	**52** (15)	**22** (10)	**45** (4)	73 (5)	80 (5)	92 (2)
	MT	Ergocryptine (5.5-6)	77 (6)	**60** (6)	**25** (9)	**50** (9)	**58 (65)**	79 (3)	92 (1)
	MT	Ergotamine (5.5-6)	89 (8)	**63** (4)	**44** (7)	**59** (8)	87 (6)	81 (7)	97 (6)
	PT	Heliotrine (9-10.5)	**22** (5)	**45** (6)	82 (2)	93 (2)	86 (3)	91 (3)	105 (3)
	PT	Monocrotaline (9-10.5)	**12** (5)	**35** (4)	75 (2)	80 (3)	83 (4)	98 (2)	99 (4)
	P/PT	Nicotine (3.1;8.2)	**20** (17)	**64** (10)	75 (2)	76 (6)	79 (5)	94 (4)	78 (8)
	P	Propamocarb (9.5)	**17** (3)	**32** (4)	78 (2)	90 (3)	88 (2)	92 (1)	94 (1)
	P	Pymetrozine (4.1)	**68** (13)	**61** (5)	71 (10)	85 (7)	108 (17)	99 (11)	80 (16)
	PT	Retrorsine (9-10.5)	**22** (9)	**47** (6)	76 (2)	86 (7)	89 (2)	99 (4)	97 (4)
	PT	Solanine-alpha (6.7)	**< 5**	**< 5**	**41** (9)	**65** (7)	81 (4)	95 (7)	95 (4)
	PT	Senkirkine (9-10.5)	**40** (4)	70 (6)	81 (3)	91 (1)	89 (1)	96 (4)	92 (2)

[1] after wetting of sample with water
[2] extraction of 2.5 g sample with 5 ml water and 15 ml organic solvent containing 1% of formic acid; MeCN = acetonitrile, MeOH = methanol, ACE = acetone
[3] QuEChERS method without d-SPE clean up
MT = mycotoxin; P = pesticide; PT = plant toxin
Figures in bold: recoveries outside range 70-120% or RSD >20%

Table 2. Comparison of recoveries for three proposed extraction methods for 172 pesticides and natural toxins in six matrices

		Recovery range				
		<30%	30-50%	50-70%	**70-120%**	>120%
Matrix	Extraction[1]	% of 172 analytes in recovery range				
Feed	MeCN	0	0	4	**96**	1
	MeOH	0	1	4	**95**	1
	ACE	0	1	2	**94**	4
Maize	MeCN	1	0	3	**92**	4
	MeOH	1	1	4	**92**	3
	ACE	1	0	2	**93**	4
Honey	MeCN[2]	0	1	8	**90**	1
	MeOH	1	0	2	**96**	1
	ACE	0	0	0	**99**	1
Meat	MeCN	2	0	5	**90**	2
	MeOH	2	2	21	**74**	1
	ACE	2	0	1	**93**	3
Egg	MeCN	4	1	4	**91**	1
	MeOH	4	2	17	**75**	2
	ACE	4	1	2	**92**	2
Milk	MeCN	1	9	17	**66**	7
	MeOH	0	2	14	**84**	1
	ACE	0	1	7	**89**	4

[1] extraction of 2.5 g sample, with 5 ml water and 15 ml organic solvent containing 1% of formic acid; MeCN = acetonitrile, MeOH = methanol, ACE = acetone.
[2] taking phase partitioning into account (see text).

Table 3. Comparison of matrix effects observed using three proposed extraction methods for 172 pesticides and natural toxins in six matrices

		Matrix effects[1]				
		signal suppression		**not significant**	signal enhancement	
		>50%	20-50%	**80-120%**	20-50%	>50%
Matrix	Extraction[2]	% of 172 analytes				
Feed	MeCN	30	39	**23**	4	4
	MeOH	56	26	**14**	2	2
	ACE	39	32	**12**	9	8
Maize	MeCN	15	20	**35**	9	22
	MeOH	17	16	**27**	14	25
	ACE	16	17	**27**	12	27
Honey	MeCN	0	9	**79**	11	1
	MeOH	0	4	**68**	23	5
	ACE	1	6	**73**	16	6
Meat	MeCN	7	15	**56**	14	9
	MeOH	9	20	**46**	10	15
	ACE	11	43	**36**	6	4
Egg	MeCN	1	6	**84**	6	3
	MeOH	2	11	**67**	14	6
	ACE	4	14	**75**	6	1
Milk	MeCN	2	16	**70**	10	2
	MeOH	18	35	**29**	8	9
	ACE	1	11	**57**	27	4

[1] response of standard prepared in final extract *vs* response in solvent standard, analysed using UPLC-MS/MS.

[2] extraction method: see legend Table 2.

Table 4. Recovery and matrix effects for veterinary drugs in six matrices using the proposed acetonitrile-based extraction method

	Recovery (average, n = 4)				
	<30%	30-50%	50-70%	70-120%	>120%
Matrix	% of 86 veterinary drugs				
Feed	0	3	14	84	1
Maize	0	0	2	98	0
Honey [1]	1	3	17	80	2
Meat	1	5	10	90	0
Egg	1	3	9	87	3
Milk	0	5	19	74	7

	Matrix effects				
	signal suppression		**not significant**	signal enhancement	
	>50%	20-50%	**80-120%**	20-50%	>50%
Matrix	% of 86 veterinary drugs				
Feed	33	19	**31**	6	12
Maize	7	17	**40**	22	14
Honey	0	2	**67**	24	6
Meat	2	2	**92**	2	1
Egg	1	0	**79**	16	3
Milk	0	5	**85**	6	5

[1] taking phase partitioning into account

Table 5. Summary of validation results: percentage of analytes with acceptable recoveries.

Matrix	level mg/kg	pesticides (136)	natural toxins (36)	veterinary drugs (86)	**all (258) analytes**
		% of average recoveries within 70-120% (n=5) [1]			
Honey	0.01	88	50	72	**77**
	0.02	93	61	87	**87**
	0.05	89	61	80	**82**
	0.25	93	72	84	**86**
	0.25 [2]	99	97		
Compound feed	0.01	66	39	66	**62**
	0.02	81	75	83	**81**
	0.05	91	83	90	**90**
	0.25	96	83	87	**91**
Maize	0.25	94	86	98	**93**
Meat	0.25	92	79	94	**90**
Egg	0.25	88	97	91	**89**
Milk	0.25	65	73	79	**70**
	0.25 [2]	88	91		

[1] Using proposed acetonitrile-based extraction method
[2] Using proposed acetone-based extraction method
Between brackets: number of analytes

Table 6. Summary of validation results: repeatability

Matrix	level mg/kg	Repeatability [1,2] (RSD%) n=5				
		< 5%	5-10%	10-15%	15-20%	> 20%
		% of all 258 pesticides, natural toxins, veterinary drugs				
Honey	0.01	18	50	14	8	10
	0.02	13	58	18	5	6
	0.05	44	43	6	3	4
	0.25	59	35	4	2	0
Compound feed	0.01	10	39	23	10	17
	0.02	12	48	17	10	13
	0.05	21	51	15	6	7
	0.25	73	19	3	2	3
Maize	0.25	71	20	4	2	3
Meat	0.25	51	33	11	2	4
Egg	0.25	60	30	8	0	2
Milk	0.25	39	39	15	3	4

[1] using proposed acetonitrile-based extraction method
[2] including all analytes for which a response was obtained (i.e. irrespective the recovery)

Figure 1.

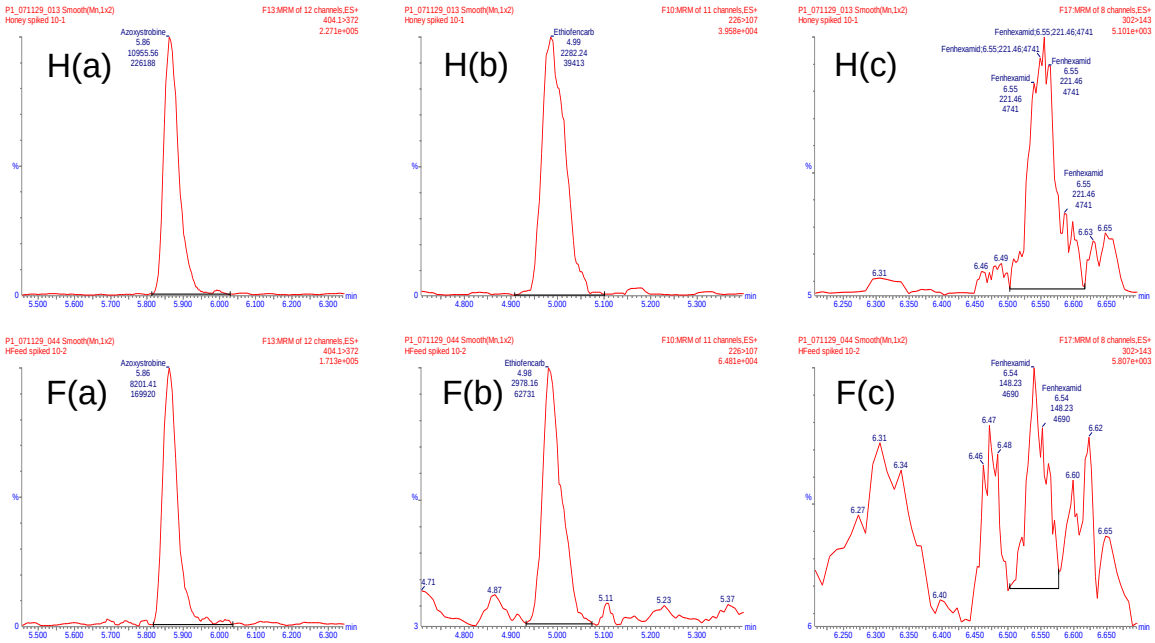


Figure 2.

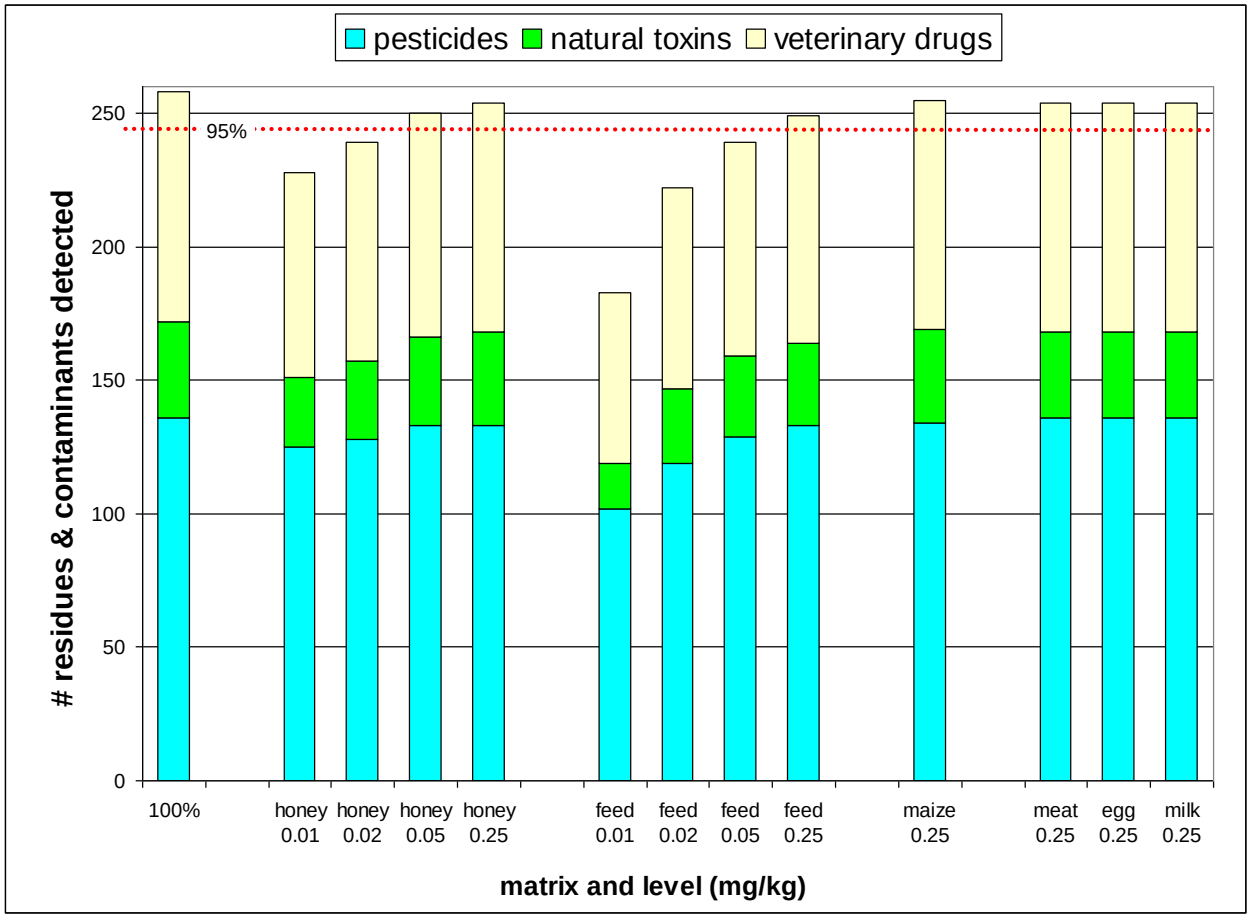
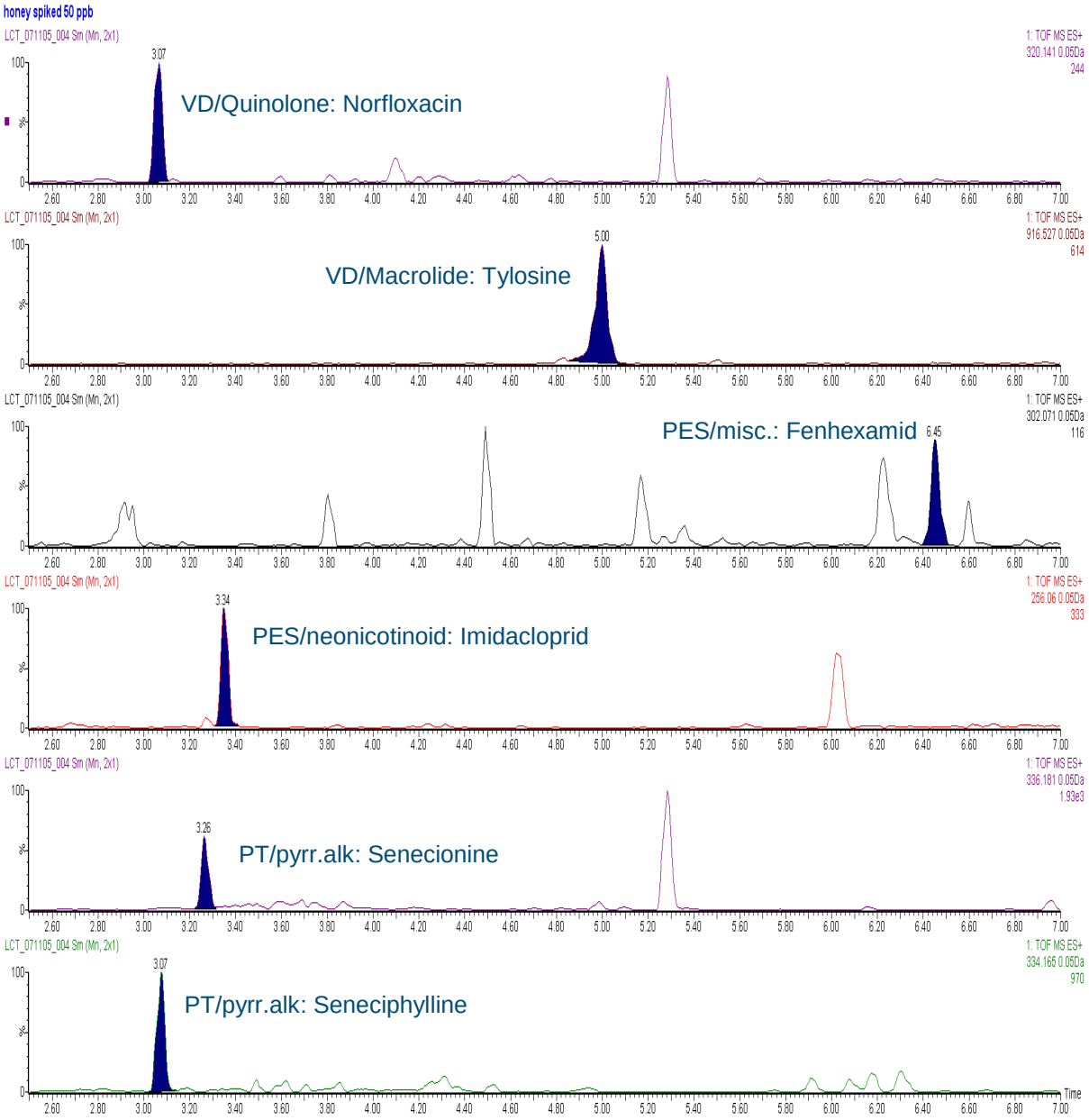

pesticides
natural toxins
veterinary drugs
residues & contaminants detected
250
200
150
100
50
0
95%
100%
honey 0.01
honey 0.02
honey 0.05
honey 0.25
feed 0.01
feed 0.02
feed 0.05
feed 0.25
maize 0.25
meat 0.25
egg 0.25
milk 0.25
matrix and level (mg/kg)

Figure 3.


honey spiked 50 ppb
LCT_071105_004 Sm (Mn, 2x1)
1: TOF MS ES+
320.141 0.05Da
244
3.07
VD/Quinolone: Norfloxacin
LCT_071105_004 Sm (Mn, 2x1)
1: TOF MS ES+
916.527 0.05Da
614
5.00
VD/Macrolide: Tylosine
LCT_071105_004 Sm (Mn, 2x1)
1: TOF MS ES+
302.071 0.05Da
116
PES/misc.: Fenhexamid
6.45
LCT_071105_004 Sm (Mn, 2x1)
1: TOF MS ES+
256.06 0.05Da
333
3.34
PES/neonicotinoid: Imidacloprid
LCT_071105_004 Sm (Mn, 2x1)
1: TOF MS ES+
336.181 0.05Da
1.93e3
3.26
PT/pyrr.alk: Senecionine
LCT_071105_004 Sm (Mn, 2x1)
1: TOF MS ES+
334.165 0.05Da
970
3.07
PT/pyrr.alk: Seneciphylline
Time