Journal of Chromatography A, 1594 (2019) 93–104

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Method validation and application of a selective multiresidue analysis of highly polar pesticides in food matrices using hydrophilic interaction liquid chromatography and mass spectrometry

Sonia Herrera López*, Jos Scholten, Barbara Kiedrowska, André de Kok

NVWA - Netherlands Food and Consumer Product Safety Authority, National Reference Laboratory for Pesticide Residues in Food and Feed, Akkermaalsbos 4, 6708 WB Wageningen, The Netherlands

ARTICLE INFO

Article history:
Received 13 November 2018
Received in revised form 6 February 2019
Accepted 11 February 2019
Available online 11 February 2019

Keywords:
Highly polar pesticides
Glyphosate
HILIC
Mass spectrometry
Selective
Multiresidue analysis

ABSTRACT

The effectiveness of highly polar pesticides in agriculture is well known, while their low costs contribute to the frequent use. On the other hand, their physicochemical properties make their analytical determination a challenging task. The aim of this study is the evaluation of a methanol-based extraction method with a simple clean-up step using a selective multiresidue LC–MS/MS method for 14 highly polar pesticides and their metabolites. For the clean-up step, several sorbents from different brands, with diverse mechanisms of action, were tested. Different dilution factors for the final extract were also evaluated in order to check the impact on the matrix effects. The optimised method was validated for matrices from different commodity groups. Recovery studies performed with grapes, lettuce, orange, oat and soya beans showed absolute average recoveries in the range 70–120% with relative standard deviation values below 20% for almost all the pesticides tested. The matrix effects observed were very different in each matrix and for each individual pesticide evaluated. Therefore, isotopically labeled procedural internal standards were used for all compounds in order to correct for recovery and matrix effects. Method Limits of Quantification for most analyte-matrix combinations were 0.02 or 0.05 mg kg^{-1}. The final optimised method appeared to be reproducible and robust in routine analysis of a wide variety of fruits, vegetables and cereals. Monitoring results are presented to show the occurrence of the compounds studied in real samples. The residue concentrations ranged from 0.023 to 30 mg kg^{-1} for the analytes detected.

1. Introduction

Pesticides form a large group of substances widely used in agriculture to protect crops from pests. Although their use has increased the food production, which is necessary to feed the increasing population, millions of agricultural workers experience unintentional pesticide poisonings or exposure each year. Programs to control the exposure, especially in developing countries, are limited and as a consequence unintentional contamination can occur [1–4]. In developed countries, the programs to control the exposure and the presence of pesticides residues in food and feed are well established and work well. In the European Union, Multi-Annual Control Programmes for pesticide residues (MACP) are carried out by all Member States to guarantee compliance with maximum residue levels (MRLs) of pesticides and to evaluate the consumer exposure to pesticide residues in food of plant and animal origin [5]. Although for the majority of pesticides MRLs are well defined, there still exists a group of pesticides for which their metabolites are not yet included in the residue definition. For example, the residue definition of glyphosate and its metabolites is a good example, as the residue definition is under re-evaluation [6].

There is a wide range of pesticides that can be analysed using multi-residue methods. Those methods usually cover hundreds of pesticides and they are mainly based on extraction with acetonitrile (as quick, easy, cheap, effective, rugged, and safe [QuEChERS] method) [7], ethyl acetate (Swedish Ethyl acetate [SweEt] method) [8], and acetone (Netherlands Luke [NL]-method) [9], followed by Liquid chromatography (LC) and Gas chromatography (GC) analysis. However, there is a group of pesticides with particular physical-chemical properties, which need different approaches (single residue methods) to be determined [10–12]. Non-selective highly polar herbicides, including glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), have been analysed predominantly using LC methods and a pre-column derivatisation

* Corresponding author.
E-mail address: s.herreralopez@nvwa.nl (S. Herrera López).

https://doi.org/10.1016/j.chroma.2019.02.024
0021-9673/

step with 9-fluorenylmethyl chloroformate (FMOC-Cl) [13–15] or post-column derivatisation [16] with o-phthalaldehyde (OPA) and mercaptoethanol. One important limitation of the FMOC derivatisation step used in pesticides analysis is the uncertainty of the derivatisation efficiency [14]. The scope of these methods, using FMOC, is limited to compounds susceptible to be derivatised. "N-acetyl" metabolites are a good example of compounds which are difficult to derivatise. Another disadvantage is the relatively long time needed for such a procedure, which is also less simple. Recently, several alternative methodologies have been proposed for direct and sensitive analysis of polar compounds. Ion chromatography (IC) was successfully used in studies for different kind of matrices: cereals, fruits and vegetables [11,17]. Three critical points of such an approach are: i) the need for additional instrumentation in the laboratory, ii) poor analytical performance obtained for the glyphosate metabolite, AMPA; iii) significant differences in retention times between matrices for these compounds [11,13]. Polar pesticides have been analysed also using graphitised carbon (Hypercarb) and hydrophilic interaction liquid chromatography (HILIC) columns. A Hypercarb column was tested by Domingos et al. for soya products [18] analysing four polar pesticides: fosetyl, maleic hydrazide, chlorate and perchlorate. The main drawback of these columns is the need for frequent reconditioning during analysis, which is time consuming and increases overall costs of analysis. Different HILIC columns were tested for ethephon, glufosinate, glyphosate, N-acetyl-glufosinate, AMPA and 3-methylphosphinicopropionic acid (MPPA) by Vass et al. [12]. The Obelisc N and Obelisc R columns, with unique mixed-mode stationary phases (HILIC/ Weak Anion-eXchange (WAX)), from SIELC company, were also included in that study. Using the Obelisc N column, a successful separation of polar pesticides was achieved, but poor retention time stability and deterioration of the column caused by matrix components was observed, as was also mentioned by Botero et al. [19] in their work. From the latest results published with the Obelisc N column, it is possible to conclude that this column is performing well in terms of separation and retention, but it is deteriorating in a short time period, which discourages its routine use [14,20,21].

In this study, a methanol-based extraction method followed by a LC–MS/MS analytical method using an Obelisc N HILIC column was developed and validated for 14 highly polar pesticides (parents and metabolites) in five food matrices, according to the European Union (EU) SANTE Guideline [22]. In this method, no derivatisation step was required for detection of glyphosate and the other target compounds. Good retention time stability was observed for all the evaluated pesticides. The column life time could be increased dramatically by optimizing the final dilution factor of the sample extracts and the composition of injection solvent and mobile phase solvents. Isotopically labeled internal standards were used for recovery and matrix effect correction. The validated method showed good robustness. Therefore, this method was successfully implemented in the routine laboratory of the National Reference Laboratory for Pesticides Residues in Food and Feed in The Netherlands.

2. Experimental

2.1. Chemicals and reagents

Analytical-grade standards of pesticides used in this study (in total, 7 pesticides and 7 degradation products) of high purity (>98%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany) except, the metabolites of glufosinate and glyphosate from Toronto Research Chemicals (TRC, North York, Canada), AMPA from Sigma–Aldrich (Steinheim, Germany) and bromide, chlorate and perchlorate from Inorganic ventures (Christiansburg, Virginia). Isotope–labeled internal standards, AMPA ^{13}C ^{15}N, ethephon D_4, fosetyl–aluminium D_{15}, 2-hydroxyethyl phosphonic acid (HEPA) D_4, glufosinate D_3 hydrochloride, N-acetyl-glufosinate D_3, 3-methylphosphinicopropionic acid (MPPA) D_3, glyphosate 1,2-$^{13}C_2$, ^{15}N, N-acetyl-glyphosate D_3, were purchased from Dr. Ehrenstorfer and from Toronto Research Chemicals. Phosphonic acid- $^{18}O_3$, $^{18}O_3$-chlorate, $^{18}O_4$-perchlorate were supplied by the EURL-SRM in Stuttgart, Germany. For the optimisation of operational conditions (ion-source-dependent parameters, and for MS/MS operation), working solutions of individual standards were prepared in methanol or water at 100 ng mL^{-1}. For the linearity study, working solutions of a mixture of the standards were prepared at different concentration levels in MeOH:H_2O (50:50, v/v) and these were kept at 4 °C. UHPLC-grade acetonitrile and LC-grade methanol were supplied by Biosolve (Dieuze, France) and Merck (Darmstadt, Germany), respectively. HPLC-grade water from a Water Purification System of Millipore (Burlington, MA, United States) was used. Formic acid (≥ 99%) was purchased from VWR (Lutterworth, United Kingdom) and trifluoroacetic acid (TFA) (≥ 99%) from Merck.

2.2. Sample preparation and extraction procedure

The validation study was carried out with blank samples of different commodity groups: grapes, orange, lettuce, oat and soya beans. These were purchased randomly in a local store for biological products, in Wageningen, The Netherlands. The samples were homogenised and then stored at −20 °C until spiking and sample extraction. Samples were prepared and processed according to the SANTE Guideline for pesticide residues analysis in food and feed [22]. Blank samples were examined to confirm absence of the target pesticide residues and used to develop and validate the proposed method. The methanol-based extraction method was validated according to the SANTE Guideline [22]. Internal standards were used for all compounds for recovery and matrix effect correction. For N-acetyl-AMPA and bromide, isotopically labeled standards were not commercially available, therefore the same internal standards as for ethephon and chlorate, respectively, were used. In the final step of the sample preparation, the extract was filtered using a 0.2 μm polyvinylidene fluoride (PVDF) syringe filter from Pall Corporation (New York, United States) and then diluted with a mixture of ACN-H_2O (60:40), containing 0.2% trifluoroacetic acid (TFA).

2.2.1. Procedure for fruits and vegetables

5.0 g of sample were weighed in 50-mL polypropylene centrifuge tubes and fortified at 0.25 mg kg^{-1} with a isotopically labeled internal standard (ILIS) solution of 10 μg mL^{-1}. Then 5 mL of ultra-pure water and 10 mL of MeOH with 1% of formic acid were added. The tubes were shaken in an automatic axial shaker (AGYTAX®, Cirta Lab S.L., Madrid, Spain) during 5 min. The extracts were centrifuged at 4150 R.C.F. for 10 min at 10 °C and filtered, as mentioned above, and an aliquot was finally diluted 12.5 times prior to injection into the LC–MS/MS system. The final matrix concentration is thus 0.02 g mL^{-1}.

2.2.2. Procedure for cereals

2.0 g of sample were weighed in 50-mL polypropylene centrifuge tubes and initially wetted with 10 mL of high-performance liquid chromatography (HPLC)-grade water for 30 min at room temperature. Thereafter, the samples were fortified at 0.5 mg kg^{-1} with ILIS solution of 10 μg mL^{-1}, and then 10 mL of MeOH with 1% of formic acid were added. The tubes were shaken in an automatic axial shaker during 5 min. The extracts were stored in the freezer at −18 °C during 3 h. The extracts were centrifuged at 4150 R.C.F. for

Table 1
Optimised LC–MS/MS (MRM) parameters for target compounds.

Compound	Q1 Mass (Da)	Q3 Mass (Da)	Ion Ratio (solvent)	DP (volts)	CE (volts)
AMPA-Quant	110	63		−30	−25
AMPA-Qual	110	79	0.70	−30	−32
AMPA-IS	112	63		−30	−25
Bromide-Quant	81	81		−30	−80
Bromide-Qual	79	79	0.85	−30	−80
Chlorate-Quant	82.9	66.9		−60	−30
Chlorate-Qual	84.9	68.9	0.37	−60	−30
Chlorate-IS	88.9	70.9		−60	−30
Ethephon-Quant	143	107		−45	−12
Ethephon-Qual1	145	107	0.35	−45	−12
Ethephon-Qual2	143	79	0.52	−45	−25
Ethephon-IS1	147	79		−30	−25
Ethephon-IS2	147	111		−30	−12
Fosetyl-Quant	109	81		−30	−18
Fosetyl-Qual	109	63	0.37	−30	−32
Fosetyl-IS	114	82		−30	−18
Glufosinate-Quant	180	95		−60	−24
Glufosinate-Qual1	180	85	0.84	−60	−24
Glufosinate-Qual2	180	63	0.60	−60	−38
Glufosinate-IS	183	63		−30	−38
Glyphosate-Quant	168	63		−30	−35
Glyphosate-Qual1	168	81	0.65	−30	−20
Glyphosate-Qual2	168	79	0.52	−30	−45
Glyphosate-Qual3	168	150	1.24	−30	−15
Glyphosate-IS	171	63		−30	−35
HEPA-Quant	125	95		−40	−18
HEPA-Qual1	125	107	0.18	−40	−15
HEPA-Qual2	125	79	2.51	−40	−28
HEPA-IS	129	79		−30	−28
MPPA-Quant	151	63		−10	−58
MPPA-Qual1	151	78	0.49	−10	−28
MPPA-Qual2	151	107	1.63	−10	−22
MPPA-IS	154	136		−30	−20
MPPA-IS2	154	110		−30	−20
N-acetyl-AMPA-Quant	151.9	110		−30	−17
N-acetyl-AMPA-Qual1	151.9	63	0.62	−30	−27
N-acetyl-AMPA-Qual2	151.9	134	0.30	−30	−16
N-acetyl-glufosinate-Quant	222	136		−30	−28
N-acetyl-glufosinate-Qual	222	63	0.45	−30	−80
N-acetyl-glufosinate-IS	225	63		−30	−80
N-acetyl-glyphosate-Quant	210	150		−30	−16
N-acetyl-glyphosate-Qual1	210	63.1	0.57	−30	−40
N-acetyl-glyphosate-Qual2	210	124.1	0.88	−30	−25
N-acetyl-glyphosate-IS	213.1	63.1		−30	−40
N-acetyl-glyphosate-IS2	213.1	125.2		−30	−25
Perchlorate-Quant	98.9	82.9		−85	−36
Perchlorate-Qual	98.9	66.9	0.16	−85	−50
Perchlorate-IS	106.8	89		−85	−38
Phosphonic acid-Quant	81	79		−30	−22
Phosphonic acid-Qual	81	63	0.35	−30	−34
Phosphonic acid-IS	87	85		−30	−22

10 min, filtered, and an aliquot was finally diluted 10 times prior to injection into the LC–MS/MS system. The final matrix concentration is thus 0.01 g mL^{-1}.

2.3. LC-ESI-QTRAP-MS analysis

A hybrid quadrupole/linear ion trap mass spectrometer system (6500 + QTRAP, Sciex Instruments, Concord, Ontario, Canada) with an electrospray ion (ESI) source, coupled to a LC-system (Shimadzu, Kyoto, Japan), consisting of two Nexera X2 LC-30AD LC-pumps and a SIL-30AC autosampler, was used for method development and validation. The LC analysis was performed with a HILIC-column, Obelisc N (5 μm, 100 Å; L = 150 mm, ID = 2.1 mm), supplied by SIELC (Wheeling, Illinois, USA). The sample extracts in the autosampler were kept at 15 °C. The column temperature was maintained at 35 °C. The LC runs were performed with mobile phase A, water with 1% formic acid, and mobile phase B, ACN. The gradient used, ranged from 80 to 20% of mobile phase B in 1 min. Then the 20% of B mobile phase was kept during 11 min. Finally, the mobile phase was programmed to the initial condition (80% of B) in 0.2 min, which was kept for 2.8 min, to condition the column for the next injection. The total chromatographic run time (injection-to-injection) was thus 15 min. The injection volume was 15 μL and the operational flow rate was set at 500 μL min^{-1}. Fig. S1 in the supplementary material shows the chromatographic peaks of the studied pesticides at 5 ng mL^{-1}. The ionisation source settings were: ion spray voltage (IS), −4000 V; temperature, 550 °C; curtain gas flow, 25 L min^{-1}; collision gas, medium; ion source gases, at a pressure of 40 and 70 psi, respectively and negative ionisation mode. Nitrogen was used as the nebuliser gas, curtain gas and collision gas.

The LC-ESI-QTRAP-MS system was used in the triple-quad mode and operated in the MRM (multiple reaction monitoring) mode, with a unit mass resolution set for Q1 and Q3. Declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP), were optimised using flow injection analysis (FIA). Optimal MS/MS parameters values are shown in Table 1. Identification was based on the criteria set in the EU SANTE document [22]: acquisition of at least two selected reaction monitoring

(SRM) transitions, retention time tolerance of ± 0.1 min and multiple reaction monitoring (MRM) ratio of product ions response (MRM_2/MRM_1) with a tolerance of ±30%, taking the retention time and response ratio of the standard in solvent as the reference value. Whenever possible, an extra SRM transition was optimised, to be used for the full identification in case of interference of the main qualifier ion by some specific matrix components. The ion ratios of the qualifier and quantifiers ions (for the standards in solvent) are given in Table 1. Analyst software version 1.6.3 was used for data acquisition and MultiQuant version 3.0.1 for data processing.

2.4. Method validation

The method was validated for five matrices (grapes, lettuce, orange, oat and soya beans) representing four different commodity groups, as stated in the EU SANTE document [22]. The method validation performance criteria were evaluated by assessing the extraction efficiency, linearity of the calibration curve, matrix effects, trueness (as % recovery), precision (as repeatability % RSDs), method quantitation limits (LOQ_m), following the EU SANTE Guideline on analytical quality control and validation procedures [22]. Spiked extracts of blank matrices of grape, lettuce, orange, oat and soya beans were used to validate the selective multiresidue method.

The linearity of the calibration curve (determination coefficient, r^2) and the instrument detection limits (LOD_i) for both standards in solvent and standards in matrix have been determined, via injecting standard solutions at seven concentration levels: 0.1, 0.5, 1, 5, 10, 50, 100 ng mL^{-1} with six replicate injections for each calibration level.

Matrix effects were assessed by comparison of the slopes of the calibration curves of matrix-matched standards with the slopes of the calibration curves of standards in solvent. Matrix effects (ME) were calculated using the following equation:

$$\mathrm{ME} = \left(\frac{\text{slope of calibration curve standard in matrix}}{\text{slope of calibration curve standard in solvent}} \right) \times 100\,(\%)$$

The accuracy (trueness and precision) of the method under repeatability conditions was determined by analysing recovery samples at 4 different spike levels, 20, 50, 100 and 500 μg kg^{-1} with 6 replicates (n = 6) for each spike level. The trueness (as % recovery, n = 6) and precision (as % RSDr, n = 6) of the recovery experiments were determined. For validation purposes, recoveries were calculated without and with using the ILIS for quantitation, so that the absolute recoveries are clearly visible.

The method LOQ has been defined as the lowest spike level of the recovery study that can be quantified with acceptable trueness (recovery, 70–120%) and precision (RSD < 20%), as described in the EU SANTE document [22]. The reporting limit (RL) is defined as the lowest level at which residues can be reported as absolute number. It is equal to, or higher than the LOQ. Internal standards were added at the beginning of the extraction step, in order to correct for matrix effects and recovery losses.

Reproducibility (inter-day) data will be obtained via the ongoing validation of the method by collection of recovery and RSD_R data from the QC-samples in routine analysis series, as stated in the SANTE document [22].

2.5. Proficiency test and real samples analysis

The methodology described above was applied to samples from different proficiency tests (PT), EUPT-SRM12 (strawberry puree), FAPAS PT-09109 (oat) and EUPT-SRM13 (soya beans) and Z-score values for the results were determined. The method was also evaluated analysing samples from routine series of the Dutch monitoring programme (e.g. grapes, orange, pear, peach, mushrooms, pepper, mango, apples, mandarin, and broccoli).

2.6. Optimised procedure for routine analysis

For the routine analysis, the quantification was performed according to the isotope-labeled internal standard calibration method with standards in solvent. For the quantification, the ratio of the peak areas of the MS / MS quantification transition of the pesticides and the isotope-labeled standards were used. At least two calibration standard sets were injected into each series (at the beginning and end). In the case of a long series, the samples were split into two parts, preceded and followed by a calibration standard set (see Table 1S in the supplementary material, a template for one batch of real samples in a routine analysis).

The linearity of the calibration line is checked by back calculating of the calibration standard with the calibration curve, using the criterion of a maximum deviation of 20%.

For the QC in each series, the internal standards are checked in the standards and in the samples. The recovery samples of the analytes must be between 80–120%. The matrix effect is also calculated by comparing the detector response of the analytes in matrix extract and in solvent at the same concentration level.

3. Results and discussion

3.1. Liquid chromatography optimisation. Mobile phases selection

The first conditions that we tested for the 14 target compounds with a very high polarity and wide range of physicochemical properties (see Table 2S) were those recommended by the European Union reference laboratory for single-residue methods, (EURL-SRM) in Stuttgart (Germany) in the QuPPe Method [23]. The various EURL-SRM methods contain up to ten of the fourteen target compounds that we intended to include in our method. In the EURL-SRM method, a Hypercarb column and acetic acid as modifier in the mobile phase were used. Our experience with this column/mobile phase combination was not satisfactory in terms of reproducibility and sensitivity. In addition, we could not get all the compounds in the same chromatographic run with those conditions. Due to the notorious lack of reproducibility and the need of frequent reconditioning for the Hypercarb column, we decided to try various different HILIC columns instead and our final choice was the Obelisc N column. A water/acetonitrile gradient and the effect of different additives, ammonium acetate, ammonium formate, acetic acid, formic acid and trifluoroacetic acid were tested, in order to obtain the best separation and sensitivity for all the compounds included in our method. As mobile phase B, acetonitrile was always used, while the changes were carried out with the mobile phase A. The following modifiers were tested: 1% acetic acid and 0.1 M ammonium acetate (pH = 4.3), 1% acetic acid and 10 mM ammonium acetate (pH = 3.5) and only with 1% acetic acid (pH = 2.8). Bad separation was observed with all these combinations and some compounds were missing, such as N-acetyl-glyphosate. Considering that the pKa values of all the compounds present in the method range from 2 to 10.8, it was decided to apply stronger acidic conditions and for that reason, other stronger acids than acetic acid (pKa = 4.75) were tested as modifier. The options were formic acid (pKa = 3.75) or TFA (pKa= -0.25). Trifluoroacetic acid is commonly used for pH-lowering, but it is not highly recommended when working with a MS detector and an electro spray ion source (ESI) due to the generated ion suppression. Therefore, we decided to work with formic acid in the mobile phase and to use TFA in the dilution/injection solvent at a concentration of 0.2%, in order to have reproducible strong acidic conditions in the autosampler vial.

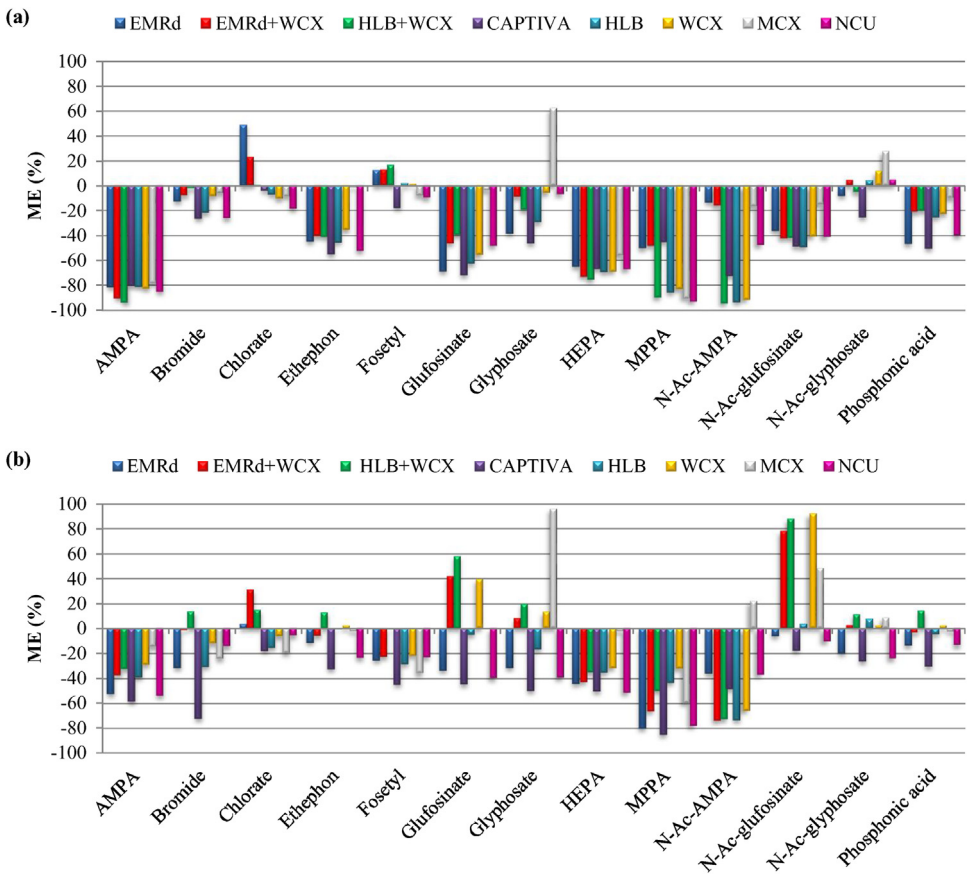

Fig. 1. Matrix effects observed in extracts of (a) soya beans and (b) oat, for all the clean–up sorbents evaluated for the selected compounds.

This way, all the analytes are kept in one unique chemical form, avoiding the conversion between forms, due to the fluctuations of the pH of different matrices. Formic acid was tested in the mobile phase A at different concentrations, ranging from 0.1% to 1%, alone or in combination with different amounts of ammonium formate. Finally, the best results in terms of stability, reproducibility and sensitivity were achieved using just 1% formic acid.

3.2. Clean-up steps evaluation

Five different clean-up sorbents were tested and compared to be included in the procedure for cereals and matrices as soya beans: Enhanced Matrix Removal (EMR), Captiva EMR-Lipid (CAPTIVA), hydrophilic-lipophilic balance (HLB), Weak Cation-exchange (WCX) and Mixed-mode cation-exchange (MCX). Two different approaches were evaluated, dispersive and cartridge solid phase extraction, for cereals as oat and rye, and soya beans matrix. HLB, WCX, MCX and CAPTIVA were evaluated as cartridges. EMR, EMR in combination with WCX and EMR with WCX were tested as dispersive solid phase extraction (dSPE) in the clean-up step.

HLB, EMR and CAPTIVA were evaluated for the removal of non-polar interferences, and WCX, MCX were tested for the removal of other ions in the matrices which could interfere with the target pesticides. The sorbents were tested individually and by combining sorbents for non-polar interferences with sorbents for polar interferences. During all the experiments, visually cleaner extracts were obtained after using the clean-up sorbents. Fig. 1 shows the matrix effect using all the sorbent combinations in the clean-up for soya beans (a) and oat (b). In general, a negative matrix effect was observed for soya beans, independently from the type of sorbent. No significant differences were generally observed between the various sorbents tested for the clean-up step. For soya beans, it should be highlighted that a positive matrix effect was observed for glyphosate and its metabolite, N-acetyl-glyphosate, using MCX in the clean-up step. In cereals, the same effect was observed for these compounds (see Fig. 1b, example for oat) and additionally, also a positive matrix effect was observed for chlorate, glufosinate and N-acetyl-glufosinate with MCX sorbent individually or in combination with EMR and HLB. However, using MCX individually, glufosinate was lost in the clean-up step in soya beans. Because it was not possible to find one suitable sorbent for all the compounds included in this study and evaluated matrices, no clean-up was finally used in the definitive protocol. However, this clean-up evaluation study, carried out with different sorbents, provides the necessary information that may be used in routine analysis to confirm some positive findings for a specific matrix-compound combination.

3.3. Extract dilution factor study

Due to the fact, that no significant differences were observed for the various SPE-sorbents in the clean-up evaluation, dilution of the final extract was tested as an alternative approach to decrease the matrix effect. The reduction of matrix effects was evaluated for three different dilution factors for the final extract concentration, 10-, 20- and 50-fold (corresponding with a matrix concentration of 0.1, 0.05, 0.02 g mL^{-1}). Fig. 2 (a) shows the effect of the dilution factor for AMPA, one of the compounds which presented the highest negative matrix effect in the five matrices studied, in grapes extract. The matrix effect was minimised when increasing the final dilution factor up to 50-fold. This approach worked well for fruit

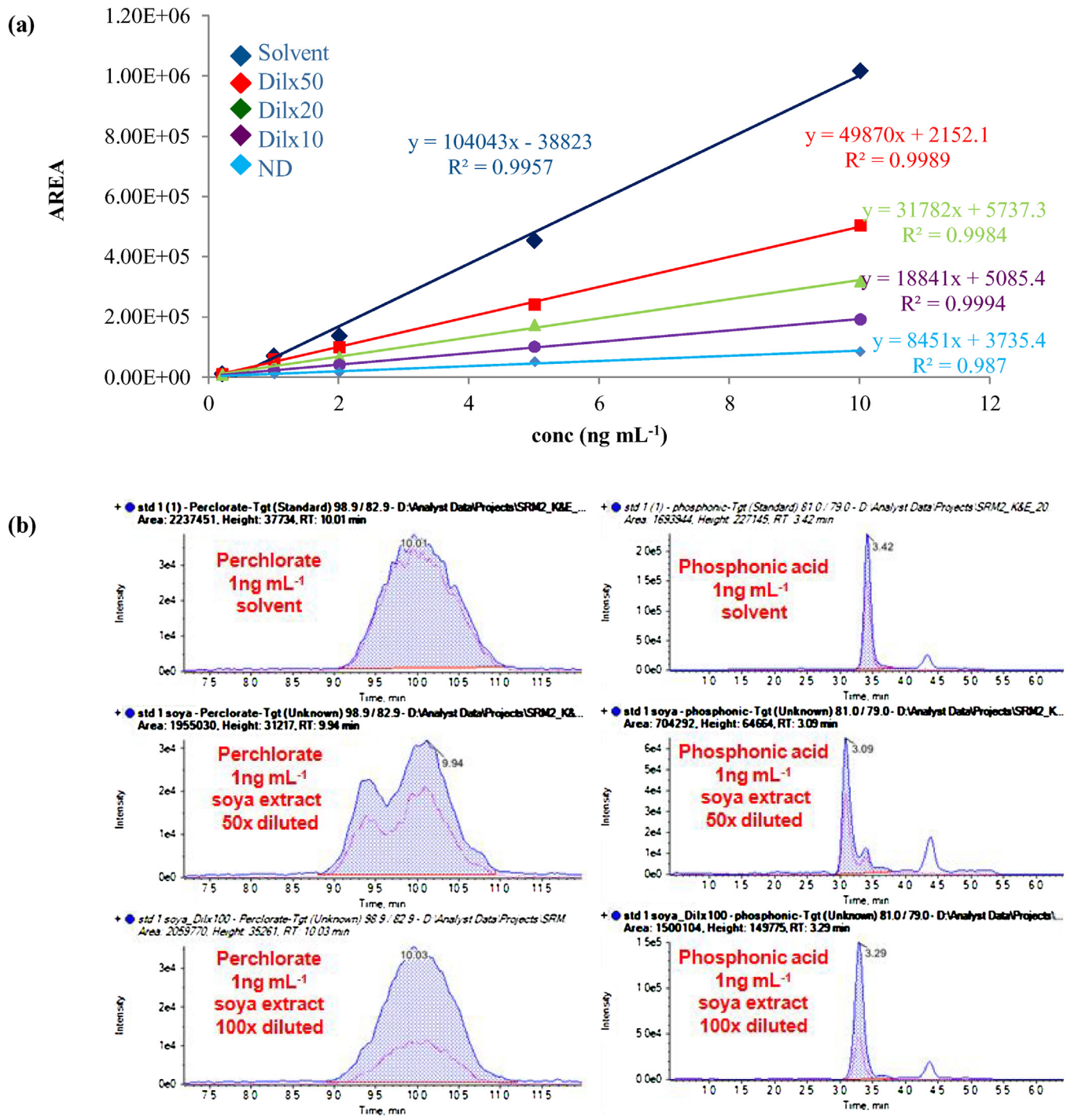

Fig. 2. **(a)** Effect of the final extract dilution factor on detector response for AMPA in grape extract matrix, (b) effect of dilution factor on retention time and peak shape for perchlorate and phosphonic acid in soya beans extract.

and vegetables matrices. However, for the soya bean matrix, it was clear that a higher dilution factor up to 100 was needed. Fig. 2 (b) displays the peaks for phosphonic acid and perchlorate in solvent (upper trace), in matrix extract, diluted 50 times (middle trace), and diluted 100 times (lower trace). A shift in the retention time for phosphonic acid, and a bad peak shape for perchlorate and phosphonic acid was observed for a dilution factor of 50. Both issues were solved using a dilution factor of 100. Thus, the dilution factor in the final method was 50 for fruit and vegetables (matrix concentration, 0.02 g mL^{-1}) and 100 for cereals and soya beans (matrix concentration, 0.01 g mL^{-1}).

3.4. Analytical method validation

3.4.1. Recovery and limits of quantification

The results of the recovery experiments using the ILIS are presented in Table 2 and those without using the ILIS (the "absolute recoveries") in Table 3S in the supplementary material. The recoveries are presented as mean percentage of six replicates for the four spike levels, 0.02, 0.05, 0.1 and 0.5 mg kg^{-1}. For bromide and phosphonic acid, evaluated recovery levels were 0.2, 0.5, 1 and 5 mg kg^{-1} due to their higher MRLs and generally high concentration levels in food matrices. Almost all analytes in the tested fruits and vegeta-

Table 2
Accuracy data (as % recovery) and precision data (as repeatability RSDr, n = 6) at 0.02, 0.05, 0.1, 0.5 mg kg^{-1} for grapes, lettuce, orange, oat and soya beans.

	Recoveries and RSDs (%), using Isotope-labeled internal standard															
	Grapes								Lettuce							
Spike level (mg kg^{-1})	0.02		0.05		0.1		0.5		0.02		0.05		0.1		0.5	
Pesticides	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr
AMPA	112	7	114	3	111	5	109	2	100	12	99	8	98	4	102	2
Bromide	74	16	79	3	77	5	82	3	83	13	105	16	108	4	105	4
Chlorate	96	4	102	3	98	2	102	2	109	6	108	4	107	3	104	2
Ethephon	99	8	101	13	103	6	101	5	96	8	104	7	103	2	103	3
Fosetyl	100	5	99	11	98	3	99	2	104	3	105	5	106	3	107	2
Glufosinate	96	9	102	18	99	7	100	5	98	11	98	10	100	5	100	5
Glyphosate	101	14	104	17	101	7	104	4	113	11	105	9	101	6	98	6
HEPA	n.d.	–	104	6	96	4	97	2	n.d.	–	99	5	87	7	89	4
MPPA	n.d.	–	n.d.	–	117	9	120	6	*87**	5	99	9	101	4	104	3
N-acetyl-AMPA	92	8	88	3	85	5	85	5	98	3	99	6	100	5	105	2
N-acetyl-glufosinate	109	11	104	3	101	4	99	2	101	14	108	6	110	6	112	3
N-acetyl-glyphosate	102	10	102	6	102	2	101	2	99	11	93	7	95	4	93	2
Perchlorate	93	4	97	11	97	5	97	1	113	3	107	5	105	2	105	2
Phosphonic acid	111	6	106	1	103	2	101	2	105	5	104	4	104	1	105	2

	Recoveries and RSDs (%), using Isotope-labeled internal standard							
	Orange							
Spike level (mg kg^{-1})	0.02		0.05		0.1		0.5	
Pesticides	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr
AMPA	111	19	105	11	103	6	104	3
Bromide	82	26	89	7	92	3	93	1
Chlorate	104	12	100	4	96	2	99	2
Ethephon	108	14	100	9	102	6	103	3
Fosetyl	97	12	98	4	99	4	99	2
Glufosinate	103	20	98	6	98	5	94	3
Glyphosate	n.d.	–	103	8	94	5	84	5
HEPA	n.d.	–	85	7	86	4	88	4
MPPA	n.d.	–	75	13	86	9	88	3
N-acetyl-AMPA	86	19	90	5	93	6	95	4
N-acetyl-glufosinate	90	14	94	7	96	5	98	4
N-acetyl-glyphosate	94	9	92	5	92	9	96	2
Perchlorate	n.d.	–	108	7	103	11	102	6
Phosphonic acid	96	10	99	5	98	3	101	2

	Recoveries and RSDs (%), using Isotope-labeled internal standard															
	Oat								Soya beans							
Spike level (mg kg^{-1})	0.02		0.05		0.1		0.5		0.02		0.05		0.1		0.5	
Pesticides	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr	REC	RSDr
AMPA	n.d.	–	93	11	98	7	94	4	n.d.	–	113	19	119	5	120	6
Bromide	70	48	90	19	91	12	83	3	96	12	100	18	110	10	118	9
Chlorate	120	3	106	3	104	5	102	3	120	5	120	15	114	6	112	7
Ethephon	105	11	100	11	99	7	103	4	117	7	113	20	110	11	114	10
Fosetyl	101	7	100	4	102	4	102	2	91	4	102	17	107	4	111	7
Glufosinate	118	14	107	6	104	5	99	7	106	20	114	17	115	4	116	7
Glyphosate	n.d.	–	n.d.	–	118	7	108	11	93*	22	83*	30	93*	11	96	6
HEPA	n.d.	–	110*	9	100	2	90	3	n.d.	–	n.d.	–	99	8	102	4
MPPA	n.d.	–	71	6	79	3	91	3	*84*	17	99	17	87	4	96	9
N-acetyl-AMPA	119	5	112	4	112	3	118	4	119	6	115	17	115	3	119	6
N-acetyl-glufosinate	98	11	97	6	97	5	100	2	118	9	104	20	114	5	118	8
N-acetyl-glyphosate	113	2	99	6	105	8	103	3	112	11	101	20	109	7	110	7
Perchlorate	101	8	102	3	102	7	101	2	n.d.	–	114	19	116	5	110	6
Phosphonic acid	103	6	101	2	101	2	100	2	117	9	110	18	113	1	115	6

n.d.: not detected.
(*) LOQ without full identification, due to missing response of the second transition.

bles showed a satisfactory value of the absolute recovery according to the criteria from the EU SANTE Guideline [22] (see Table 3S). At the lowest evaluated level, 0.02 mg kg^{-1}, HEPA and MPPA were not detected in the five matrices (except MPPA in soya beans). The lack of sensitivity of the qualifier transitions for those two metabolites, made a full identification impossible at this concentration level. The recoveries for N-acetyl-glufosinate and perchlorate in grapes at 0.02 mg kg^{-1} did not fulfil the EU SANTE criteria range (70–120%), 124 and 60% respectively. MPPA at levels of 0.1 and 0.5 mg kg^{-1} also presented recoveries below 70%. In lettuce, AMPA and MPPA showed recoveries around 60%, but with good RSD values ≤20%. In the case of orange and oat, glufosinate, MPPA, N-acetyl-glufosinate and perchlorate presented recoveries below 70%, but also here the repeatability was good, with RSD values (n = 6) below 20%. In the whole recovery study carried out, perchlorate in orange and soya beans and glyphosate in oat, were the pesticides which showed

Table 3
The determination coefficient (r^2) of the calibration curve for standards in matrix and solvent. Linear range, validated method LOQ (from recovery study) and matrix effect (%) without correction with internal standards for grapes, lettuce, orange, oat and soya beans.

	GRAPES							
	r2 solvent	r2 matrix	Linear range (ng mL^{-1}) solvent	Working range conc (*) (mg kg^{-1})	Linear range (ng mL^{-1}) matrix	Working range conc (**) (mg kg^{-1})	LOQ (mg kg^{-1})	ME (%)
AMPA	0.9994	0.9992	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−60
Bromide	0.9976	0.9979	2-1000	0.1-50	2-1000	0.1-50	0.2	3
Chlorate	0.9974	0.9983	0.2-100	0.01-5	0.2-100	0.01-5	0.02	0
Ethephon	0.9915	0.9904	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−4
Fosetyl	0.9991	0.9996	0.2-100	0.01-5	0.2-100	0.01-5	0.02	0
Glufosinate	0.9971	0.9925	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−32
Glyphosate	0.9956	0.9953	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−19
HEPA	0.9993	0.9929	0.2-100	0.01-5	0.5-100	0.025-5	0.05	−56
MPPA	0.9928	0.9928	0.2-100	0.01-5	1-100	0.05-5	0.1	−85
N-acetyl-AMPA	0.9962	0.9978	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−28
N-acetyl-glufosinate	0.9980	0.9942	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−21
N-acetyl-glyphosate	0.9939	0.9965	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−3
Perchlorate	0.9910	0.9926	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−5
Phosphonic acid	0.9985	0.9989	2-1000	0.1-50	2-1000	0.1-50	0.2	−3
	LETTUCE							
	r2 solvent	r2 matrix	Linear range (ng mL^{-1}) solvent	Working range conc (*) (mg kg^{-1})	Linear range (ng mL^{-1}) matrix	Working range conc (**) (mg kg^{-1})	LOQ (mg kg^{-1})	ME (%)
AMPA	0.9980	0.9998	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−55
Bromide	0.9996	0.9995	2-1000	0.1-50	2-1000	0.1-50	0.2	5
Chlorate	0.9996	0.9996	0.2-100	0.01-5	0.2-100	0.01-5	0.02	3
Ethephon	0.9988	0.9989	0.2-100	0.01-5	0.2-100	0.01-5	0.02	2
Fosetyl	0.9984	0.9976	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−1
Glufosinate	0.9915	0.9994	0.2-100	0.01-5	0.2-100	0.01-5	0.02	82
Glyphosate	0.9989	0.9990	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−26
HEPA	0.9991	0.9975	0.2-100	0.01-5	0.5-100	0.025-5	0.05	−72
MPPA	0.9868	0.9994	0.5-100	0.025-5	0.5-100	0.025-5	0.05^{+}/0.02^{***}	35
N-acetyl-AMPA	0.9995	0.9994	0.2-100	0.01-5	0.2-100	0.01-5	0.02	3
N-acetyl-glufosinate	0.9940	0.9990	0.2-100	0.01-5	0.2-100	0.01-5	0.02	44
N-acetyl-glyphosate	0.9998	0.9997	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−1
Perchlorate	0.9970	0.9958	0.5-100	0.025-5	0.2-100	0.01-5	0.02	−5
Phosphonic acid	0.9993	0.9996	2-1000	0.1-50	2-1000	0.1-50	0.2	7
	ORANGE							
	r2 solvent	r2 matrix	Linear range (ng mL^{-1}) solvent	Working range conc (*) (mg kg^{-1})	Linear range (ng mL^{-1}) matrix	Working range conc (**) (mg kg^{-1})	LOQ (mg kg^{-1})	ME (%)
AMPA	0.9984	0.9956	0.2-100	0.01-5	0.5-100	0.025-5	0.02	−76
Bromide	0.9984	0.9970	2-1000	0.1-50	2-1000	0.1-50	0.5	6
Chlorate	0.9983	0.9961	0.2-100	0.01-5	0.2-100	0.01-5	0.02	14
Ethephon	0.9984	0.9961	0.2-100	0.01-5	0.2-100	0.01-5	0.02	1
Fosetyl	0.9957	0.9927	0.2-100	0.01-5	0.2-100	0.01-5	0.02	4
Glufosinate	0.9984	0.9974	0.2-100	0.01-5	0.2-100	0.01-5	0.05	12
Glyphosate	0.9981	0.9977	0.2-100	0.01-5	0.5-100	0.025-5	0.05	−29
HEPA	0.9972	0.9980	0.2-100	0.01-5	0.5-100	0.025-5	0.05	−47
MPPA	0.9974	0.9990	0.5-100	0.025-5	0.5-100	0.025-5	0.05	−52
N-acetyl-AMPA	0.9984	0.9982	0.2-100	0.01-5	0.2-100	0.01-5	0.02	−8
N-acetyl-glufosinate	0.9984	0.9970	0.2-100	0.01-5	0.2-100	0.01-5	0.02	5
N-acetyl-glyphosate	0.9982	0.9968	0.2-100	0.01-5	0.2-100	0.01-5	0.02	6
Perchlorate	0.9898	0.9880	0.5-100	0.025-5	0.2-100	0.01-5	0.05	0
Phosphonic acid	0.9986	0.9974	2-1000	0.1-50	2-1000	0.1-50	0.2	5
	OAT							
	r2 solvent	r2 matrix	Linear range (ng mL^{-1}) solvent	Working range conc (*) (mg kg^{-1})	Linear range (ng mL^{-1}) matrix	Working range conc (**) (mg kg^{-1})	LOQ (mg kg^{-1})	ME (%)
AMPA	0.9992	0.9999	0.1-100	0.01-10	0.5-100	0.05-10	0.05	−68
Bromide	0.9993	0.9997	5-1000	0.5-100	1-1000	0.1-100	0.5	4
Chlorate	0.9990	0.9986	0.1-100	0.01-10	0.1-100	0.01-10	0.02	40
Ethephon	0.9992	0.9990	0.1-100	0.01-10	0.1-100	0.01-10	0.02	4
Fosetyl	0.9987	0.9989	0.1-100	0.01-10	0.1-100	0.01-10	0.02	4
Glufosinate	0.9993	0.9992	0.1-100	0.01-10	0.1-100	0.01-10	0.02	28
Glyphosate	0.9984	0.9980	0.5-100	0.05-10	0.5-100	0.05-10	0.1	34
HEPA	0.9994	0.9975	0.5-100	0.05-10	0.5-100	0.05-10	0.1^{+}/ 0.05^{***}	−25
MPPA	0.9988	0.9996	0.5-100	0.05-10	0.5-100	0.05-10	0.05	51
N-acetyl-AMPA	0.9998	0.9996	0.1-100	0.01-10	0.1-100	0.01-10	0.02	0
N-acetyl-glufosinate	0.9988	0.9991	0.1-100	0.01-10	0.1-100	0.01-10	0.02	46
N-acetyl-glyphosate	0.9956	0.9986	0.1-100	0.01-10	0.1-100	0.01-10	0.02	12
Perchlorate	0.9948	0.9954	0.1-100	0.01-10	0.1-100	0.01-10	0.02	2
Phosphonic acid	0.9993	0.9995	1-1000	0.1-100	1-1000	0.1-100	0.2	8

Table 3 (*Continued*)

	SOYA BEANS							
	r2 solvent	r2 matrix	Linear range (ng mL^{-1}) solvent	Working range conc (*) (mg kg^{-1})	Linear range (ng mL^{-1}) matrix	Working range conc (**) (mg kg^{-1})	LOQ (mg kg^{-1})	ME (%)
AMPA	0.9995	0.9993	0.1-100	0.01-10	0.1-100	0.01-10	0.05	−85
Bromide	0.9989	0.9990	1-1000	0.1-100	1-1000	0.1-100	0.2	−3
Chlorate	0.9979	0.9989	0.1-100	0.01-10	0.1-100	0.01-10	0.05	5
Ethephon	0.9967	0.9968	0.1-100	0.01-10	0.1-100	0.01-10	0.02	−3
Fosetyl	0.9948	0.9969	0.1-100	0.01-10	0.1-100	0.01-10	0.02	−4
Glufosinate	0.9986	0.9971	0.1-100	0.01-10	0.1-100	0.01-10	0.02	−2
Glyphosate	0.9981	0.9984	0.5-100	0.05-10	1-100	0.1-10	0.5+/ 0.02***	−24
HEPA	0.9989	0.9984	0.5-100	0.05-10	1-100	0.1-10	0.1	−66
MPPA	0.9995	0.9994	0.5-100	0.05-10	0.5-100	0.05-10	0.02	−50
N-acetyl-AMPA	0.9981	0.9986	0.1-100	0.01-10	0.1-100	0.01-10	0.02	−13
N-acetyl-glufosinate	0.9987	0.9983	0.1-100	0.01-10	0.1-100	0.01-10	0.02	17
N-acetyl-glyphosate	0.9974	0.9998	0.1-100	0.01-10	0.1-100	0.01-10	0.02	−6
Perchlorate	0.9993	0.9942	0.1-100	0.01-10	0.1-100	0.01-10	0.05	21
Phosphonic acid	0.9990	0.9987	1-1000	0.1-100	1-1000	0.1-100	0.2	3

(*) corresponding concentration in product, based on standards in solvent.
(**) corresponding concentration in product, based on standards in matrix extract.
(***) LOQ, without full identification, due to missing response of the second transition.
(+) RL, Reporting limit at LOQ fulfilling all the identification criteria.

the lowest absolute recoveries <35%. As not all pesticides fulfilled the required criteria and this will probably never be achieved for such a diverse group of difficult analytes, for a good recovery, procedural isotopically labeled internal standards were used for the quantification to correct for the recovery losses in the final method. When using the ILIS, all the recoveries fulfilled the criteria from the EU SANTE guideline (see Table 2), except for HEPA, MPPA and glyphosate at the level 0.02 mg kg^{-1}.

The LOQs were determined based on the recoveries obtained with quantitation with the ILIS (see Table 3). In grapes and lettuce, 70% of the compounds had LOQs at 0.02 mg kg^{-1}, and the rest of the compounds had higher LOQs. Either they were intentionally validated at higher levels (phosphonic acid and bromide), or had a poor sensitivity, especially for the qualifier transition. Therefore, HEPA or MPPA could not be validated at the lowest level. In orange, the percentage of compounds with LOQs at 0.02 mg kg^{-1} was 50%, but almost 80% of the compounds have LOQs below or equal to 0.05 mg kg^{-1}. It is worth to mention that the limits of identification (LOI) of glyphosate in oat and soya beans were set at 0.1 and 0.5 mg kg^{-1}, respectively, due to the interferences from the matrices in the qualifier transition at the low levels, making a proper identification impossible. Because the quantifier transition was perfectly detected at 0.02 mg kg^{-1} and matching with the transition from the glyphosate internal standard, this level was considered as a screening detection limit for glyphosate in soya beans.

3.4.2. Linearity and matrix effect

The linearity of the calibration curve for both standards in solvent and in matrix-extracts was evaluated. Calibration curves were constructed with seven concentration levels, from 0.2 to 100 ng mL^{-1} (corresponding to 0.01 – 5 mg kg^{-1}) in fruit and vegetables and from 0.1 to 100 ng mL^{-1} (corresponding to 0.01–10 mg kg^{-1}) for cereals and soya beans. The levels for bromide and phosphonic acid were ten times higher. The response for standards in solvent and in matrix-extracts was linear with a determination coefficient (r^2) higher than 0.99 in the tested range for all the pesticides studied. Only MPPA and perchlorate in solvent showed a determination coefficient of 0.9868 and 0.9880, respectively (Table 3). The linearity was also checked comparing the back-calculated concentration of the standards with the real concentrations. The criterion of a maximum deviation of 20% was met in all cases.

The assessment of the matrix effect (ME) was carried out by comparing the slopes of the calibration curves from the standards in solvent and matrix. The values are included in Table 3. Matrix effects within the range of -85% to +82% were observed. In grapes and lettuce, 57% of the studied analytes showed insignificant ME, <20%. Another 21% and 22% showed medium and strong ME, within the range of 20–50% and >50%, respectively. In orange, 10 compounds had low ME, 2 had a medium and the other 2 a strong ME. In the case of oat and soya, 50 and 64% of the analytes, respectively, had low to negligible matrix effect.

AMPA showed a strong negative matrix effect in all the matrices evaluated. Glufosinate and its metabolites, MPPA and N-acetyl-glufosinate, also showed similar matrix effects in each matrix tested. These three compounds had a positive matrix effect in lettuce, however, they had a negative matrix effect in grapes. In this latter matrix, MPPA had one of the strongest matrix effects of this study (-85%) (see Fig. 3).

It was also observed that the matrix effect was totally depending on the individual matrix and not on a commodity group. This was noticed for compounds such as glufosinate and MPPA. Glufosinate showed an opposite matrix effect in two matrices from the same commodity group, grapes and orange, -32 and 12%, respectively. Grapes and orange belong to the commodity group with high water and acid content. In lettuce, another matrix with high water content, glufosinate showed a strong positive matrix effect, 82%. MPPA had a high negative matrix effect in grapes, -85%, and in orange, -52%, but in lettuce this compound has medium positive matrix effect, +35%. Due to the variable results from the matrix effect evaluation, isotopically labelled internal standards were required in the final method for the correction of the matrix effects.

3.5. Stability of retention times

Poor retention time stability and limited robustness using HILIC columns were mentioned in previous publications when highly polar pesticides were analysed [19,24]. The retention time stability was evaluated carefully in this study using the Obelisc N column. Good retention time stability for all the pesticides was obtained for over 1000 injections of different kind of matrices. Fig. 4 shows, for example, the retention time for glyphosate in different matrices, grapes, orange, lettuce, oat, soya beans, lemon, pepper and in solvent. The retention time values for glyphosate were plotted against the injection number (1000 injections). Slight difference in reten-

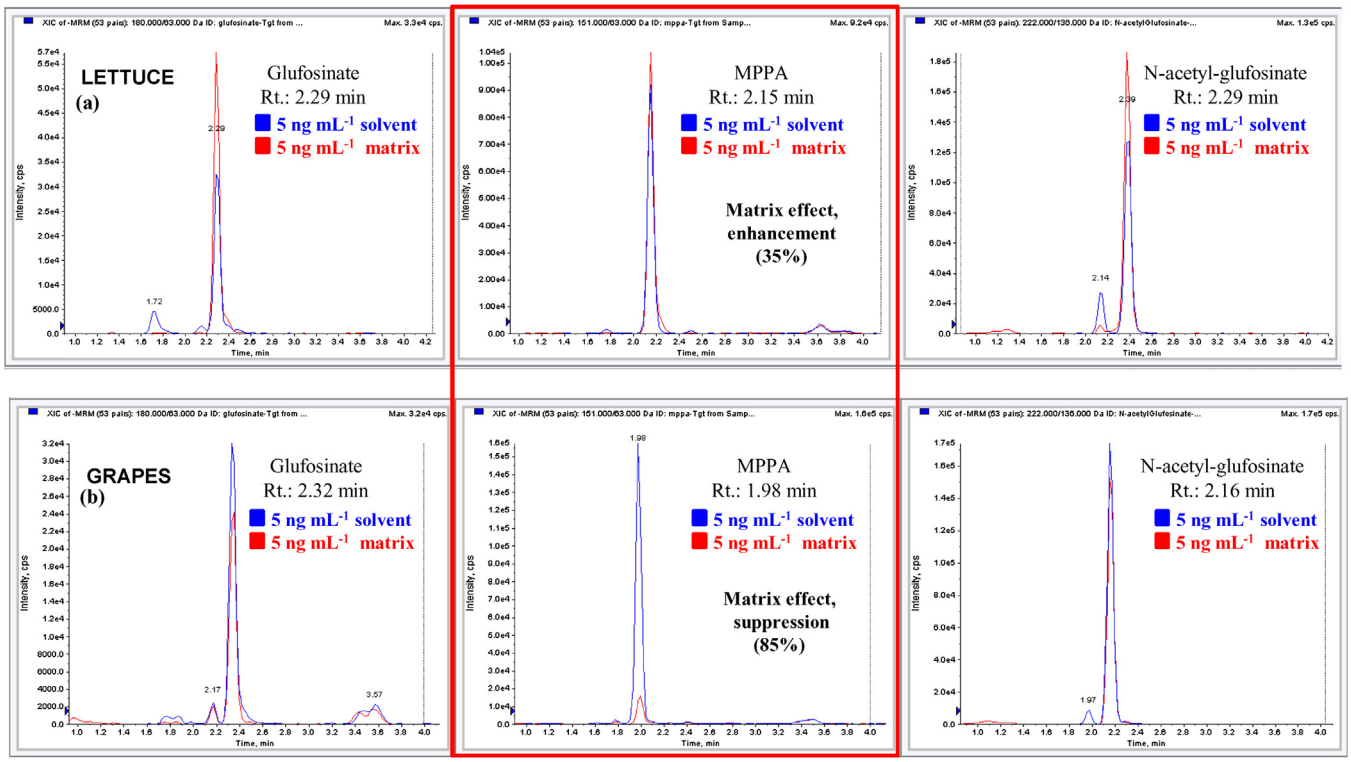

Fig. 3. Extracted ion chromatograms showing the overlapping peaks of glufosinate, MPPA and N-acetyl-glufosinate in solvent and matrix extract, **(a)** lettuce, **(b)** grapes.

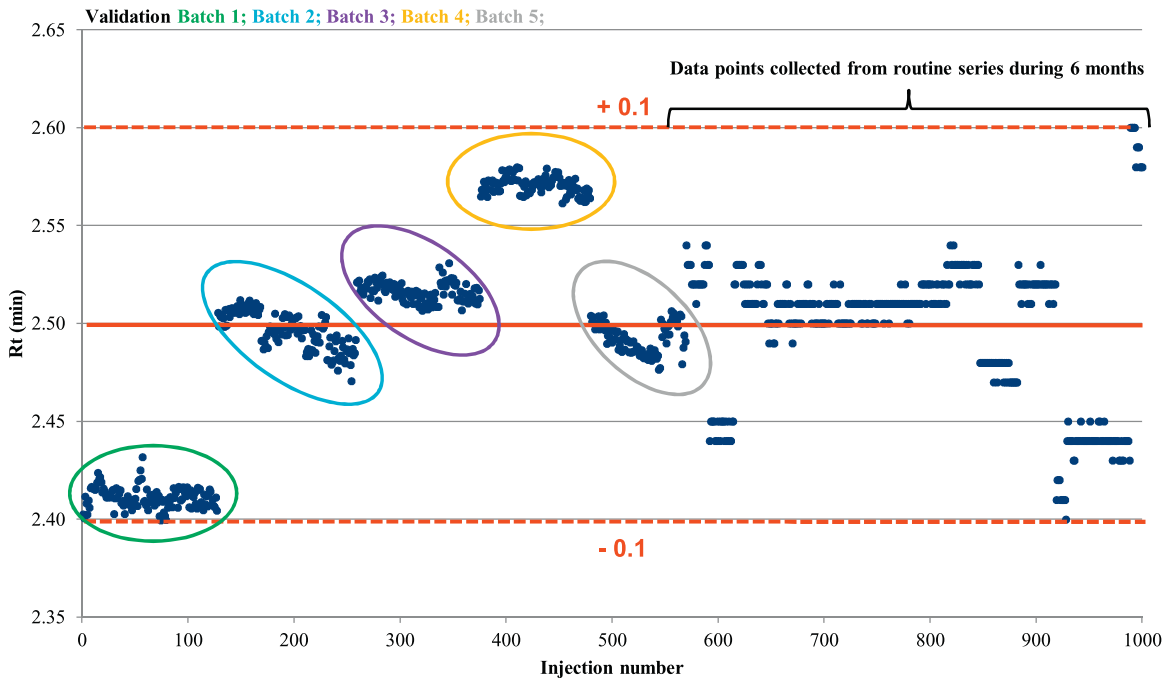

Fig. 4. Retention time stability for glyphosate in solvent and in different matrix extracts and in different batches of samples. A total of 1000 injections were performed on different days.

tion times could be observed between different runs / batches of samples, but within each individual batch, retention times were remarkably stable. Retention time values fulfilled the stability criterion for LC–MS/MS (± 0.1 min) in the EU guideline. However, it is very important to apply a good conditioning and reconditioning of the column if necessary, in order to keep the good stability of the retention times. Additionally, differences in the retention times between three different Obelisc N columns were verified. For most of the compounds, the differences between individual columns were below 1 min. Bromide, chlorate, fosethyl, N-acetyl-glyphosate and phosphonic acid showed slightly higher differences in the retention times, but all still below 2 min. Perchlorate was the compound with the highest difference in retention times between the three columns.

Table 4
Pesticide residues found in Dutch monitoring samples (fruit and vegetables), using the developed selective multiresidue method.

Monitoring year	Commodity	Country of origin	Number of samples analysed	Number of positive findings	Concentration range (mg kg $^{-1}$)			
					Glufosinate	Ethephon	HEPA	Phosphonic acid
2017	Orange	Egypt	18	2				0.18-5.5
		Morocco	5	4				0.10-0.73
		Spain	9	8				0.57-1.9
	Pear	Argentina	2	0				
		Belgium	2	0				
		Chile	1	1				0.74
		The Netherlands	11	7				0.80-15.7
		Peru	1	0				
		South Africa	2	0				
	Grapes	Brazil	5	5		0.054-0.24		0.027-13.9
		Chile	6	6		0.028-0.83		0.44-5.7
		Egypt	66	66		0.069-0.77	0.025-0.16	0.023-30
		France	1	1				2.3
		India	1	1				4.2
		Unknown	2	1				1.4
		Peru	1	1				8
		South Africa	14	13		0.044-0.43		0.49-13.2
	Berries	Unknown	1	1		0.12		

Monitoring year	Commodity	Country of origin	Number of samples analysed	Number of positive findings	Concentration range (mg kg $^{-1}$)			
					Glufosinate	Ethephon	HEPA	Phosphonic acid
2018	Apples	The Netherlands	1	0				
		Italy	1	1				9.9
		New Zealand	1	0				
	Broccoli	Spain	1	0				
	Cultivated fungi	The Netherlands	1	1				0.04
	Mandarins	Spain	1	1				26
	Mangoes	Peru	1	1		0.11		
	Oranges	Egypt	20	2				0.18-5.5
		Morocco	5	4				0.099-0.73
		Spain	12	11				0.57-8.1
		Turkey	2	2				0.65-0.68
	Passion fruit	Uganda	1	0				
	Peaches	Chile	1	0				
	Pears	Argentina	2	0				
		Belgium	2	0				
		Chile	1	1				0.74
		The Netherlands	12	7				0.81-15.7
		Peru	1	0				
		South Africa	2	0				
		China	1	0				
	Peppers	Morocco	1	1				3.8
		The Netherlands	2	0				
		Spain	3	0				
		Turkey	5	3				0.09-0.17
		Israel	2	0				
	Pomegranate	India	1	1				0.47
	Table grapes	Brazil	5	5		0.05-0.24		0.027-13.9
		Chile	11	9		0.028-0.23	0.043	0.28-5
		Egypt	66	66		0.069-0.77	0.023-30	0.025-0.16
		France	1	1				2.3
		India	6	6		0.14		4.2-15
		Unknown	2	1				1.4
		Peru	2	2		0.23	0.04	8-8.6
		South Africa	26	26	0.063	0.034-0.67	0.027-0.080	0.085-13.2
	Tomatoes	Unknown	1	0		0.12		
	Star apple	Dominican Republic	1	0				

3.6. Analysis of real samples and proficiency test samples (EUPT and FAPAS)

The performance of the method was evaluated by participating in EU proficiency tests of the European Union Reference Laboratory (EU-PTs) for single residue methods, in 2017 and 2018. In the first PT sample, EUPT-SRM12 (strawberry), three of the 17 pesticides present in the test material were included in our validated method, glyphosate, N-acetyl-glyphosate and phosphonic acid. Acceptable z-scores were obtained for these three compounds: -0.1, -0.3 and 0.2, respectively. In the second PT sample, EUPT-SRM13 (soya beans), out of the 15 compounds present in the sample, 7 were detected, bromide (0.6), glufosinate (0.1), glyphosate (0.7), MPPA (0.6), N-acetyl-glyphosate (-0.2), perchlorate (0.4) and phosphonic acid (1.1), all also with z-scores within the range ±2. Finally, the method was tested by participating in a FAPAS proficiency test for cereals (oat). The z-score obtained for glyphosate was in this case 0.8.

Furthermore, 352 samples from the Dutch monitoring programme were analysed in 2017–2018 (see Table 4). The fruit and vegetable samples analysed originated from 19 countries and they represented different commodity groups. Almost 77% of the

samples were positive for at least one pesticide residue. Phosphonic acid (included in the residue definition of fosetyl) was detected in almost all the positive samples. The residue concentration ranged from 0.023 to 30 mg kg^{-1}. The highest concentration was detected in grapes from Egypt (30 mg kg^{-1}), but this was still not an exceedance of the MRL (100 mg kg^{-1}). Ethephon and HEPA were also detected, but only in grapes. In the same matrix, also glufosinate at a level of 0.06 mg kg^{-1} was detected in samples from South Africa. Of all the monitoring samples, only mangoes from Peru with a level of 0.11 mg kg^{-1} exceeded the EU-MRL for ethephon (0.05 mg kg^{-1}). The method proved to be very stable and robust in routine analysis.

4. Conclusions

A fast, simple method for the determination of 14 difficult highly polar pesticides has been developed and validated for grapes, lettuce, orange, oat and soya beans. In the clean-up study carried out, no significant differences were observed between the different sorbents tested. No single sorbent performed well for all pesticide-matrix combinations. Therefore, no clean-up step was included in the final method. A large dilution factor for the final extract was used in the method to minimise the matrix effect and to avoid retention time shift, peak splitting or bad peak shapes. The stability of the retention times was achieved by regular conditioning of the column and thanks to the high dilution factors in the final method. This procedure avoids injection of high matrix concentrations onto the column and therefore rapid column degradation. Method validation data for both the polar pesticides and their metabolites in grapes, orange, lettuce, oat and soya beans fulfil the EU SANTE requirements, when internal standards were used. Quantification using isotopically labelled internal standards is required for correction for matrix effects and recovery losses for some compounds, such as HEPA and MPPA. The good z-scores obtained from the participation in three different proficiency tests revealed that the method was performing well. The analysis of samples from the Dutch monitoring programme showed the reliable applicability of the method in routine analysis. Ethephon, HEPA and phosphonic acid were mainly detected in most of the fruit and vegetable samples analysed.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version at doi:https://doi.org/10.1016/j.chroma.2019.02.024.

References

[1] J. Jeyaratnam, Acute pesticide poisoning: a major global health problem, World Health Stat. Q. 43 (1990) 139–144.
[2] M.C.R. Alavanja, N.L. Sprince, E. Oliver, P. Whitten, C.F. Lynch, P.P. Gillette, N. Logsden-Sacket, C. Zwerling, Nested case-control analysis of high pesticide exposure events from the Agricultural Health Study, Am. J. Ind. Med. 39 (2001) 557–563.
[3] M.C.R. Alavanja, Pesticides use and exposure extensive worldwide, Rev. Environ. Health 24 (2009) 303–309.
[4] H. Guo, H. Wang, J. Zheng, W. Liu, Jian Zhong, Q. Zhao, Sensitive and rapid determination of glyphosate, glufosinate, bialaphos and metabolites by UPLC–MS/MS using a modified Quick Polar Pesticides Extraction method, Forensic Sci. Int. 283 (2018) 111–117.
[5] European Commission, Maximum Residue Levels. https://ec.europa.eu/food/plant/pesticides/max_residue_levels_en.
[6] EFSA-Europe, Review of the Existing Maximum Residue Levels for Glyphosate According to Article 12 of Regulation (EC) No 396/2005, 2018.
[7] M. Anastassiades, S.J. Lehotay, Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce, J. AOAC Int. 86 (2003) 412–431.
[8] T. Pihlström, G. Blomkvist, P. Friman, U. Pagard, B.-G. Österdahl, Analysis of pesticide residues in fruit and vegetables with ethyl acetate extraction using gas and liquid chromatography with tandem mass spectrometric detection, Anal. Bioanal. Chem. 389 (2007) 1773–1789.
[9] A. Lozano, B. Kiedrowska, J. Scholten, M. Kroon, A. de Kok, A.R. Fernández-Alba, Miniaturisation and optimisation of the Dutch mini-Luke extractionmethod for implementation in the routine multi-residue analysis of pesticides in fruits and vegetables, Food Chem. 192 (2016) 668–681.
[10] H.G.J. Mol, R.C.J. van Dam, Rapid detection of pesticides not amenable to multi-residue methods by flow injection–tandem mass spectrometry, Anal. Bioanal. Chem. 406 (2014) 6817–6825.
[11] L. Rajski, F.J. Díaz Galindo, V. Cutillas, A.R. Férnandez-Alba, Coupling ion chromatography to Q-Orbitrap for the fast and robust analysis of anionic pesticides in fruits and vegetables, J. AOAC Int. 101 (2018) 352–359.
[12] A. Vass, J. Robles-Molina, P. Pérez-Ortega, B. Gilbert-López, M. Dernovics, A. Molina-Díaz, J.F. García-Reyes, Study of different HILIC, mixed-mode, and other aqueous normal-phase approaches for the liquid chromatography/mass spectrometry-based determination of challenging polar pesticides, Anal. Bioanal. Chem. 408 (2016) 4857–4869.
[13] S. Goscinny, H. Unterluggauer, J. Aldrian, V. Hanot, S. Masselter, Determination of glyphosate and its metabolite AMPA (Aminomethylphosphonic acid) in cereals after derivatization by isotope dilution and UPLC-MS/MS, Food Anal. Method 5 (2012) 1177–1185.
[14] H. Guo, L.S. Ritera, C.E. Wujcika, D.W. Armstrong, Direct and sensitive determination of glyphosate and aminomethylphosphonic acid in environmental water samples by high performance liquid chromatography coupled to electrospray tandem mass spectrometry, J. Chromatogr. A 1443 (2016) 93–100.
[15] I. Hanke, H. Singer, J. Hollender, Ultratrace-level determination of glyphosate, aminomethylphosphonic acid and glufosinate in natural waters by solid-phase extraction followed by liquid chromatography–tandem mass spectrometry: performance tuning of derivatization, enrichment and detection, Anal. Bioanal. Chem. 391 (2008) 2265–2276.
[16] M. Ibáñez, O.J. Pozo, J.V. Sancho, F.J.L. López, F. Hernández, Residue determination of glyphosate, glufosinate and aminomethylphosphonic acid in water and soil samples by liquid chromatography coupled to electrospray tandem mass spectrometry, J. Chromatogr. A 1081 (2005) 145–155.
[17] K. Granby, S. Johannesen, M. Vahl, Analysis of glyphosate residues in cereals using liquid chromatography-mass spectrometry (LC-MS/MS), Food Addit. Contam. 20 (2003) 692–698.
[18] R. Domingos Alves, R. Romero-González, R. López-Ruiz, M.L. Jiménez-Medina, A. Garrido Frenich, Fast determination of four polar contaminants in soy nutraceutical products by liquid chromatography coupled to tandem mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 8089–8098.
[19] A.M. Botero-Coy, M. Ibáñez, J.V. Sancho, F. Hernández, Direct liquid chromatography–tandem mass spectrometry determination of underivatized glyphosate in rice, maize and soybean, J. Chromatogr. A 1313 (2013) 157–165.
[20] C. Ming-Xue, C. Zhao-Yun, J. Yan, Z. Zhi-Wei, Direct determination of glyphosate and its major metabolite, aminomethylphosphonic acid, in fruits and vegetables by mixed-mode hydrophilic interaction/weak anion-exchange liquid chromatography coupled with electrospray tandem mass spectrometry, J. Chromatogr. A 1272 (2013) 90–99.
[21] N. Yoshioka, et al., Rapid determination of glyphosate, glufosinate, bialaphos, and their major metabolites in serum by liquid chromatography-tandem mass spectrometry using hydrophilic interaction chromatography, J. Chromatogr. A 1218 (2011) 13675–13680.
[22] European Commission, Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticides Residues Analysis in Food and Feed, SANTE/11813/2017, 2017.
[23] EURL for Single Residue Method. Quick Method for the Analysis of numerous Highly Polar Pesticides in Foods of Plant Origin via LC-MS/MS involving Simultaneous Extraction with Methanol (QuPPe- Method). http://www.eurlpesticides.eu/userfiles/file/EurlSRM/meth_QuPPe-PO_EurlSRM.pdf.
[24] N. Chamkasem, T. Harmon, Direct determination of glyphosate, glufosinate, and AMPA in soybean and corn by liquid chromatography/tandem mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 4995–5004.