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Development of a QuEChERS-Based Method for the Simultaneous Determination of Acidic Pesticides, Their Esters, and Conjugates Following Alkaline Hydrolysis

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Supporting Information

ABSTRACT: An analytical procedure was developed allowing the simultaneous determination of acidic pesticides and their conjugates by addition of an alkaline hydrolysis step into the European Union (EU) version of the QuEChERS method. The procedure resulted additionally in hydrolysis of most esters of phenoxy acids. On the basis of information from metabolism studies and the hydrolytic conditions employed in supervised field trials as well as results on the influence of physical and chemical parameters (temperature, time, type of solvent, type of matrix), alkaline hydrolysis for 30 min at 40 °C was deemed a good compromise for the determination of residues of 2,4-D, dichlorprop, fluazifop, haloxyfop, MCPA, and MCPB. The applicability of the proposed method was tested by analyzing food samples with incurred residues in six German laboratories not involved in method development. Up to 6 times higher residues are measured by using the QuEChERS extraction procedure with the newly developed alkaline hydrolysis step.

KEYWORDS: *acidic pesticides, QuEChERS method, alkaline hydrolysis, LC-MS/MS, EU reference method*

INTRODUCTION

For several pesticides, the European Union (EU) residue definitions for monitoring established within the Regulation on maximum residue levels (MRLs) of pesticides in or on food and feed of plant and animal origin[1] include esters and conjugates of acids. At present, this applies, among others, to 2,4-D, 2,4-DB, acibenzolar-S-methyl, dichlorprop(-P), fluazifop-P-butyl, fluroxypyr, haloxyfop(-R), MCPA, and MCPB. The consideration of conjugates for setting of MRLs follows the Guidance Document on the Definition of Residue[2] prepared by the Organization for Economic Co-operation and Development (OECD). Inclusion of conjugates in the MRL residue definition is recommended when they contribute significantly to the total radioactive residue in metabolism studies in plants, livestock, or rotational crops and is justified "... due to their potential to be converted back to a biologically active compound following hydrolysis in the mammalian digestive system".

Conjugation in plants may take place with a multitude of natural compounds (e.g., sugars, polysaccharides, amino acids, proteins, and plant cuticles containing epoxy groups.[3−5] The chemical structures of these conjugates are thus often not fully identified in metabolism studies. In such cases, the MRL regulation refers to conjugates in general rather than to specific compounds with defined structures (e.g., glucosides or glucuronides). In addition, conjugation with endogenous macromolecules may result in the formation of nonextractable residues.[6] Nevertheless, on the basis of the OECD Guidance Document on Pesticide Residue Analytical Methods,[7] analytical methods for pesticide residues should be able to measure all components included in the residue definition. If the residue definition includes conjugated residues, the method must include appropriate techniques for releasing the conjugated moiety. Appropriate techniques to release conjugated residues are mentioned in the Residue Chemistry Test Guidelines of the U.S. Environmental Protection Agency (EPA):[8] "Whenever there are indications of the formation of bound components which may not be recovered by the extracting solvent but are of toxicological concern, modifications should be made in the procedure that will free and recover the liberated components. One such modification would be the initial hydrolysis of the treated crop under acidic, alkaline, or enzymatic conditions to free the components."

The aim of this study was to develop an analytical method for the liberation of acidic pesticides from their esters and conjugates. Analytical methods used within the framework of residue trials should also conform to methods used for MRL enforcement to ensure the correct (i.e., uniform) quantification of residues.

Unfortunately, metabolism and residue studies are rarely published in the open literature by manufacturers of pesticides. Publications on the nature of the conjugates formed during metabolism of acidic pesticides in plants are also rare. The most extensive information available is on 2,4-D. The most

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Table 1. Conditions for Hydrolysis Used in Published Methods for Acidic Pesticides Regulated with Esters and Conjugates

pesticides	amount of reagent added (per g of sample)	time and temperature for hydrolysis	extraction solvent (per g of sample)	point of addition of extraction solvent	author/reference
2,4-D	H_2SO_4	100 °C	diethyl ether	after hydrolysis	Crosby[38]
MCPA	0.6 mmol of NaOH	120 min/100 °C	12 mL of diethyl ether	after hydrolysis	Chow et al.[24]
2,4-D	6 N H_2SO_4	100 °C	not stated	after hydrolysis	Nelson et al.[25]
2,4-D, dichlorprop	enzymatic hydrolysis	120 min/50 °C	3 mL of diethyl ether/petroleum ether	after hydrolysis	Løkke[26]
2,4-D	1.25 mmol of NaOH	30 min/80 °C	22.5 mL of diethyl ether	after hydrolysis	Cessna[39]
2,4-D, 2,4-DB, dichlorprop, MCPA, MCPB	0.6−3.0 mmol of NaOH	120 min/100 °C	12−60 mL of acetone/dichloromethane (2/1, v/v)	after hydrolysis	Specht et al.[40]
2,4-D	2.1 mmol of H_2SO_4	60 min/100 °C	1.5 mL of benzene	after hydrolysis	Bristol et al.[41]
2,4-D, 2,4-DB, dichlorprop, MCPA, MCPB	1.25 mmol of NaOH	60 min/90 °C	10 mL of dichloromethane	after hydrolysis	Gilsbach et al.[31]
fluazifop-butyl	0.3−1 mmol of NaOH	90 min/60 °C	0.5−1.6 mL of dichloromethane	after hydrolysis	Clegg[19]
2,4-D, 2,4-DB, dichlorprop	4 mmol of NaOH	45 min/100 °C	75 mL of acetone/dichloromethane (4/3, v/v)	after hydrolysis	Steinwandter[42]
2,4-D	0.075 mmol of K_2CO_3	20 min/100 °C	9 mL of acetone/cyclohexane/ethyl acetate (5/2/2, v/v/v)	after hydrolysis	Anastassiades et al.[28,43]
2,4-D, 2,4-DB, clopyralid, dicamba, dichlorprop, MCPA	1.0 mmol of NaOH	>10 h/room temperature	15 mL of trichloromethane	after hydrolysis	Schaner et al.[44]
2,4-D, dicamba, dichlorprop, fluazifop, fluroxypyr, haloxyfop, MCPA	0.3 mmol of NaOH	30 min/80 °C	2 mL of ethyl acetate + 0.5% acetic acid	after hydrolysis	Östlund[45]
2,4-D, dichlorprop, fluazifop, fluroxypyr, MCPA	0.3 mmol of NaOH	30 min/room temperature	2 mL of acetonitrile	after hydrolysis	Santilio et al.[46]
not specified	0.3 mmol of NaOH	30 min/room temperature	2 mL of acetonitrile	after hydrolysis	EU reference laboratory for pesticides in cereals and feeding stuffs[47]
2,4-D, 2,4-DB, clopyralid, dicamba, dichlorprop, fluroxypyr, haloxyfop, MCPA, MCPB	0.3 mmol of NaOH	30 min/room temperature	5 mL of acetonitrile + 1% formic acid	after hydrolysis	Sack et al.[32]

important conjugates of 2,4-D in monocotyledonous crops such as grasses and cereals are glycoside esters of the parent compound[9−11] or glycosides of hydroxylated metabolites.[5,12] Susceptible dicots such as broadleaf weeds, soybean, sunflower, and tobacco also possess these same detoxification mechanisms but to a lesser degree.[5,13] These plants primarily conjugate 2,4-D with amino acids.[13] Both dichlorprop and its 4-hydroxy derivatives undergo conjugation with diglucoses via the carboxyl group and the hydroxyl group, respectively.[14] Similar to 2,4-D, amino acid conjugates of dichlorprop are not observed in cereals and grass.[15,16] Fluazifop-butyl partly degrades to the free acid fluazifop,[17,18] which is also conjugated in the plant.[19] Condensed information on the metabolism of 2,4-D,[20] haloxyfop,[21] dicamba,[22] and MCPA[23] is available from reports of the WHO/FAO Joint Meeting on Pesticide residues (JMPR). The JMPR evaluations of 2,4-D, haloxyfop, and MCPA mention the fast conversion of esters into free acids after application and emphasize the importance of conjugates (typically glycosides), which release the free acids after hydrolysis. The liberation of conjugated acids is possible by alkaline, acidic, or enzymatic hydrolysis or combinations thereof. One of the first studies was conducted with incurred residues of MCPA in wheat.[24] The authors noted a higher level of MCPA after alkaline hydrolysis compared to hydrolysis under acidic conditions. A second early study on incurred 2,4-D residues in potatoes tested acidic hydrolysis only and noted a significant enhancement of free 2,4-D.[25] Nonextractable residues of 2,4-D in wheat straw and forage mainly released 2,4-D (free acid) by strong alkaline hydrolysis.[11] A combination of acidic and enzymatic hydrolysis was found to be most appropriate by Løkke in his investigation on residues of dichlorprop and 2,4-D in cereals.[26] Chkanikov et al. noted a similar recovery of 2,4-D from its glycosides under alkaline and acidic conditions.[10] By contrast, amino acid conjugates, which are minor metabolites in cereals, were hydrolyzed by acids only. The JMPR report for haloxyfop[21] mentioned the necessity of "mild" alkaline hydrolysis to release the free acid from glycosides or triglycerides. Glycosidic conjugates of diclorprop were hydrolyzed with the enzyme cellulose.[15,16] A summary of these and many subsequent studies on the hydrolysis of conjugates is given in Table 1, in which it can be observed that most authors preferred alkaline hydrolysis, which was mainly applied before extraction with organic solvents. Only one author compared hydrolysis before and after the extraction step and found fewer problems and better performance with hydrolysis after extraction.[27]

Several of the methods presented in Table 1 were used for samples with incurred residues, and results are compared to extractions without hydrolysis. After applying acidic hydrolysis to immature cereal plants, 2,4-D levels increased 3-fold compared to the levels determined without hydrolysis.[10] In potato tubers the increase following acidic hydrolysis was up to 6.8-fold.[25] Following alkaline hydrolysis of citrus fruit, the 2,4-D levels were reported to increase 1.6−2.1-fold in oranges,[28] 2.4−6.3-fold in grapefruit, and 2.8−5.7-fold in lemons.[29] In immature wheat plants MCPA levels were reported to increase 6-fold[24] after alkaline hydrolysis. In whole wheat grain, which was treated with MCPA in the field and with mecoprop postharvest, the increase of MCPA upon alkaline hydrolysis was 7-fold compared to only 1.4-fold in the case of mecoprop.[30] A QuEChERS-based method entailing an alkaline hydrolysis directly after water addition to the sample was employed in these cases. The detected residue of dichlorprop in wheat grain from residue studies increased from 2.3 to 7.0 mg/kg

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(approximately 3-fold) after alkaline hydrolysis.[31] In peanut butter, sunflower seeds, white bread, cornbread, and oranges, increased levels of residues of 2,4-D and clopyralid were detected after alkaline hydrolysis.[32]

Today, the analysis of acidic pesticides is possible by liquid chromatography−tandem mass spectrometry (LC-MS/MS) and no longer requires derivatization. An excellent overview of the LC-MS/MS methods used for acidic pesticides was presented by Sack et al.[32] Extraction and cleanup procedures based on the well-known QuEChERS method[33−35] may be used if the partitioning into acetonitrile from the water phase is supported by a lower pH value.[36,37] Furthermore, the typical cleanup by dispersive SPE with primary−secondary amine sorbent (PSA) should not be used in order to avoid losses of free acids.[32] Due to the high popularity and broad use of the QuEChERS method, it was decided that the reference method developed here should follow this procedure as closely as possible. Conjugates of acidic pesticides are unfortunately not available as analytical standards or reference compounds and could thus not be used for method development. Therefore, it was decided to use esters of the acidic pesticides for hydrolysis experiments. From prior experience we expected that the saponification of esters requires harsher conditions than the hydrolysis of conjugates.

MATERIALS AND METHODS

Chemicals. Pesticide standards used in this work (purity > 96%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany), Sigma-Aldrich Chemie (Seelze, Germany), High Purity Compounds (Cunnersdorf, Germany), or Honeywell Specialty Chemicals (Seelze, Germany) and are listed in the Supporting Information (Table S1).

Deuterium-labeled pirimicab-d_6 (purity = 99.8%) and nicarbazin (1,3-bis(4-nitrophenyl)urea-4,6-dimethyl-2(1*H*)-pyrimidinone (1:1) (purity = 99.4%) used as internal standards and QuEChERS extraction salts (4 mg of magnesium sulfate, 1 g of sodium chloride, 1 g of trisodium dihydrate, 0.5 g of disodium hydrogencitrate sesquihydrate, cataog no. 55227-U, were purchased from Sigma-Aldrich Chemie.

Deuterium-labeled 2,4-D-d_3 (purity = 99.0%) and TRIS (tris-(hydroxymethyl)aminomethane) (purity = 97.0%) used as internal standards were purchased from Dr. Ehrenstorfer. Hydrochloric acid (HCl) 1 M, acetic acid 1 M, sodium hydroxide (NaOH) 5 and 15 mol/L, potassium hydroxide (KOH) solution 1 mol/L, sulfuric acid (H_2SO_4) 2.5 mol/L (5 N), 5 mol/L (10 N), and phenolphthalein (3,3-bis(4-hydroxyphenyl)-1(3*H*)-isobenzofuranone) indicator solution (ACS, Reg. Ph Eur., 0.1% in ethanol) were purchased from Merck KGaA (Darmstadt, Germany).

The following solvents and chemicals were used: acetonitrile (ULC/MS grade, Biosolve BV, Valkenswaard, The Netherlands), acetic acid glacial (HPLC grade, Fisher Scientific, UK), formic acid (99%, ULC/MS grade, Biosolve BV), water (deionized, from a Milli-Q Advantage A10 system, Millipore).

Preparation of Standard Solutions. Individual stock solutions of pesticide esters and acids were prepared at 1 or 0.1 mg/L. Working solutions of pesticide esters were prepared from a mixed stock solution at 2.5 μg/mL by dilution with acetonitrile/water (1:1 v/v) or blank matrix extract resulting in final concentrations from 5 to 250 ng/mL for each pesticide ester. Working solutions of pesticide acids were prepared from a mixed stock solution at 1.25 μg/mL by dilution with acetonitrile/water (1:1, v/v) or blank matrix extract resulting in final concentrations from 12.5 to 400 ng/mL.

Estimation of the Lowest Amount of Hydroxide Required To Maintain pH ≥12 (Tests on Sodium Hydroxide Consumption). Food samples from a local supermarket were homogenized at low temperature, and the pH value of the samples was measured. The homogenized sample was weighed (5.0 ± 0.1 g) in an Erlenmeyer flask. The samples were tempered for at least 15 min in a water bath, and 10.0 mL of 1 M NaOH solution was added to the sample homogenate. The samples were shaken for 2 min and allowed to stand for 10 min. Phenolphthalein indicator solution in ethanol was added. The amount of base was titrated with 1 M HCl until the color of the indicator turned pink.

Hydrolysis of Phenoxy Acid Esters and Fluroxypyr-meptyl in Acetonitrile/Water Solutions. The experiments were performed in five separate screw-cap vials. One milliliter of a mixed pesticide ester working solution (250 ng/mL of each pesticide ester in acetonitrile/water, 1/1, v/v) and 10.0 μL of internal standard solution (TRIS and pirimicarb-d6, 1 μg/mL each) were added to the vials and vortexed for 10 s. To each vial was added 100 μL of 1 M KOH, and the solutions were vortexed for 10 s. The reaction was stopped after 60 s, 5 min, 10 min, 30 min, or 60 min by the addition of 125 μL of acetic acid (1 mol/L). The slightly higher quantity of acetic acid for neutralization ensured a pH <6 in the final solution. Immediately after stopping the reaction, the solutions were again vortexed for 10 s. The experiments at 40 °C were performed in a water bath. The pH value of the reaction mixture after neutralization was checked by appropriate pH indicator strips. An aliquot of the reaction mixture was filtered and transferred into a HPLC vial.

Preliminary Tests of QuEChERS Extraction Combined with Alkaline Hydrolysis of Esters of Phenoxy Acids. Organic food samples from a local supermarket were extracted according to the following procedure. Amounts of 10.0 ± 0.1 g (cucumber, grapefruit); 5.0 ± 0.05 g (whole wheat flour, lentils), and 2.0 ± 0.02 g (black tea) homogenized sample material were weighed in 50 mL disposable screw-capped polypropylene centrifuge bottles. For whole wheat flour and lentils 10.0 mL of cold distilled water was added, immediately vortexed, and soaked for 15 min. At this stage samples were fortified with mixed pesticide ester standard solution resulting in a final level of 0.25 mg/kg pesticide ester each and internal standard mixture. To sample homogenates of wheat flour, lentils, cucumber, and tea were added 10.0 mL of acetonitrile and 0.7 mL of 5 N NaOH for simultaneous extraction and hydrolysis. For the grapefruit sample, 10.0 mL of acetonitrile and 0.7 mL of 15 N NaOH were added. The samples were mixed intensively for 10 s and shaken for 30 min in a water bath showing a temperature of 40 ± 0.5 °C. The reaction was stopped by the addition of 0.35 mL of 5 M H_2SO_4 except for grapefruit, for which 0.7 mL of 5 M H_2SO_4 was added. The pH value was checked after neutralization. After the addition of magnesium sulfate, sodium chloride, and buffering citrate salts, the mixture was shaken intensively and centrifuged for phase separation. Aliquots of the organic phases for all samples except cucumber and tea were stored for 2 h in a freezer (−25 °C). After additional centrifugation, the clear supernatants were transferred to HPLC vials. Blank matrix extracts for preparation of matrix-matched standards were prepared according to the same procedure without fortification. One portion of each of the five commodities was fortified with the pesticide ester standard mixture and extracted without addition of sodium hydroxide and subsequent neutralization with sulfuric acid to control the recovery of the fortified pesticide esters and to check whether some free acids are formed when the normal QuEChERS procedure is applied.

QuEChERS Extraction of Samples with Incurred Residues (Final Reference Method). Food samples with incurred residues of acidic pesticides were provided by Eurofins SOFIA GmbH, Berlin, Germany, and Eurofins Dr. Specht Laboratories, Hamburg, Germany. Some fresh samples previously identified by the CVUA Stuttgart to contain residues of acidic pesticides were also tested. Dry samples (cereal grain, rapeseed, lentils, dried tomatoes, and dried orange) were stored at room temperature in the dark. Fresh samples were stored at −25 °C in a freezer.

Amounts of 10.0 ± 0.1 g (Brussels sprouts, grapefruit, lime), 5.0 ± 0.05 g (wheat grain, beans, lentils, peas, soybeans, rapeseed), or 2.0 ± 0.02 g (dried orange, dried tomato) homogenized sample material were weighed in 50 mL disposable screw-capped polypropylene centrifuge bottles. For dry samples (lentils, beans, wheat, peas, and dried orange, dried tomato), 6.0 or 10.0 mL of cold distilled water was added and soaked for 15 min. Internal standard mixture (0.1 mL) was added to the samples. For extraction and simultaneous hydrolysis 10.0

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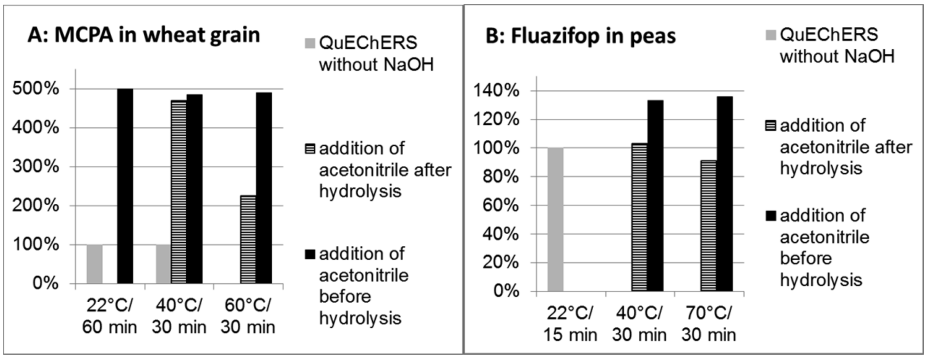

Figure 1. Relative amount of MCPA detected in EU proficiency test sample C1-SRM2 (wheat) (A) and fluazifop in peas (B) depending on conditions used for extraction and hydrolysis.

mL of acetonitrile and 1 mL of 5 N NaOH (wheat grain, beans, lentils, peas, and dried orange) were added. For grapefruit samples, 2 mL of 5 N NaOH were added. The samples were mixed for 10 s intensively and shaken for 30 min in a preheated water bath at a temperature of 40 ± 0.5 °C. The reaction was stopped by the addition of 1 mL of 2.5 M H_2SO_4 except for grapefruit (1.4 mL of 2.5 M H_2SO_4). After the addition of magnesium sulfate, sodium chloride, and buffering citrate salts, the mixture was shaken intensively and centrifuged for phase separation. Aliquots of the organic phases for all samples were stored for 2 h in a freezer (−25 °C). After additional centrifugation, the clear supernatants were transferred to HPLC vials and were ready for analysis. Nonhydrolyzed sample extracts were prepared in the same way, omitting the addition of sodium hydroxide and subsequent neutralization. Instead of shaking for 30 min at 40 °C, samples were shaken mechanically for 15 min at room temperature.

LC-MS/MS Analysis. Liquid chromatography was achieved using an Agilent 1190 system as well as an Agilent Infinity 1290 system. Each system consisted of a binary pump, a vacuum solvent degasser unit, a column oven, and a temperature-controlled automated liquid sampler (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). The Agilent 1190 system was interfaced to an AB SCIEX API 4000 triple-quad (AB SCIEX Instruments) mass spectrometer and controlled by Analyst 1.5.1 software.

The Agilent Infinity 1290 system was interfaced to an AB SCIEX API 5500 QTrap (AB SCIEX Instruments) and controlled by Analyst 1.6.2 software. Both mass spectrometers were equipped with a TUBO V Ion source. The separation was performed using Synergi Fusion-RP columns, 150 mm × 2 mm internal diameter, and 4 or 2.5 μm particle sizes (Phenomenex, Aschaffenburg, Germany) coupled with the corresponding precolumn (4 mm × 2 mm). The flow rate was 0.2 mL/min or 0.15 mL/min. The injection volume was 20 μL. Mobile phase A was methanol/water (1/4, v/v), and mobile phase B was methanol/water (9/1, v/v), both of which contained 5 mM ammonium formate. Directly after injection, elution was performed with 100% eluent A for 8 min. The gradient was raised from 100% eluent A to 100% eluent B within 3 min. For a further 12 min the elution was performed with 100% eluent B. Within 2 min the solvent was changed to 100% A. The total run time was 25 min.

Electrospray ionization (ESI) in positive or negative mode for analytes was used. For the API 4000 triple-quad system separate runs for analytes with ESI in positive mode and negative mode were made. For the API 5500 QTRAP, the scheduled MRM mode was used (MRM detection window, 120 s; target scan time, 0.6 s). Gas flow, gas temperature, and voltages were set as needed for the particular instrument to obtain optimum sensitivity (see Table S2 of the Supporting Information for details).

Validation of the Method. To determine the concentrations of the acids and residual esters, calibration lines were calculated from the peak areas of calibration solutions. The concentrations of the calibration solutions of the esters ranged from 5 to 250 ng/mL and for acids from 12.5 to 300 ng/mL. The peak area ratio of the standard and the internal standard was used for calculation. The correlation coefficients of the corresponding regression lines were always ≥0.99. Standard solutions in (hydrolyzed) blank matrix extracts were used to prepare matrix-matched calibration standards.

RESULTS AND DISCUSSION

Conditions of Hydrolysis and Selection of Most Relevant Pesticides. There are two diverging concepts for the hydrolysis of conjugates and bound residues. In the first case, an attempt is made to hydrolyze all conjugates and bound residues. This requires a stepwise hydrolysis to avoid degradation of liberated compounds, which may occur if too harsh conditions are used from the beginning. The second concept is to hydrolyze the main part of conjugates (and bound residues) in a milder one-step procedure. As the aim was to develop a procedure for the hydrolysis of esters and conjugates that is widely applicable and acceptable, several factors were taken into account. Previous experiments with conjugates have shown that they are relatively quickly hydrolyzed and do not require strong hydrolysis conditions. Applying longer extraction times and higher temperatures did not increase the determined residue levels of free acids. The hydrolysis yield of various, especially sterically hindered, esters was an important aspect to check. Published data can be found in Table 1. Summarizing the results, it becomes clear that alkaline hydrolysis was used in nearly all cases. There was no clear preference with respect to the temperature of the hydrolysis. Because hydrolytic treatments of crop samples widely differed by pesticide, we decided that the proposed reference method should focus on average hydrolysis conditions applied for the most relevant acidic pesticides with regard to residue findings.

The selection of the most relevant pesticides was done by using data from the German Food Monitoring. For detailed explanation and graphical results please refer to Figure S1 of the Supporting Information. The pesticide most often found was 2,4-D. Fluazifop and haloxyfop followed at ranks two and three with 343 and 83 positive findings, respectively. MCPA/MCPB, dichlorprop, and fluroxypyr were found in ≤0.02% of investigated samples. 2,4-DB was never reported.

Hydrolysis of Conjugates and Esters Depending on Temperature, Matrix, and Solvent. *Lowest Amount of Hydroxide Needed for Hydrolysis.* Different kinds of fruits and vegetables were treated with sodium hydroxide to estimate the lowest amount of base required to maintain pH ≥12 used in field studies. The remaining amount of hydroxide after 30 min of treatment at 40 °C was measured by titration with 1 mol/L aqueous HCl. Sample materials such as apple, avocado, carrot, cauliflower, cucumber, grapes, kohlrabi, iceberg lettuce, onions,

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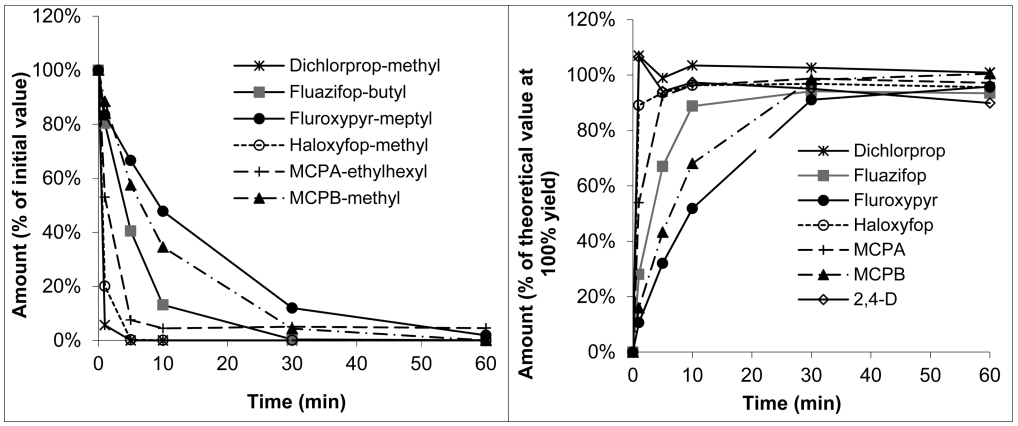

Figure 2. Alkaline hydrolysis of pesticide esters in acetonitrile/water at room temperature: (left) degradation of esters; (right) formation of acids.

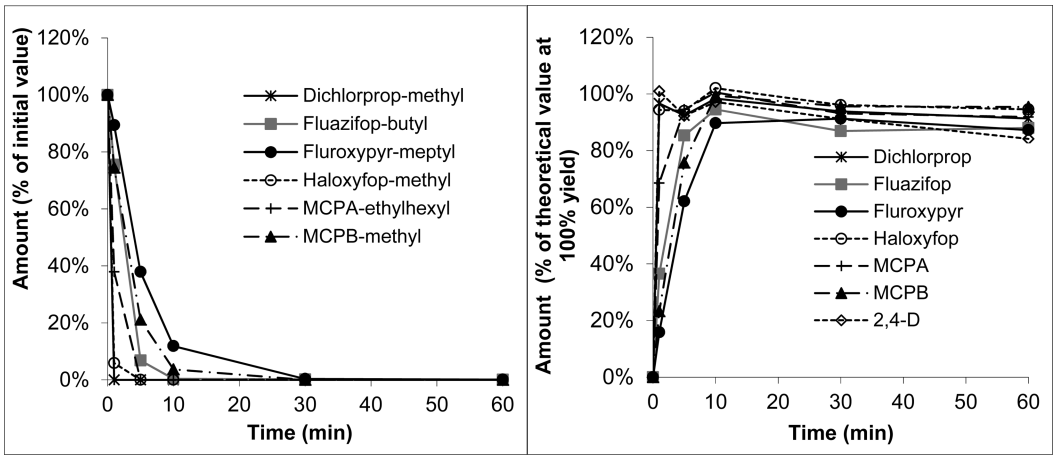

Figure 3. Alkaline hydrolysis of pesticide esters in acetonitrile/water at 40 °C: (left) degradation of esters; (right) formation of acids.

oregano, and potato "consumed" between 0.03 and 0.2 mmol/g sample. Only highly acidic fruits such as limes and lemons consumed >0.2 mmol/g. The highest amount (0.66 mmol) of NaOH was consumed by 1 g of limes. All measured sample pH values and the individual NaOH consumption data are listed in Table S4 of the Supporting Information. The pH value of the homogenized samples is not a sufficient indicator for NaOH consumption. Summarizing the treatment with sodium hydroxide, our data show that a maximum of 2 mmol of NaOH per gram of sample is required to maintain a pH ≥12 for hydrolysis of conjugates (and esters) in 1 g of sample material.

Timing of Hydrolysis Step (before or after Addition of Acetonitrile). Experimental tests of the hydrolysis of conjugates were conducted with cereal grains containing incurred residues of MCPA at a level of 0.284 mg/kg. The sample material was produced in 2007 for the EU proficiency test C1-SRM2.[30] The second test was conducted with peas containing residues of fluazifop at a level of 0.362 mg/kg (without hydrolysis). Samples were analyzed by three different methods: (a) by the standard QuEChERS method without a hydrolysis;[33] (b) by a modified QuEChERS version entailing an alkaline hydrolysis module, which includes the addition of 2 mmol of NaOH/g sample directly after addition of water, that is, prior to the addition of acetonitrile; and (c) by a modified QuEChERS version entailing an alkaline hydrolysis module, which includes the addition of 2 mmol of NaOH/g sample after the addition of water and acetonitrile. Each of the three methods was conducted at three different extraction/hydrolysis temperatures (room temperature and 40 and 60 °C). The results presented in Figure 1 obtained with both sample materials indicate the advantage of conducting the hydrolysis step after the addition of acetonitrile. Interestingly, the obtained amounts of free phenoxy acids were higher at 40 °C (or lower temperature). This is most likely related to the fact that hydrolysis at higher temperatures results in more extensive coagulation of the material, causing parts of the material to be inaccessible to hydroxide. When the hydrolysis was conducted after the addition of acetonitrile, coagulation was clearly less pronounced. We assume that the strong coagulation at 60 °C when acetonitrile was added after hydrolysis resulted in an incomplete wetting of wheat grain with NaOH solution causing the reduced liberation of free MCPA. The results indicated that an amount of 0.5−2 mmol of NaOH/g sample and a temperature of 40 °C are sufficient to liberate conjugated phenoxy acids.

Hydrolysis of Esters in the Absence of Sample Matrix. Herbicide esters are reported to quickly hydrolyze into their free acids within the plants or the soil and to further form metabolites and conjugates within the plants. There are, however, bulky esters, which are more resistant to hydrolysis in plants and which can thus still be detected in the harvested products (e.g., fluazifop-butyl). For many of the herbicides there are numerous esters on the market. To avoid additional measurements of numerous esters, their nearly quantitative conversion to the respective acids would reduce the need for

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additional measurements of these esters. Unfortunately, our tests have shown that especially bulky esters of acidic pesticides require quite harsh conditions for hydrolysis. Nevertheless, detection of esters in food samples analyzed for MRL compliance or in residue trials is rare because of the fast degradation of esters to the free acids.[20,21] As demonstrated in Figure 1, the conjugates are typically easily hydrolyzed with the yield of the released free acids reaching a plateau already at relatively mild hydrolysis conditions. Esters of the pesticides can thus be regarded as "worst-case" model compounds for tests of hydrolytic conditions. Three sets of experiments were performed including (1) hydrolysis of pesticide esters in acetonitrile/water depending on temperature, time, and the added amount of base; (2) investigations of the influence of plant matrix on reaction rate; and (3) application of the final procedure to samples with incurred residues. In set 1 the degradation of pesticide esters and the concurrent formation of acids in alkaline acetonitrile/water were measured from 1 to 60 min (a) at room temperature and (b) at 40 °C. In the absence of sample matrix, the added amount of 0.1 mmol of NaOH/mL solvent was sufficient to obtain a pH >12. Reaction mixtures were measured separately for pesticide esters and acids by LC-MS/MS without cleanup or salting-out steps. Figure 2 shows the degradation of esters and simultaneous appearance of acids at room temperature. After 10 min, the residual quantities of esters were below 5% with the exception of fluazifop-butyl, fluroxypyr-meptyl, and MCPB-methyl. For these esters, the hydrolysis rate was much slower and 60 min was needed to convert ≥90% of the esters to the respective free acid.

To investigate the impact of higher temperature on hydrolysis rate, the hydrolysis experiments were repeated at 40 °C. Degradation of esters and formation of acids were much faster (see Figure 3). After 30 min, residual esters were no longer detectable. A hydrolysis time of 30 min seems to be sufficient in the absence of matrix. The mean recoveries expressed as the sum of detected esters and acids were >90% for all pesticides in these experiments.

Hydrolysis of Esters in the Presence of Sample Matrix. The influence of sample matrix on the hydrolysis was tested by spiking different food samples with esters and hydrolyzing them at 40 °C for 30 min as described above. It was expected that high water content commodities, for example, cucumber, represent the simplest matrix. The samples used for testing the alkaline hydrolysis during QuEChERS extraction were chosen according to the proposed commodity groups stated in the OECD guidance document.[7] The selected samples represented the commodity groups of high water content (cucumber), high protein content (lentils), high starch content (wheat flour), and high acid content (grapefruit). Black tea was the representative sample for commodities that are difficult to analyze. The added amount of sodium hydroxide was 0.35 mmol/g sample for cucumber. Because of the half sample weight in combination with an unchanged volume of sodium hydroxide, the added alkali was raised to 0.7 mmol of hydroxide/g sample for lentils and wheat flour. For grapefruit, a higher amount of alkali was added (1.1 mmol of hydroxide/g) to account for the higher acidity of the sample. The detected amounts of residual esters demonstrated a nearly complete hydrolysis for most investigated compounds for cucumber only. For grapefruit, relatively low amounts of residual esters between only 23 and 33% of the starting value were detected for fluroxypyr-meptyl and MCPB-methyl. The results in Figure 4 indicate that (a) the type of analyte and (b) the type of matrix have an influence on the yield of the hydrolysis step.

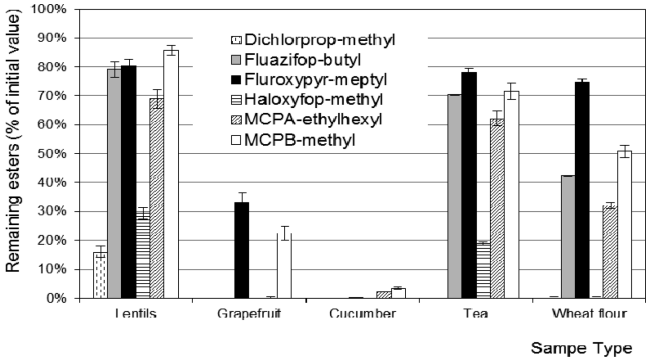

Figure 4. Percentage of detected residual esters measured by LC-MS/MS after alkaline hydrolysis for 30 min at 40 °C and QuEChERS extraction of different plant commodities spiked with pesticide esters (0.25 mg/kg).

In an additional experiment we have compared the recovery of remaining intact esters after fortification to cucumber when applying hydrolysis before and after the addition of acetonitrile. As can be seen in Table 2, this experiment confirmed that hydrolysis after the addition of acetonitrile is much more effective, with hydrolysis yields being virtually quantitative after 30 min at 40 °C.

Results of Samples with Incurred Residues. The final extraction procedure for different sample types is given in Figure 5. The figure shows that compared to QuEChERS without hydrolysis in the worst case (acidic samples with high water content) 3.4 mL of additional water, that is, 2 mL with NaOH and 1.4 mL with H_2SO_4, is added to acidic samples with high water content. In all other cases, hydrolysis results in a surplus of ≤2 mL of water. Our results show that this additional water amount does not prevent the complete phase separation between water and acetonitrile after the addition of QuEChERS salts (magnesium sulfate and sodium chloride).

The applicability of the proposed reference method for the release of conjugates was tested by analyzing 20 different food samples with incurred residues of acidic pesticides. It should be noted that six of these samples contained multiple residues. The analytical results for the pesticides determined after QuEChERS extraction and after application of the reference method are given in Table 3. The highest increase was detected in citrus fruits (grapefruit, orange, and lime). For cereals and dry legumes, the results were more variable. It is interesting that even in samples of the same matrix (e.g., wheat grain) a difference of up to 115% was observed. A possible reason for this variability might be that the amount of conjugated bound residues depends on the time between application and harvest, the so-called preharvest interval, the type of formulation employed (salt, ester, acid), and the time elapsing between harvest and sampling/analysis.

Additionally, the developed reference method was tested in an interlaboratory trial. Five samples (dried lentils, mungo beans, linseed, yellow peas, and rapeseed) containing incurred residues were sent to six German food monitoring laboratories not involved in the method development as well as to two laboratories involved in the method development. The laboratories analyzed the samples by the QuEChERS multi-residue method with and without the hydrolysis module. The

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Table 2. Impact of Alkaline Hydrolysis at Various Temperatures and Extraction Times on Various Esters Spiked on Cucumber with Hydrolysis Being Applied (a) prior to the Addition of Acetonitrile and (b) after the Addition of Acetonitrile at a Spiking Level of 0.2 mg/kg

	hydrolyzed before acetonitrile addition, % of starting value			hydrolysed after acetonitrile addition, % of starting value	
	30 min, 40 °C	60 min, 40 °C	30 min, 80 °C	30 min, 40 °C	60 min, 40 °C
2,4-dichlorprop-ethylhexyl	104	65	11	6	0
fluazifop-butyl	22	12	0	0	0
fluroxypyr-meptyl	70	29	3	0	0
haloxyfop-ethoxyethyl	11	6	0	0	0
MCPA butoxyethyl	3	2	0	0	0
mecoprop-1-octyl	110	70	12	0	0

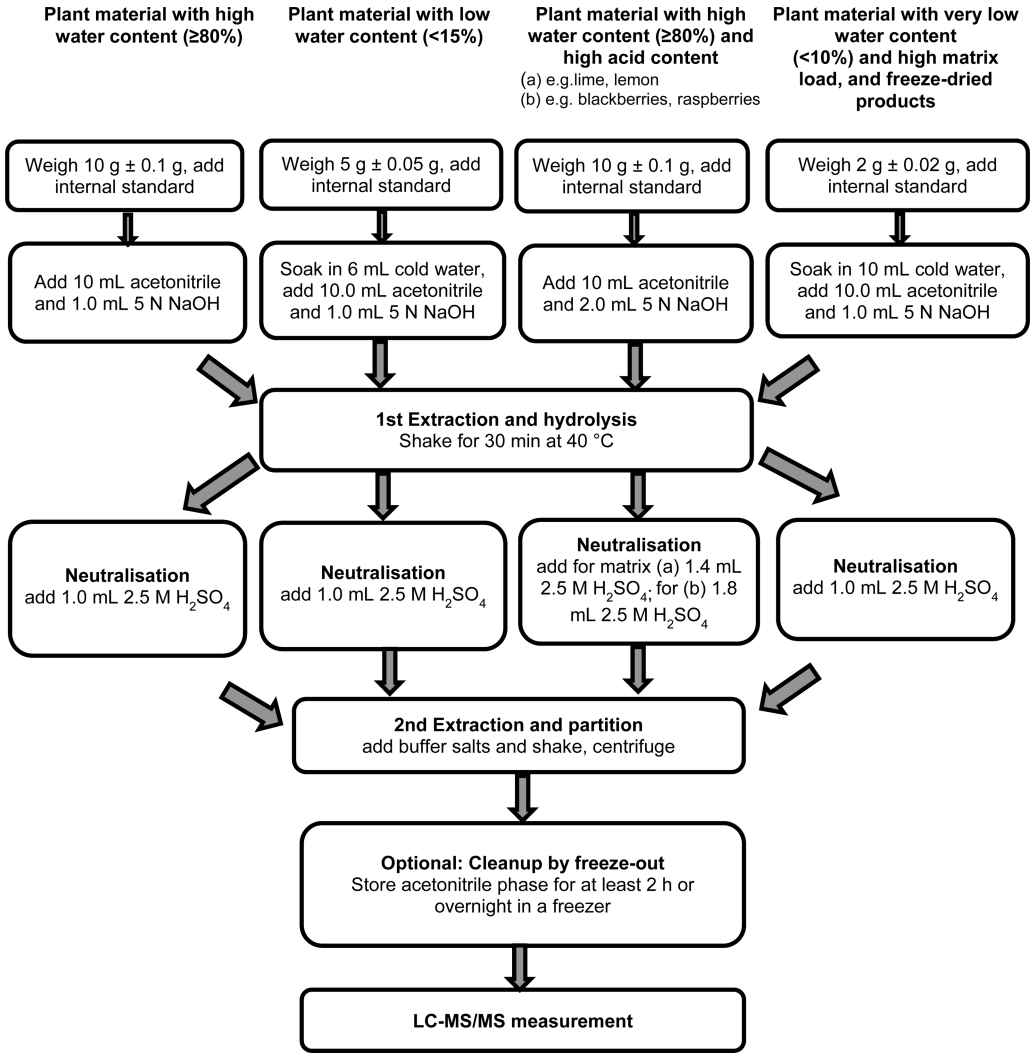

Figure 5. Flowchart of the proposed reference method.

measured residue for a pesticide without hydrolysis is set as 100%. The detected residues of 2,4-D in dried lentils were 108 ± 10% after hydrolysis, with 10% being the standard deviation. For haloxyfop in mungo beans, the mean residues compared to nonhydrolyzed samples rose to 119 ± 22%. The corresponding data for fluazifop were 88 ± 21% in rapeseed, 127 ± 18% in peas, 129 ± 42% in mungo beans, 132 ± 36% in dried lentils, and 729 ± 170% in linseed, respectively.

Summarizing the results, the proposed extension of the widely accepted QuEChERS method by an alkaline hydrolysis modules is applicable for the determination of residues of 2,4-D, dichlorprop(-P), fluazifop(-P), fluroxypyr, haloxyfop(-R), MCPA, and MCPB. It was developed considering the results of metabolism studies with these pesticides. As hydrolysis is conducted in the presence of the matrix bulk, the method allows the liberation of residues conjugated to nonextractable endogenous macromolecules, which is also done in most residue studies. The early addition of the extraction solvent (acetonitrile) before hydrolysis reduces the time needed for hydrolysis and allows the application of lower temperatures. Esters of phenoxy pesticides have been chosen as suitable surrogates for the investigations because defined conjugates

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Table 3. Results of Determination of Acidic Pesticides in Samples with Incurred Residues

sample	commodity	sample type	pesticide	amount (QuEChERS extraction), mg/kg	amount (reference method), mg/kg	ratio (QuEChERS = 100%)
1	lentil, sample 1	dry, high starch content	fluazifop	0.087	0.112	129
1	lentil, sample 1	dry, high starch content	2,4-D	0.10	0.14	138
2	lentil, sample 2	dry, high starch content	haloxyfop	0.051	0.053	104
2	lentil, sample 2	dry, high starch content	2,4-D	0.042	0.041	98
3	lentil, sample 3	dry, high starch content	2,4-D	0.078	0.077	99
4	mungo bean seed, sample 1	dry, high starch content	haloxyfop	0.613	0.665	108
4	mungo bean seed, sample 1	dry, high starch content	fluazifop	0.042	0.057	136
5	beans dried, sample 1	dry, high starch content	haloxyfop	0.403	0.455	113
5	beans dried, sample 1	dry, high starch content	fluazifop	0.024	0.033	138
6	wheat grain, sample 1	dry, high starch content	2,4-D	0.059	0.127	215
7	wheat grain, sample 2	dry, high starch content	2,4-D	0.164	0.212	129
8	wheat grain, sample 3	dry, high starch content	2,4-D	0.075	0.068	91
9	yellow pea, sample 1	dry, high starch content	2,4-D	0.034	0.037	109
9	yellow pea, sample 1	dry, high starch content	fluazifop	0.053	0.071	134
10	yellow pea, sample 2	dry, high starch content	2,4-D	0.107	0.104	97
11	yellow pea, sample 3	dry, high starch content	fluazifop	0.362	0.482	133
12	tomato, dried	dry	fluazifop	0.036	0.041	114
13	soybean	high fat content	fluazifop	0.43	0.52	121
14	rapeseed, sample 1	high fat content	fluazifop	0.49	0.49	100
14	rapeseed sample 1	high fat content	2,4-D	0.006	0.02	333
15	orange, dried	acidic	2,4-D	0.063	0.089	141
16	orange, dried	acidic	2,4-D	0.015	0.029	193
17	grapefruit,	acidic	2,4-D	0.06	0.41	683
18	grapefruit	acidic	2,4-D	0.078	0.188	241
19	lime, fresh	acidic	2,4-D	0.398	0.657	165
20	Brussels sprout	high water content	fluazifop	0.027	0.027	100

were not available as reference materials. Results obtained with samples containing only esters of phenoxy pesticides have shown that the chosen hydrolysis conditions allow at least their detection and often also their quantification as free phenoxy acids. Because the proposed hydrolysis conditions of the new method reflect conditions used in analysis of samples from residue studies quite well, a better matching of MRL setting and MRL enforcement can be reached. The developed method is proposed as a reference method for alkaline hydrolysis to further harmonize the analysis of residues of acidic pesticides in plant materials in the European Union.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b05407.

Tables S1−S4 include information on pesticide standards used in this work (pesticide standards, structural formulas, molecular weights, and suppliers), MS-MS parameters including transitions, retention times of pesticides and internal standards, gas and source parameters for AB Sciex 5500 Qtrap as well as experimental results on hydroxide consumption by various food commodities. Table S5 lists hydrolysis conditions used in residue studies conducted for MRL setting including references and explanation of content of table. Figure S1 includes a description of analysis of data from German food monitoring data including graphical presentation of results. Figures S2 and S3 give additional results for samples with incurred residues. Figure S4 includes a description of determination of nonvolatile coextracted matrix including a graphical presentation of results (PDF)

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Notes

The authors declare no competing financial interest.

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