EURL-SRM - Analytical Observations Report

Concerning the following…

- **Compounds**: 2-Phenylphenol (a.k.a Orthophenylphenol, Biphenyl-2-ol, 2PP, OPP)
- **Commodities**: Plant Origin (Pears, Oranges, dried beans)
- **Extraction Methods**: AcH-QuEChERS, AH-QuEChERS, QuEChERS
- **Instrumental analysis**: LC-MS/MS and GC-MS/MS

Analysis of 2-Phenylphenol (sum) via AcH-QuEChERS - involving acidic Hydrolysis - and of 2-Phenylphenol Glucoside via CEN-QuEChERS

Version 1
(03.02.2025)

1. Aims

The aim of this study was to elaborate a QuEChERS-based methodology that covers the full residue definition of 2-phenylphenol (in the following abbreviated as **OPP**) applying for food of plant origin. This residue definition, which has been established in 2018, entails free OPP and its conjugates thus calling for a hydrolysis step to release conjugated OPP. Method development in this direction was for a long time hindered by the non-availability of OPP-conjugates. Recently, the glucose conjugate of OPP (in the following abbreviated as **OPP-Glc**) became commercially available allowing to check both its breakup behavior under different conditions as well as its amenability to a direct analysis via CEN-QuEChERS, the most commonly used method in pesticide laboratories. Another aim was to check the share of conjugated OPP in products with incurred residues and to find out whether glucose conjugates vastly predominate, as this would potentially paving the way for a direct analysis of OPP and OPP-Glc, thus circumventing the hydrolysis step.

OPP is mostly found in citrus fruits, which may also contain acidic pesticides which also require de-conjugation during analysis (e.g. 2,4-D and 2,4-DP), which also require the use of a method involving a de-conjugation step. It was therefore also of interest to find out whether these compounds could be de-conjugated by the same approach. The suitability of alkaline and acidic hydrolysis in breaking up both types of conjugates in one go was therefore one of the aims of this study. As a first step, the focus was on a purely chemical approach leaving enzymatic de-conjugation for a later stage.

2. Background information

OPP is an EU-approved fungicide with a long history of usage. In the past, OPP used to be classified as a food additive within the EU, and was allowed to be used for the preservation of citrus fruits under the codes E231

(for the free phenol) and E232 (for the sodium salt = SOPP)[1]. As far back as 2003[2] it was decided to define OPP (and SOPP) as an active substance for plant protection and to remove it from the food additives list. But it took more than a decade for this decision to be fully implemented. In 2010, OPP was officially classified as an active substance for plant protection by including it into Annex I of Directive 91/414/EEC[3,4]. Following a transition period, OPP was eventually also removed from the list of permitted food additives in 2014.
Within the EU plant protection framework, OPP is currently only registered for the post-harvest treatment of citrus fruits where it is used to impede the growth of penicillium mould. Five EU countries (CY, GR, ES, HR and PT) have currently authorizations in place. Application of OPP on citrus fruits may take place either by dipping the fruits into aqueous OPP solutions or into OPP-containing wax suspensions. Outside the EU, OPP applications are also reported for other types of fruits, such as pome fruits, stone fruits, pineapples and melons, as well as for fruiting vegetables, such as aubergines and sweet peppers[5,6,7]. Disinfection of eggs with OPP was also reported[8].

OPP used as a biocide: OPP is not only effective against fungi (including yeasts) but also against bacteria and certain viruses. It is therefore also used in combination with other disinfectants in biocidal products with applications in e.g. households, farms and food processing sites. Approved OPP-containing biocidal preparations with food-relevant applications include products for disinfection of equipment, containers, surfaces and pipes associated with the production, transport, storage or consumption of food, drinking water, and feed (PT4), products for human/veterinary hygiene (PT1/PT3), and products for preserving polymers and paper (PT-9). OPP is furthermore allowed to be used as a preservative of certain pesticide formulations (PT6)[7]. It is reportedly also used in the production of resins, rubber chemicals and dyes.
Given its broad use in various products that may come into contact with food, the contamination of food products following biocidal uses, is not unlikely. Migration of OPP into food has been for example reported within canned beverages[9] as well through the contact of food with paper and cardboard[10]. Higher migration rates are reported when recycled paper is used.

Processing contaminant: Interestingly, OPP has also been reported being a processing contaminant in coffee, with Theurillat et al. reporting generation during roasting[11] and Menzio et al. reporting the presence of extractable fatty-acid-conjugates (lipophilic), both in green and roasted coffee[12].

Metabolism studies[13]: Studies on OPP-treated oranges revealed that 12 weeks after treatment by dipping the fruits into a 0.1% or a 0.5% solution, approx. 30% of the OPP was present in form of acid-hydrolysable conjugates. Based on experiments involving enzymatic hydrolysis with β-glucosidase, it was concluded that some of these conjugates are glucosides. In pears, the share of conjugated OPP following a 28 weeks storage after treatment with OPP (aqueous solution containing 4% SOPP) was found to be 93%. Seven different fractions of conjugates were isolated and hydrolysed and it was assumed that all these fractions are most likely sugar conjugates

[1] Directive 1995/2/EC
[2] Directive 2003/114/EC
[3] Commission Directive 2009/160/EU
[4] Directive 91/414/EEC was superseded by Regulation (EU) 1107/2009
[5] Rückstände von o-Phenylphenol in Bioprodukten; Bernhard Speiser Stand: 22. 8. 2014
[6] https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation99/23Phenylphenol.pdf
[7] See also https://www.chem-on.com.sg/image/catalog/product_catalog/TDS_Dowicide%20A.pdf
[8] https://cdn.who.int/media/docs/default-source/wash-documents/wash-chemicals/2phenylphenol-background.pdf?sfvrsn=261b56f5_3
[9] Mehmet Coelhan et al.; Determination and Levels of the Biocide ortho-Phenylphenol in Canned Beers from Different Countries - J. Agric. Food Chem. 54/16 (2006) 5731-35; Doi: 10.1021/jf060743p
[10] Lenka Votavová et al.; Occurrence of 2-phenylphenol in food paper packages - Cent. Eur. J. Chem. 12(11) 2014 1162-1168, Doi: 10.2478/s11532-014-0563-x
[11] Viviane Theurillat et al.; Traces of 2-phenylphenol in roasted coffee are not related to agrochemical residue in green coffee beans, but to generation during roasting; Food Addit Contam Part A Chem Anal Control Expo Risk Assess; 2022 Mar;39(3):525-530. Doi: 10.1080/19440049.2021.2005829.
[12] Janet Menzio et al. The challenge of o-phenylphenol detection in coffee: How "OPP-conjugates" hide their presence in green and roasted samples - Food Chemistry Vol. 404, Part A, 15 March 2023, 134597; Doi: 10.1016/j.foodchem.2022.134597
[13] https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation99/23Phenylphenol.pdf

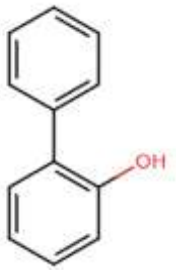
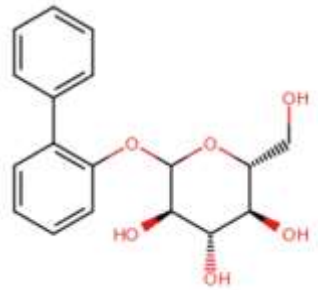
including glucose conjugate. Post-extraction solids also released OPP (< 5% of total OPP), which are assumed being bound to cellulose (a glucose polymer).

Residue definition and nature of conjugates: In contrast to food of animal origin where the EU residue definition for enforcement includes only OPP, the residue definition for food of plant origin entails OPP and its conjugates[14]. The types of conjugates entailed are, however, not specified. Nevertheless, judging from the results of the metabolism studies (see FAO report[13]), it seems that sugar conjugates are by far the most prominent at least in fruits. Sugar conjugates of phenolic compounds, such as OPP, being bound via an ether bond, are much harder to breakup compared to the ester bonds between carboxylic acids and sugars.

3. Analyte Properties

Physicochemical properties and some additional information on OPP can be found in *Table 1*.

Table 1: Chemical properties of 2-phenylphenol and 2-phenylphenol-glucoside

Name: 2-phenylphenol (CAS: 90-43-7) ; IUPAC Name: Biphenyl-2-ol		
Parameter	**Value**	
Molecular Mass	170.2 g/mol	
Formula	$C_{12}H_{10}O$	
Exact mass	170.07316 Da	
Pka	9.5 at 20°C (exp.) ; the free acid is the predominant form at the physiological pH range between 2 and 7	
LogKow	3.2 at 22.5°C (experimental)	
Water solubility	Sodium 2-phenylphenolate is very soluble 1200 g/l	
Stability	Photodegradation reported to lead to the formation of … • phenylhydroquinone (PHQ, CAS No. 1079-21-6), • phenylbenzoquinone (PBQ, CAS No. 363-03-1), • 2-hydroxydibenzofuran (2OHDBF, CAS No. 86-77-1)	
Residue definition EU	**EU:** 2-phenylphenol (sum of 2-phenylphenol and its conjugates, expressed as 2-phenylphenol) (R),(F) **Codex:** Sum of 2-phenylphenol and sodium 2-phenylphenate, free and conjugated, expressed as 2-phenylphenol	
2-phenylphenol is approved in…	CY, EL, ES, HR, PT	
Toxicity	OPP has been reported of exhibiting low acute toxicity but there are indication for carcinogenicity in male rats[15]	
ADI / ARfD	0.4 mg/kg bw per day / - mg/kg bw (WHO)[8]	
Name: 2-phenylphenol-glucoside (CAS: 363165-10-0)		
Parameter	**Value**	
Molecular Mass	332.3 g/mol	
Formula	$C_{18}H_{20}O_6$	
Exact mass	332.12599 Da	
Pka	12.2 (acidic), (computed by *chemicalize.com*) i.e. non-ionized form predominates to pH 12.5	
LogP	1.05 (computed by *chemicalize.com*)	
Residue definition EU	See 2-phenylphenol	
Conversion Factors	OPP-Glc ➔ OPP (0.51); OPP ➔ OPP-Glc (1,95)	

[14] *EU residue definition for enforcement: "2-phenylphenol (sum of 2-phenylphenol and its conjugates, expressed as 2-phenylphenol)"*

[15] *WHO - Water-safety-and-quality: Chemical-fact-sheets-2022/2-phenylphenol-and-its-sodium-salt-fact-sheet-2022 (Hyperlink)*

4. Chemicals and Consumables

The used materials and apparatuses are listed in CEN-QuEChERS (EN-15662). The suppliers of the used analytical standards are shown in *Table 2*

Table 2: Sources of Analytical standards (exemplary).

Compound	CAS	Company	Order No.
2-phenylphenol	90-43-7	LGC (Dr. Ehrenstorfer)	DRE-C11890000
2-phenylphenol-glucoside	363165-10-0	HPC Standards GmbH	DRE-C11890100
2-phenyl-phenol $^{13}C_6$	287389-48-4	Sigma-Aldrich Chemie GmbH	606286
2-phenyl-phenol D_5	64420-98-0	LGC (TRC)	P335872
Propyzamid D_3	1219805-79-4	CDN Isotopes	D-6431

Disclaimer: Names of companies are given for the convenience of the reader and do not indicate any preference by the EURL-SRM towards these companies and their products

Stock solutions (e.g. at 1 mg/mL) are prepared in acetonitrile, taking the purity of the standard substances into account. Working solutions are also prepared in acetonitrile. Stock and working solution of both OPP and OPP-Glc show good stability over many months when stored in the refrigerator. OPP is reportedly photosensitive, thus exposure to sunlight should be reasonably minimized.

The OPP-$^{13}C_6$ used was found to contain a sizable share of native OPP in the range of ~2%. Adding 0.25 µg of this ILIS to an analytical portion will result in ~0.005 µg native OPP added to the sample. In the case of 10 g/5g/2g sample portions the contamination will be in the range of 0.0005/0.001/0.0025 mg/kg, which, especially in the case of 2 g sample portions, is quite relevant, considering that the MRLs of many dry products are set at a just 4-fold higher value (0.01 mg/kg). Typically, this bias is corrected by a shifted calibration curve (intercept), but still care is needed to avoid false positives.

For preparing the 10N NaOH solution, fill ca. 150 mL of water into a 200 mL volumetric flask and gradually add 80 g of NaOH pellets preferably using a styrer (careful: exothermic reaction!). After the solution has cooled down to ambient temperature fill-up to volume with water. For preparing 10 N H_2SO_4, fill ca 130 mL of water and gradually add 55,52 mL of 96% H_2SO_4 (d=1,84 g/mL) sulfuric acid. After the solution has cooled down to ambient temperature fill-up to volume with water.

5. Sample Preparation and Measurement

For the analysis of OPP and intact OPP-Glc, sample homogenates are extracted according to the CEN-QuEChERS method (citrate-buffered, EN-15662). Samples are homogenized by cryogenic milling using dry ice.

The AcH-QuEChERS deviates from CEN-QuEChERS as follows:

For water containing fruits and vegetables use 5 g analytical portions. For dry commodities (e.g. cereals and pulses) you may adjust the weight of the sample portions to e.g. 2 or 2.5 or-5 g considering the clumping behavior of the matrix and the sensitivity that needs to be achieved. Prior to extraction/hydrolysis, water needs to be added to all sample types: For commodities with >85% moisture (e.g. apples) add 1 mL of water; for commodities between 70 and 85% moisture (e.g. potatoes) add 2 mL of water; for dry commodities (independently of the analytical portion size) add 6 mL of water.

After the addition of 10 mL acetonitrile add 2 mL of 10 N H_2SO_4, close the vial, shake briefly and heat overnight (16-24 h) at 70°C in a shaking water bath or a heated shaker. Make sure that the material is moving during shaking and does not clump. If it clumps increase agitation speed and the tilt angle of the vial. Reducing sample weight will also reduce such effects. When the hydrolysis step is over, cool down the vials (e.g. in a water bath), open them, add 2 mL 10N NaOH to neutralize as well as citrate buffer salts (as in CEN-QuEChERS).

Continue the QuEChERS procedure normally. A flowchart is shown in ***Figure 1***

The extracts (acetonitrile phase) are directly subjected to separation and measurement via LC-MS/MS. Both OPP and OPP-Glc are analyzed in the negative mode. For OPP-Glc the formate adduct is used as parent ion.

OPP may be also analyzed, more sensitively, using GC-MS/MS, but care is needed to a) reduce the influence of OPP artefacts; and b) reduce interaction of OPP with active surfaces, which may cause severe peak tailing. For OPP, ILISs are available (see ***Table 2***), but a generic IS, such as propyzamide-D_3 may also be used (e.g. 100 µL of a 10 µg/mL standard). The ILISs are added to the sample portion prior to hydrolysis and extraction. Exemplary LC-MS/MS conditions are given in ***Table 3*** for OPP and in ***Table 4*** for OPP-Glc.

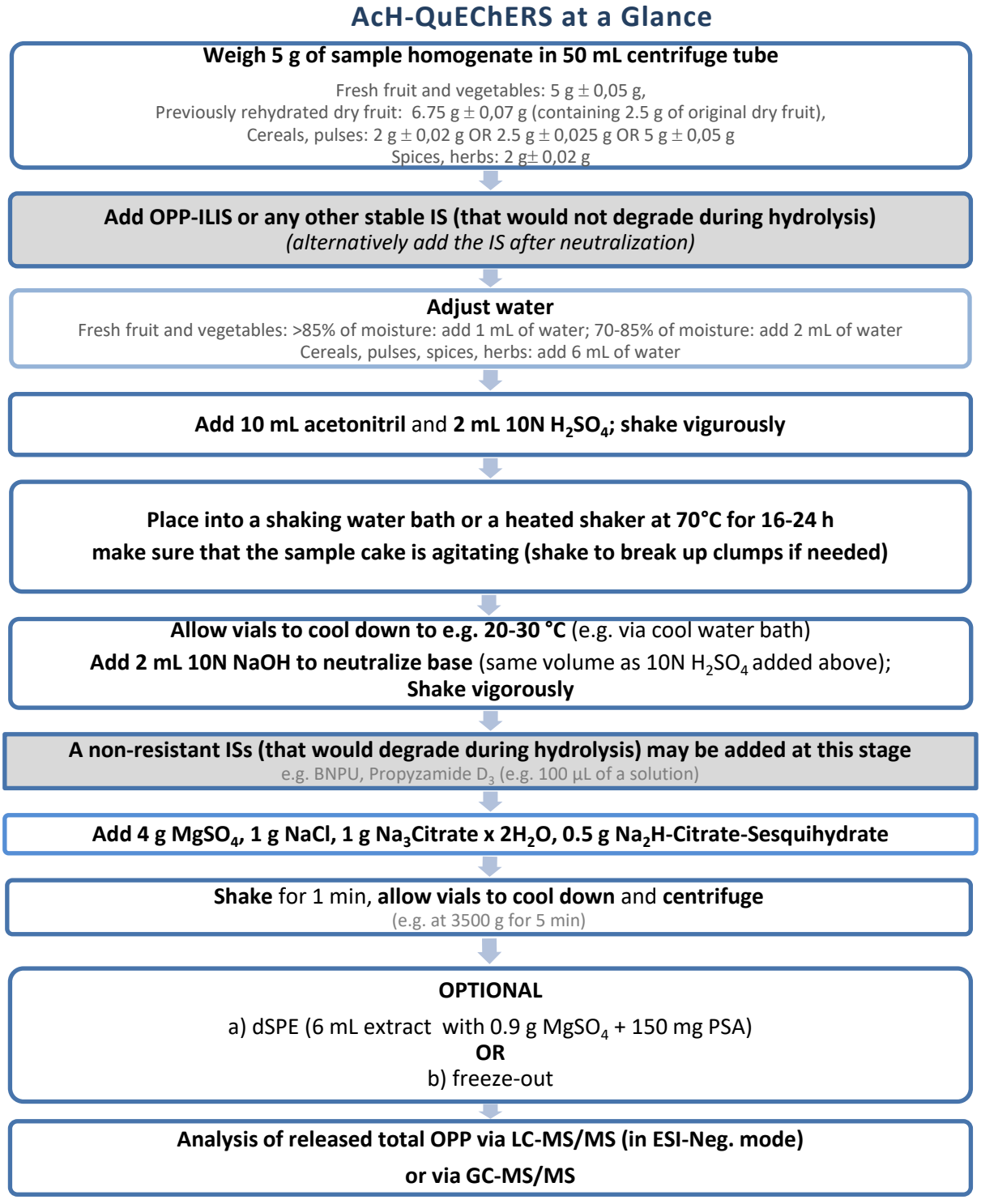


Figure 1: *Flow chart of the AcH-QuEChERS for the analysis of OPP (sum)*

Table 3: Instrumentation and method details for the analysis of OPP, OPP D_5 or OPP $^{13}C_6$

Instrument parameters	Conditions					
LC-MS/MS System (exemplary)	(LC: Shimadzu Nexera LC40; MS: Sciex QTrap 5500+)					
Column/temperature	Acquity UPLC BEH C18,1.7 µm; 2.1 x 100 mm					
Pre-column	Pre-column Van Guard BEH C18, 1.7 µm					
Eluent A	0.01 % acetic acid in water (with 5% acetonitrile)					
Eluent B	0.01 % acetic acid in acetonitrile					
Elution	**%A**	**Flow [mL/min]**	**Time [min]**			
	80	0.4	0.00			
	70	0.4	4.00			
	10	0.4	7.00, hold till 8.50			
	80	0.4	8.60, hold till 14.50			
Injection volume	2 µL					
Acquired mass transitions (m/z)	**Compound**	**Mass transitions and their MS-parameters**				
		Q 1 (m/z)	**Q 3 (m/z)**	**DP[1] (V)**	**CE[2] (V)**	**CXP[3] (V)**
	OPP	169	115	-55	-44	-5
		169	141	-55	-32	-1
		169	93	-55	-36	-3
	OPP (D_5)	174	120	-55	-44	-5
	OPP ($^{13}C_6$)	175	121	-55	-44	-5
	Propyzamid-D_3 (internal standard)	257	231	-90	-20	-1
Ion source settings	**Ionisation mode**	ESI negative				
	Ion Spray Voltage	-4500 V				
	Temperature	470 °C				
	Curtain/Nebulizer/Heater Gas Flow	35 psi /60 psi / 70 psi				

DP: Declustering Potential; 2) CE: Collission Energy; 3) CXP: Cell Exit Potential

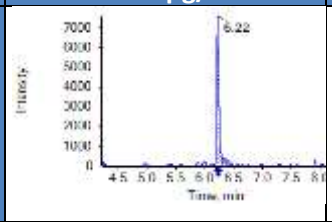
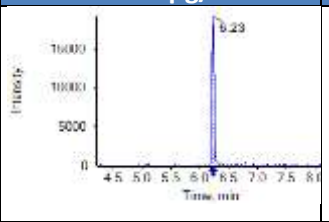
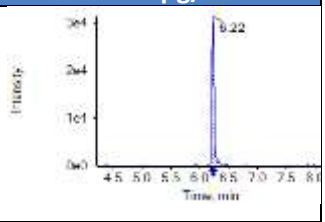
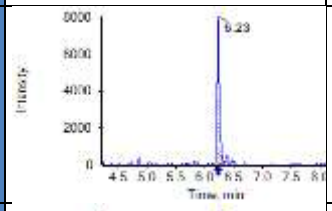
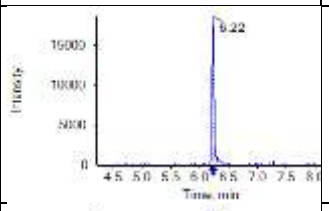
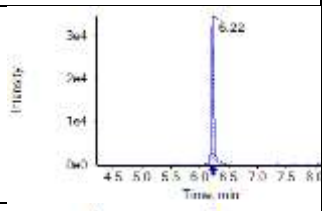
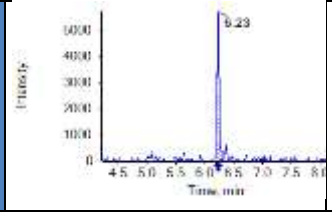
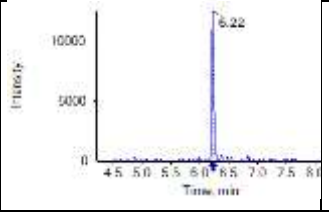
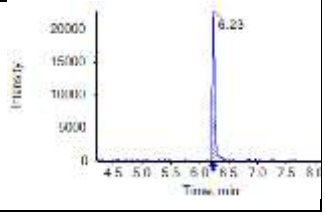
OPP (m/z) 169/115	**0.005 µg/ml**	**0.0125 µg/ml**	**0.025 µg/ml**
Spiked **acetonitrile**			
Spiked **QuECHERS** extract of Pears			
Spiked **AcH-QuECHERS** extract of Pears			

Figure 2: *Exemplary chromatograms of OPP in pure solvent and pears extracts; m/z 169/115*

Table 4: Instrumentation and method details for the analysis of OPP-Glucoside (OPP-Glc)

Instrument parameters	Conditions					
LC-MS/MS System (exemplary)	(LC: Shimadzu Nexera LC40; MS: Sciex QTrap 5500+)					
Column/temperature	Acquity UPLC BEH C18,1.7 µm; 2.1 x 100 mm					
Pre-column	Pre-column Van Guard BEH C18, 1.7 µm					
Eluent A	0.1 % formic acid in water (with 5% methanol)					
Eluent B	0.1 % formic acid in methanol					
Elution	**%A**	**Flow [mL/min]**	**Time [min]**			
	80	0.4	0.00			
	70	0.4	4.00			
	10	0.4	7.00, hold till 8.50			
	80	0.4	8.60, hold till 14.50			
Injection volume	2 µL					
Acquired mass transitions (m/z)	**Compound**	**Mass transitions and their MS-parameters**				
		Q 1 (m/z)	**Q 3 (m/z)**	**DP[1] (V)**	**CE[2] (V)**	**CXP[3] (V)**
	OPP-Glc (HCOO-adduct)	377	169	-15	-22	-15
		377	45	-15	-60	-7
		378	45	-5	-48	-7
	Propyzamid-D_3 (internal standard)	257	231	-90	-20	-1
	BNPU [4] (internal standard)	174	120	-45	-16	-7
Ion source settings	**Ionisation mode**	ESI negative				
	Ion Spray Voltage	-4500 V				
	Temperature	470 °C				
	Curtain/Nebulizer/Heater Gas Flow	35 psi /60 psi / 70 psi				

1) DP: Declustering Potential; 2) CE: Collission Energy; 3) CXP: Cell Exit Potential; 4) Bis-Nitrophenyl Urea

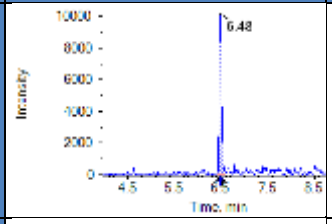
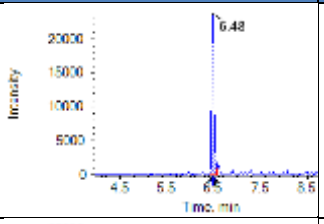
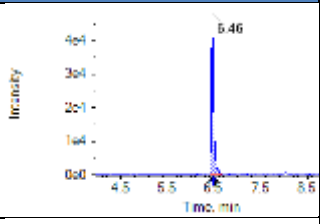
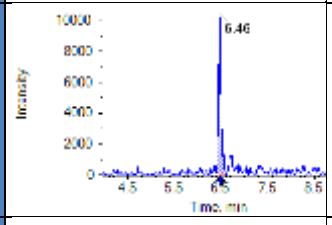
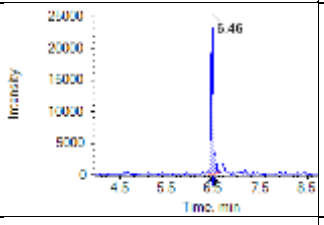
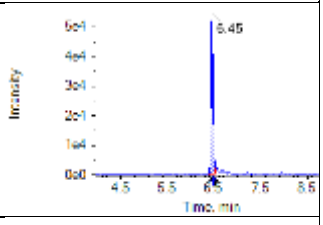
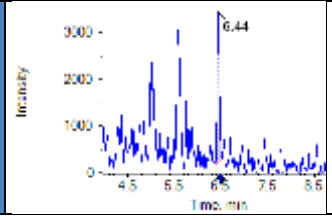
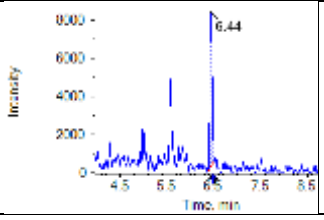
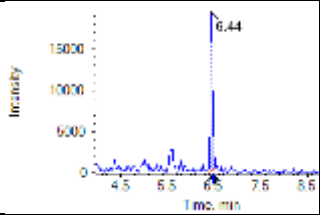
OPP-Glc (m/z) 377/169	**0.01 µg/ml**	**0.025 µg/ml**	**0.05 µg/ml**
Spiked **acetonitrile**			
Spiked **QuECHERS** extract of Pears			
Spiked **AcH-QuECHERS** extract of Pears			

Figure 3: Exemplary chromatograms of OPP-glucoside (OPP-Glc) in pure solvent and pears extracts; m/z 377/169

6. Hydrolysis of OPP-Glc

The hydrolytic behavior of OPP-Glc was tested both via acidic and alkaline hydrolysis. However, the glycosidic bond between OPP and glucose proved very resistant to alkaline hydrolysis. Acidic hydrolysis, being considerably faster was therefore pursued, while enzymatically catalyzed de-conjugations were left for a later stage.

Preliminary experiments showed that reaction speed can be considerably increased by rising the acid–to-sample-ratio. Doubling H_2SO_4 molarity from 5 to 10N and the added volume from 1 to 2 mL increased the reaction speed considerably, but also considerably increased the final volume of the aqueous phase - following neutralization. To speed-up the reaction further two options were left: a) increase the reaction temperature had to be increased beyond 60°C, which was initially preferred as it is also used for the de-conjugation of acidic herbicides temperature, and b) decrease the analytical portion size. Increasing temperature, however, was limited by the stability of the PP-falcon tubes. At temperatures >75°C, deformations of the tube caps were observed which resulted in occasional leakages during subsequent agitation steps. Although differences were observed between tubes manufacturers as regards the heat resistance, 70°C was considered a reasonable compromise. For rising the reaction speed further, it was decided to reduce sample weight (for fresh produce) from 10 g to 5 g keeping the molarity and volume of added H_2SO_4 (2 mL / 10 N).

Some exemplary results of experiments concerning the hydrolytic behavior of OPP-Glc are shown in ***Figure 4***, ***Figure 5*** and ***Figure 6***.

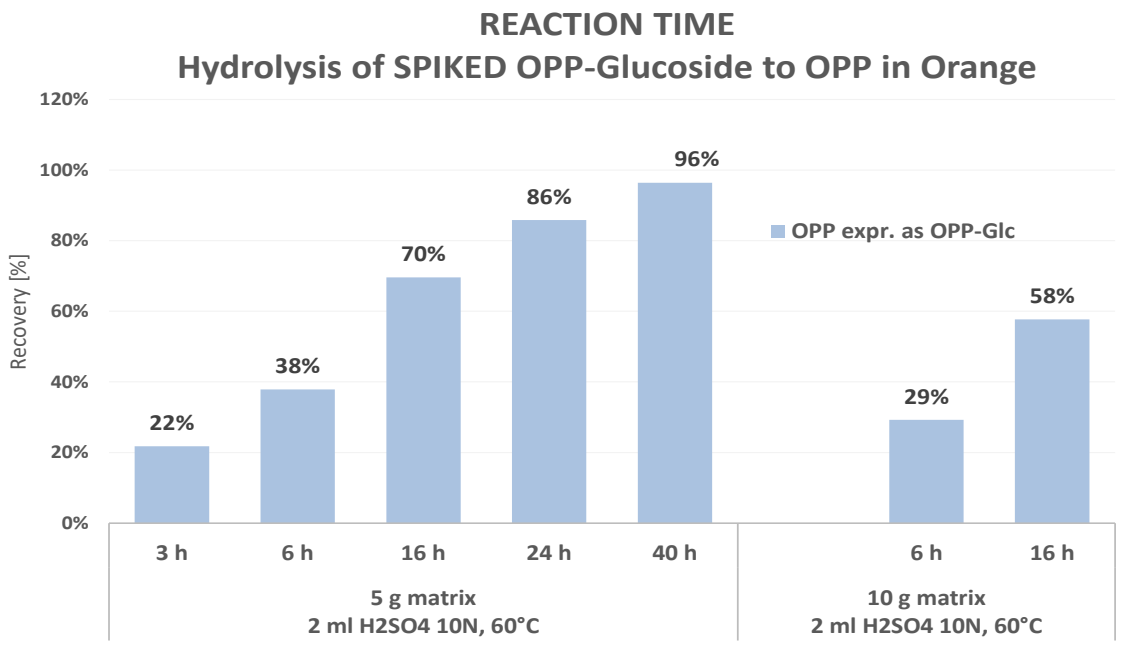


Figure 4: *Impact of hydrolysis duration and sample amount on the transformation of OPP-Glucoside (OPP-Glc) to OPP using AcH-QuEChERS. Matrix: Orange; Spiking: OPP-Glc at 0.5 mg/kg. N=3 for each tested condition. In all cases 2 mL 10N H2SO4 were added and hydrolysis took place within a heated shaker at 60°C. Reaction time varied as shown in the graph. Both OPP and OPP-Glc were measured via LC-MS/MS (see **Table 3** and **Table 4**)*

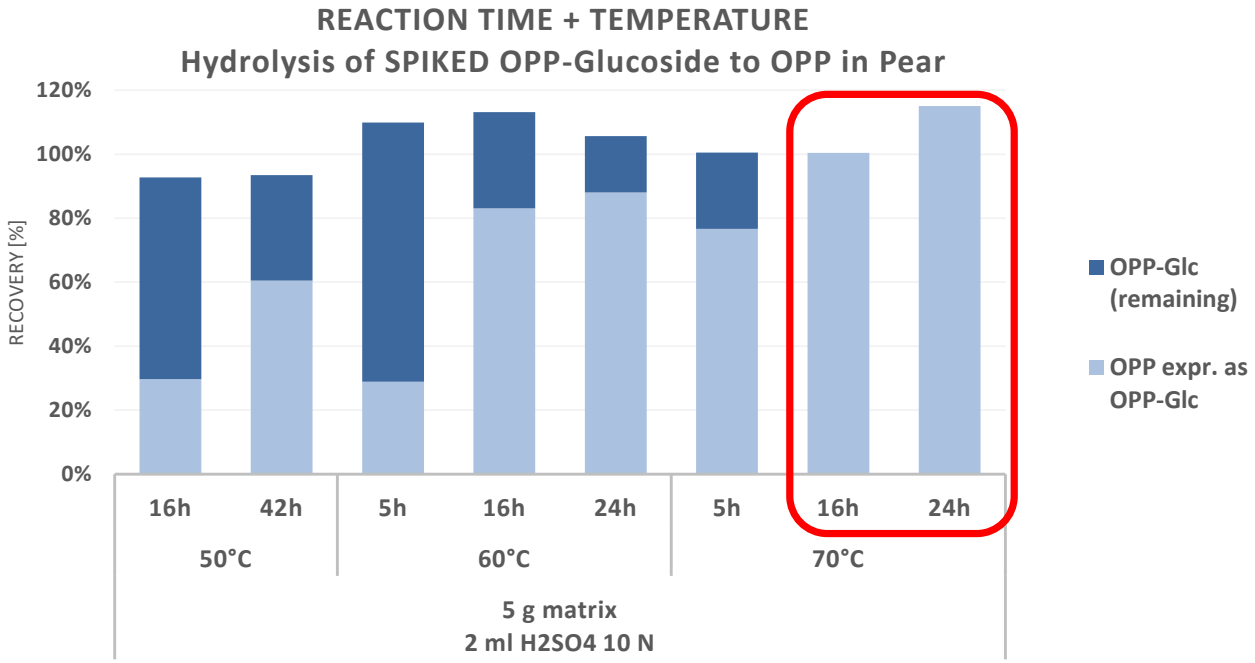


Figure 5: *Impact of hydrolysis duration and reaction temperature on the transformation of OPP-Glc to OPP using AcH-QuEChERS. Matrix: Pears; Spiking: OPP-Glc at 1 mg/kg. N=3 for each tested condition. In all cases 2 mL 10N H_2SO_4 were added to 5g sample and hydrolysis took place within a heated shaker. Reaction temperature and time varied as shown in the graph. Both OPP and OPP-Glc were measured via LC-MS/MS (see* ***Table 3*** *and* ***Table 4****).*

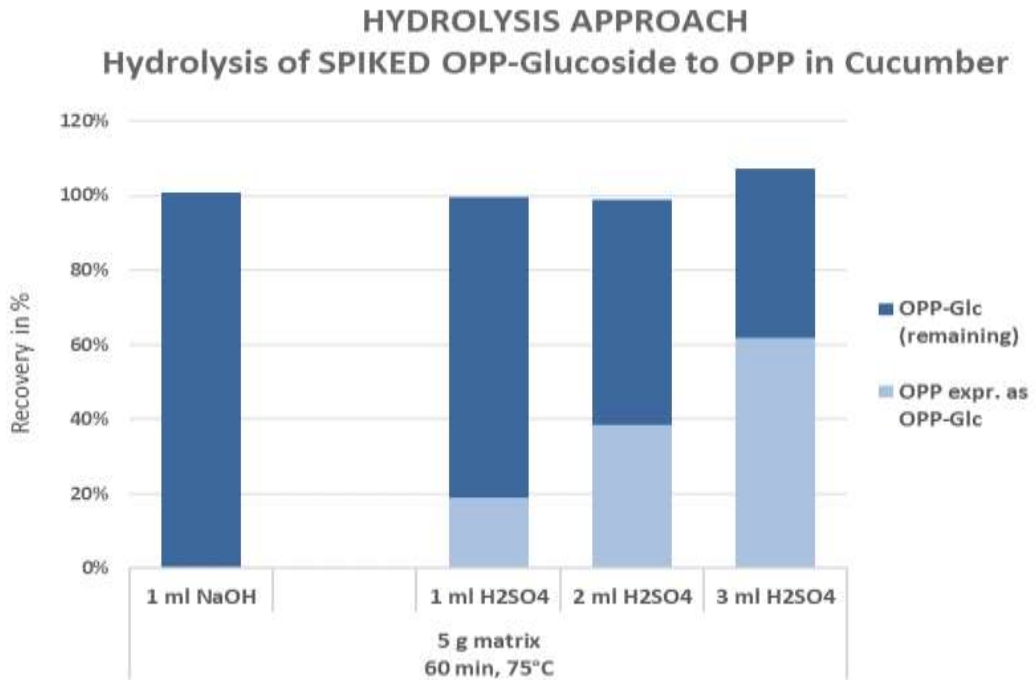


Figure 6: *Hydrolysis of OPP-Glucoside (OPP-Glc) to OPP via alkaline or acidic hydrolysis. Matrix: Cucumber; Spiking: OPP-Glc at 1 mg/kg. N=3 for each tested condition. Analysis using QuEChERS involving alkaline hydrolysis (AH) or acidic hydrolysis (AcH). Hydrolysis took place at 75°C for 60 min within a heated shaker. The amounts of base/acid used were as follows: In AH-QuEChERS (modified), 1 mL 10N NaOH was added to 5g sample (corresponds to 2 mmol/g); in AcH-QuEChERS: 1 mL or 2 mL or 3 mL 10N H2SO4 were added to 5 g sample (corresponds to 2, 4 and 6 mmol/g, respectively). Both OPP and OPP-Glc were measured via LC-MS/MS (see* ***Table 3*** *and* ***Table 4****). Note that at 75°C the PP-falcon tubes occasionally leaked due to deformation of the caps.*

7. Hydrolysis of incurred OPP-conjugates

The recovery of OPP-Glc via CEN-QuEChERS was determined at 96%. Alkaline hydrolysis (AH-QuEChERS) only marginally hydrolyzed OPP-Glc using the conditions applying for acidic herbicides. In contrast, acidic hydrolysis (AcH-QuEChERS) hydrolyzed OPP-Glc quasi quantitatively. The results of this experiment are shown in Figure 7.

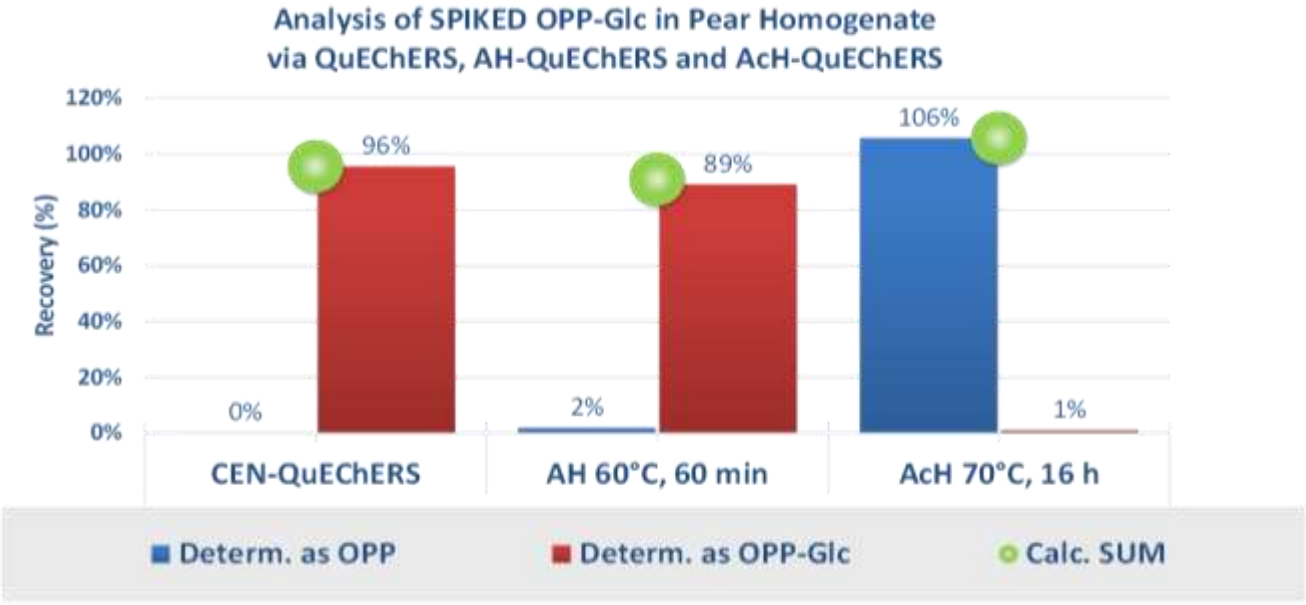


Figure 7: *Recoveries of OPP-Glc spiked on pears at 1 mg/kg. Analysis via AcH-QuEChERS (5g sample, 2 mL 10N H_2SO_4, 70°C/16h); AH-QuEChERS (5 g sample + 1 mL water + 2 mL 5N NaOH, 60°C/60 min) and CEN-QuEChERS (5g sample + 5 mL water); N=3 for each condition.*

In absence of pear samples with incurred OPP residues, immature organic pears from the market were superficially treated with an OPP-sodium salt solution. Spiking was conducted either by spreading multiple 5-10 µL portions of the SOPP solution throughout the surface of the pears (Sample 2), or by dipping the intact pears into a SOPP solution (Sample 3). The treated samples were stored in the dark at room temperature over 19 and 12 days, respectively. By that time the pears ripened and began showing signs of rotting. The pears were then coarsely cut, placed into a freezer overnight and cryogenically milled using dry ice. In parallel, non-treated pears were stored and used to prepare cryo-milled blank homogenates and extracts. The samples were extracted by QuEChERS, AH-QuEChERS and AcH-QuEChERS. Matrix-matched calibration as well as OPP-ILIS were used to minimize variability and bias. It should be noted however, that the cryogenically milled homogenate of sample 2 remained in the freezer for approximately 5 years before analysis.

Both samples were analyzed via CEN-QuEChERS and AcH-QuEChERS. Analysis focused on both free OPP and OPP-Glc. No other OPP conjugates were analyzed.

Sample 2 (see *Figure 8*) contained free OPP only in traces. Acidic hydrolysis released OPP in the range of 6.8 mg/kg. In a previous analysis 5 years earlier the determined level of free OPP in the homogenate was 0.53 mg/kg. However this analysis was run by GC and the OPP level might have been overestimated due to a release of OPP in the GC-injector. Furthermore, it cannot be excluded that free OPP might have slowly degraded or reacted otherwise over the 5-years of storage in the freezer. In the present experiment, OPP glucoside only contributed 22% to the total determinable OPP which was assumed being virtually entirely conjugated as no free OPP was detected. Following hydrolysis, there was still a small amount of intact OPP-Glc in the extract corresponding to ca 2% of OPP (sum). To some extent this might have originated from cellulose-bound OPP that was released gradually during hydrolysis. In **sample 3** (see *Figure 9*), acidic hydrolysis increased free OPP levels 4-fold. OPP-

conjugates made up for 75% of OPP (sum), while OPP-Glc made up for more than half (56%) of the conjugated OPP and 42% of the total OPP. By calculation, other types of OPP-conjugates must have contributed 44% to the conjugated OPP or 33% to the OPP (sum).

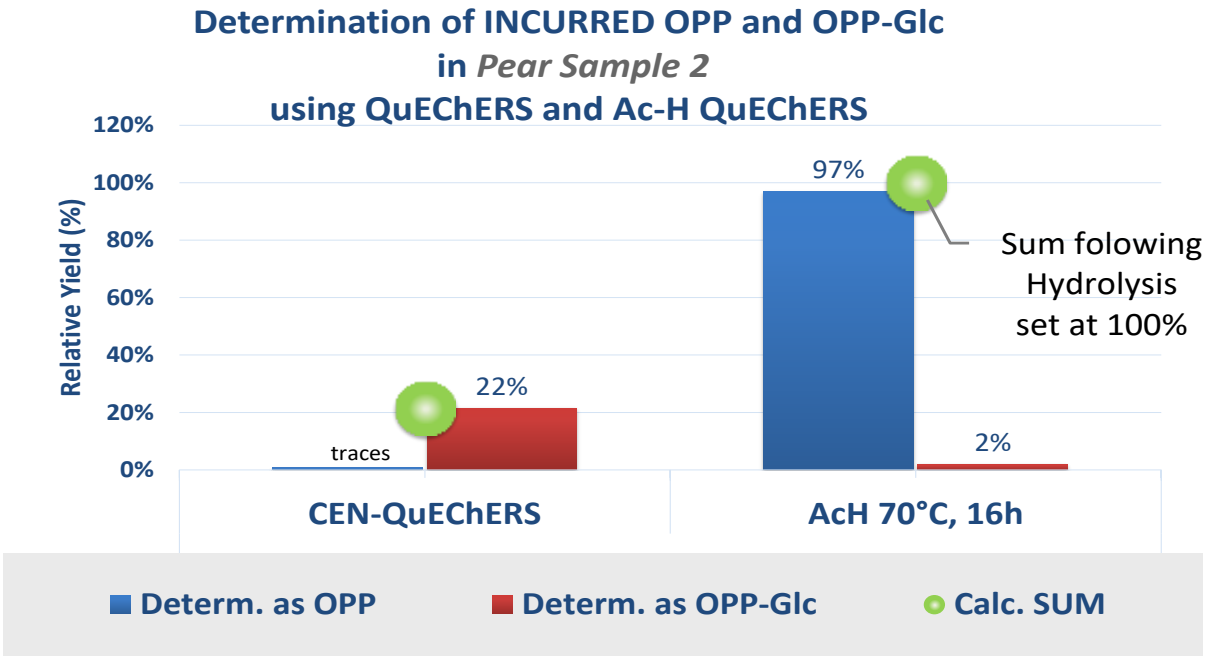


Figure 8: Relative yields of OPP and OPP-Glc obtained from a homogenate of pears which were treated with OPP in the laboratory. The homogenate was kept frozen for ca. 5 years before analysis. Among the OPP conjugates only OPP-Glc was analysed individually. AcH-QuEChERS: 5g sample, 2 mL 10N H_2SO_4, 70°C/16 h. N=3 for each condition.

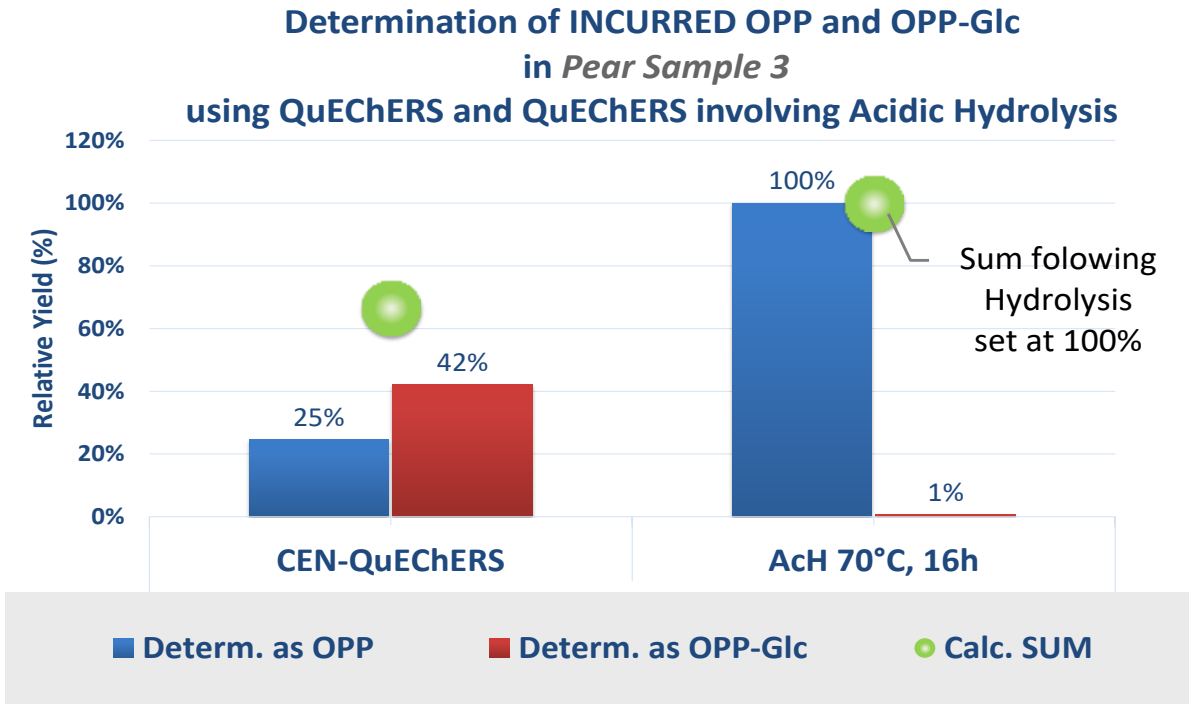


Figure 9: Relative yields of OPP and OPP-Glc obtained from a homogenate of pears which were treated with OPP in the laboratory. The homogenate was kept frozen for ca. 5 years before analysis. Among the OPP conjugates only OPP-Glc was analysed individually. AcH-QuEChERS: 5g sample, 2 mL 10N H_2SO_4, 70°C/16 h. N=3 for each condition.

Analysis of other samples entailing incurred OPP showed a very moderate increase in OPP levels following AcH-QuEChERS: In a mandarine sample, the OPP level determined using CEN-QuEChERS was 0,44 mg/kg and using AcH-QuEChERS it was 0,60 mg/kg (an increase of 35%). In the case of dried orange peel the respective shift was from 1.72 mg/kg to 2,21 mg/kg (+28%), whereas in grapefruit (which contains very little sugar on the peel the shift was only 2% (0.57 to 0.58 mg/kg). In all cases the analysis was run in triplicate while ILIS was used to correct for bias. Interestingly, no increase in the OPP levels was noticed in any of these samples when employing AH-QuEChERS, which suggests that any conjugates via ester bonds (e.g. with fatty acids) are of minor importance in these matrices. Further investigations in this direction are planned.

8. Validation data:

Validation experiments using the AcH-QuEChERS were conducted by spiking OPP-Glc to the matrix and determining the released OPP, which was then expressed as OPP-Glc.

Recovery experiments were conducted on oranges (representing commodities with high acid content) on pears and cucumbers (representing commodities with high water content) and on dried beans (representing dry commodities with low fat content)[16]. The analytes were spiked in quintuplicate to portions of sample homogenates, see Table 5. Note that with dried beans the recovery rates using 5g sample portions were lower than when 2 g portions were employed

Table 5: *Results of validation experiments on OPP-Glc following conversion to OPP via via AcH-QuEChERS (involving acidic hydrolysis for 16 h at 70°C), each n = 5. Recoveries = Rec.; Relative Standard Deviations = RSD*

Matrix	Extraction Method	Sample weight	Spiking level (mg/kg) OPP-Glc	Mass Transition (m/z)	Calculation using matrix-matched calibration	
					Mean Rec. (ILIS-corrected) (%)	RSD (±%)
Pears	AcH-QuEChERS	5g	0.1	169/115	101	7.3
				169/141	99	7.9
				169/93	101	9.6
Oranges			0.1	169/115	101	7.4
				169/141	91	8.6
				169/93	99	12.0
Dried Beans		2g	0.05	169/115	104	4,4
				169/141	97	15,4
				169/93	103	5,7
		2g	0.2	169/115	93	5.1
				169/141	98	3.3
				169/93	91	4.4
		5g	0.4	169/115	85	7.5
				169/141	86	7.3
				169/93	80	12.2

The "absolute" recovery of OPP in AcH-QuEChERS spiked as such, determined via matrix-matched calibration was 97% (n=5). It can be concluded that OPP shows a good hydrolytic stability during AcH-QuEChERS. Additional recovery experiments were also run in duplicate on miscellaneous matrices in order to check the impact of the matrix on the hydrolysis rate of OPP-Glc. In parallel to these experiments the matrix effects on OPP in extracts after hydrolysis were measured by comparison with equally concentrated standards in pure solvent. The results of these experiments are shown in ***Table 6***.

[16] According of the grouping of commodities in Document Nº SANTE/11312/2021 V2;

Table 6: Recoveries of OPP following spiking with OPP-Glc and conversion to OPP via AcH-QuEChERS (involving acidic hydrolysis for 16 h at 70°C in a shaking heater), recovery experiments in duplicate with few exceptions.

	Average Recovery (n=2), spiked OPP-Glc determined as OPP	Matrix Effects[17] on free OPP using AcH-QuEChERS (CEN-QuEChERS)
Rice 5g	108%	-8%
Wheat semolina 5g	99%	-26%
Hazelnut 2g	85%	-31%
Pears 5g	101% (n=5)	-38% (+7% in CEN-QuEChERS)
Beans 2g	94%	-45% (-26% in CEN-QuEChERS)
Linseed 2g	No OPP-Glc spiked in this experiment	-42%
Chia seeds 2g	100%	-47%
Lentils, dried, green 2g	105%	-51%
Oats 5g	96%	-60%
Oranges 5g	101% (n=5)	-67%
Millet 5g	119%	-68%
Cardamom 2g	No OPP-Glc spiked in this experiment	-81%
Paprika spice 2g	No OPP-Glc spiked in this experiment	-84%
Oregano 2g	No OPP-Glc spiked in this experiment	-88%

9. Miscellaneous hints and aspects

a. Issues with GC Analysis

Analysis of OPP via GC is tricky as it seems that OPP is formed as an artefact during injection. This aspect is being investigated. It was further found that upon injection of OPP-Glc, OPP is formed to some extent and it is strongly assumed that other sugar-conjugates of OPP also release OPP during GC injection. In presence of OPP conjugates, the analysis of free OPP is likely to lead to erroneous results. But even in absence of OPP (based on LC-MS/MS analysis results), all measured MRM-transitions of OPP showed signals at the correct RT, with a matching peak shape and at a matching ion ratio. This suggests the formation of OPP as an artefact in GC. A formation of OPP during coffee-roasting was also reported previously[11].

Another problematic issue with the GC analysis of OPP is that it tends to interact with surfaces within the GC system via its polar OH group. This leads to peak tailing. When injected in pure solvent (no competition) the tailing becomes very broad (over many minutes) at times, depending on the condition of the system. Especially in the case where levels are small, a large fraction of the peak cannot be integrated as it merges with the background. In presence of matrix and especially when adding analyte protectants (APs)[18], the OPP peak becomes sharper, at the same time OPP artefacts also become more visible. In extracts cleaned-up with PSA peak tailing is stronger. Interestingly, OPP-Glc also gives a peak in GC, but its suitability for quantification is questionable due its tendency to break-down in the injector, releasing OPP (see above).

Overall, GC-analysis of OPP is more sensitive than LC-MS/MS analysis, but also more affected by peak broadening and OPP-artefacts.

[17] Calculated using the following formula: (Area in Matrix-Based Standard/ Area in Solvent-Based Standard) -1, expressed in %. To prepare the matrix-based standards, blank matrix was extracted by AcH-QuEChERS and the extract was spiked with free OPP at the same concentration as the solvent-based standard.

[18] https://www.eurl-pesticides.eu/library/docs/srm/EURL_Observation-APs.pdf

b. Analysis of OPP via LC-MS/MS

In the past OPP was regarded not amenable to LC-MS/MS due to its poor ionization behavior. With older generation instruments the sensitivity achieved was not sufficient for routine residue analysis. But nowadays analysis down to 1 ng/mL in pure solvent is possible. Matrix effects and mass-spectrometric interferences, especially when acidic hydrolysis was previously conducted, compromise the ability to quantify at low levels (see also *Table 6*). This aspect need attention when it comes to quantification or when setting reporting limits.

c. Analysis of OPP-Glc via LC-MS/MS

OPP-Glc can be analysed as such as it shows good recovery rates by QuEChERS. However, analysis via LC-MS/MS requires attention to the eluent. Addition of formic acid promotes the formation of formiate adducts, which can be used as parents in MS/MS ion transitions.

d. Hydrolysis of esters of acidic herbicides via AcH-QuEChERS

Some commodities, and in particular citrus fruits, contain both OPP as well as carboxylic acid pesticides (e.g. 2,4-D and 2,4-DP), which also require de-conjugation during analysis. In such cases, it would be advantageous if de-conjugation covered both OPP-conjugates as well as the conjugates and esters of such acidic pesticides.

As alkaline hydrolysis does not work for OPP-conjugates, the ability of AcH-QuEChERS to breakup resistant esters of phenoxyalkanoic acids was checked. In previous experiments (not shown here) it was shown that acidic hydrolysis (using the same molarity, temperature, and time as AH-QuEChERS) is less effective than AH-QuEChERS to break up these esters, but the AcH-QuEChERS method presented here uses a higher temperature a higher acid-to-sample ratio, a higher molarity as well as an extended reaction time. It turned out that under the AcH-QuEChERS conditions even some of the most resistant esters break-up to a satisfactory degree (see *Figure 10*).

In a separate experiment 2,4-D glucoside (2,4-D-Glc) quantitatively transformed to free 2,4-D using the present AcH-QuEChERS method (Matrix: dried beans; spiking level 0.02 mg/kg; 2,4-D-Glc; avg. conv. yield to 2,4-D 101%; RSD 4%; n=5).

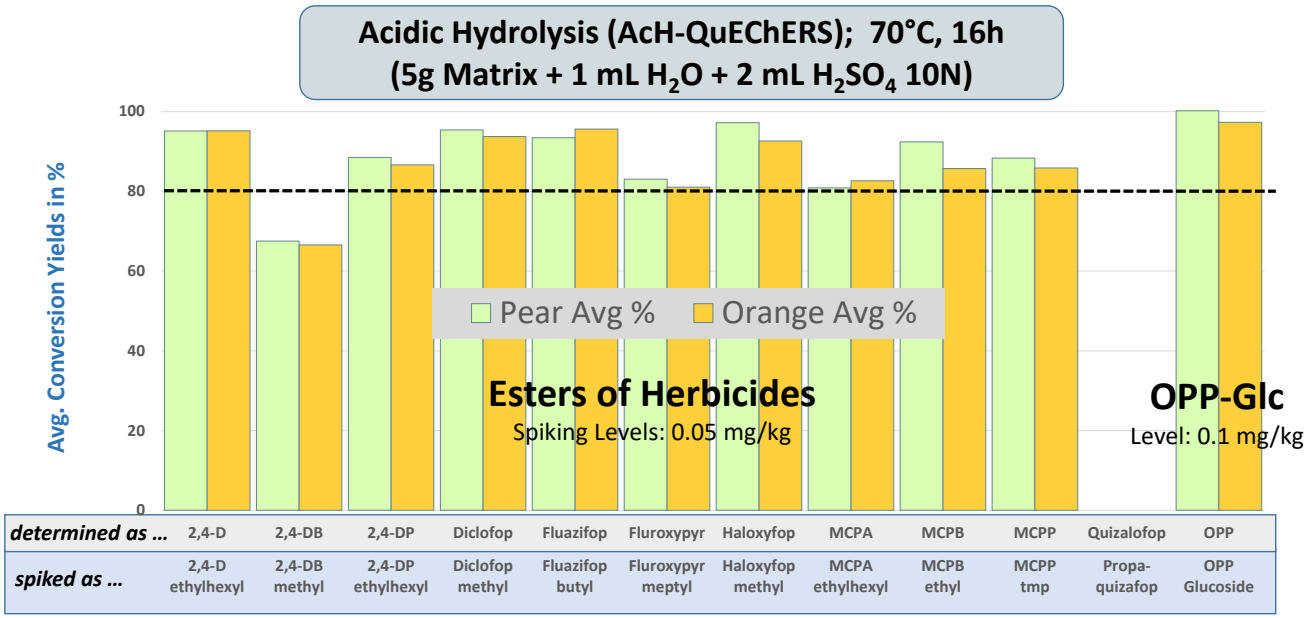


Figure 10: *Conversion yields of OPP-Glc and esters of acidic herbicides using AcH-QuEChERS (5g sample, 2 mL 10N H_2SO_4, 70°C/16 h). N=3 for each condition*

10. Conclusions

The glycosidic linkage of OPP to sugars is chemically quite resistant to alkaline hydrolysis but also to weak acidic hydrolysis (this applies to all sugar conjugates of phenols). The developed Ac-QuEChERS method entails a very strong hydrolysis approach with a high acid-to-sample ratio (5g sample + 2 mL 10 N H_2SO_4 for high water content commodities), a high temperature (70°C) and a long reaction time (16-24 h).

Validation experiments demonstrated that AcH-QuEChERS effectively breaks-up sugar conjugates. Other potential conjugates e.g. via ester bonds were not investigated but it is expected that these would also hydrolyse under such strong conditions. The duration of the reaction (16-24 h) is convenient from the practical point of view as the reaction can run unattended overnight. Unfortunately, the temperature required for AcH-QuEChERS (70°C) is higher than that of AH-QuEChERS (60°C), which does not serve a simultaneous conduction of the two methods within the same heatable shaker.

LC-MS/MS is proposed for measurement as interferences were observed in GC-MS/MS, which are suspected being OPP artefacts formed in the injector. Such signals are observed when injecting extracts of blank matrices even when no signals are detected in LC-MS/MS. This aspect needs to be investigated further. A release of OPP was also observed when injecting OPP-glucoside (OPP-Glc), possibly due to a decomposition in the injector,

Direct analysis of OPP-Glc via QuEChERS is possible as recoveries are satisfactory and measurement by LC-MS/MS is sensitive. Nevertheless, OPP (sum) cannot be simply calculated by adding up the results of OPP and OPP-Glc, as other types of conjugates form a considerable share of conjugated-OPP.

As ACH-QuEChERS was effective in breaking up esters and glucoside conjugates of phenoxyalkanoic acids, this approach might also be useful for covering the full RD of acidic pesticides such as 2,4-D. This aspects needs further investigation.

11. Document History

Action	When	Changes / Actions	Document Version
Conduction of experiments	2018-2024		
Drafting the document	Q4 2024 and Q1 2025		
Publication of V1	February 2024		V1