EURL-SRM - Analytical Observations Report

concerning the following…

- **Compound(s)**: Acidic Herbicides, their esters and conjugates
- **Commodities**: Various of plant and animal origin
- **Extraction Method(s)**: QuEChERS entailing alkaline or enzymatic hydrolysis steps
- **Instrumental analysis**: LC-MS/MS

Analysis of Acidic Pesticides Entailing Conjugates and/or Esters in their Residue Definitions

Version 1 (last update: 04.03.2020)

1. Overall Background:

Nature of conjugated residues:

Pesticides and metabolites entailing carboxy- phenol-, amino- or other reactive chemical groups, tend to undergo covalent bonds with certain molecules within plants or animals. This process often fulfils the purpose of detoxifying and/or facilitating the excretion of xenobiotic chemicals. The bound residues formed are commonly known as “conjugated residues” or “conjugates”. Typical conjugation partners in plants include sugars, sugar derivatives, amino acids, fatty acids and alcohols. The extent and nature of conjugate formation can vary considerably, not only between pesticides, but also for the same pesticide between different crop types. Even within the same crop species, the conjugation pattern of a pesticide can vary significantly, depending on numerous factors, such as the growth stage of the plant, the timing of pesticide application, the form of application (influencing the distribution within the plant), and the climatic conditions. Similar aspects apply to food of animal origin, with a different range of conjugation partners being favoured, such as sulphates, phosphates, amino acids, sugars and sugar-derivatives such as glucuronic acid. In many cases, the original pesticide or metabolite can be released when the matrix is subjected to hydrolysis.

Upon consumption of food containing conjugated compounds, these will, to some extent, hydrolyse within the human intestinal tract, thus becoming bioavailable and relevant for risk assessment. Therefore, where compounds of interest are extensively conjugated, this is taken into consideration when setting residue definitions (RDs) and MRLs. The conjugates are sometimes only considered in the RDs for risk assessment (applying conversion factors to extrapolate from the determined free form of the residue to the total residue including conjugates) and sometimes also in the RDs for enforcement. The variable degree of conjugation, even within the same type of crop, compromises the ability to set reliable conversion factors to account for conjugates for risk assessment purposes. Where the RD for enforcement purposes entails (unspecified) conjugates, labs need to consider this in analysis and apply procedures breaking up conjugates.

Table 1 gives a brief overview of RDs entailing conjugates, either of the parent molecules, or of specific metabolites or both. For some compounds (e.g. amitraz and prochloraz) the residue definitions refer to "common moieties" rather than to "conjugates". At first sight, there is some uncertainty as to whether such RDs only include the metabolites containing the common moiety or also conjugates thereof. However, as the release of a common moiety from an unspecified range of metabolites requires applying a hydrolysis step, which will inevitably also release the common moiety from conjugates, it becomes clear that these conjugated metabolites need to be considered as part of the residue definitions. In case of doubts, common moiety methods submitted by applicants for registration purposes may need to be consulted.

In rare cases, RDs include well defined conjugates (e.g. spirotetramate enol glucoside, or 6-hydroxymethylpymetrozine phosphate). Where such compounds are stable enough to be analyzed directly, a hydrolysis step is not needed.

Table 1: *Examples of residue definitions entailing conjugates*

Residue Definitions	RD applies to matrices* of …	Conjugates of … (P= Parent; M= Metabolite)	
		Specified	Unspecified
2,4-D (sum of 2,4-D, its salts, its esters and its conjugates, expressed as 2,4-D)	PO+AO		P (carboxylic)
2-phenylphenol (sum of 2-phenylphenol and its conjugates, expressed as 2-phenylphenol)	PO		P (phenolic)
Bentazone (Sum of bentazone, its salts and 6-hydroxy (free and conjugated) and 8-hydroxy bentazone (free and conjugated), expressed as bentazone)	PO+AO **		M (phenolic)
Pyridate (sum of pyridate, its hydrolysis product CL 9673 (6-chloro-4-hydroxy-3-phenylpyridazin) and hydrolysable conjugates of CL 9673 expressed as pyridate)	PO+AO		M (phenolic)
Acibenzolar-S-methyl (sum of acibenzolar-S-methyl and acibenzolar acid (free and conjugated), expressed as acibenzolar-S-methyl)	PO+AO		M (carboxylic)
Ethofumesate (Sum of ethofumesate, 2-keto–ethofumesate, open-ring-2-keto-ethofumesate and its conjugate, expressed as ethofumesate)	PO+AO		M (carboxylic)
Amitraz (amitraz including the metabolites containing the 2,4 -dimethylaniline moiety expressed as amitraz)	PO+AO		M (anilinic)***
Prochloraz (sum of prochloraz and its metabolites containing the 2,4,6-Trichlorophenol moiety expressed as prochloraz)	PO+AO		M (phenolic)***
Chlorpropham - codes 1016000 and 1030000: chlorpropham and 3-chloro-4-hydroxyaniline conjugates, expressed as chlorpropham;	AO		M (anilinic)
Spirotetramat and its 4 metabolites BYI08330-enol, BYI08330-ketohydroxy, BYI08330-monohydroxy, and BYI08330 enol-glucoside, expressed as spirotetramat	PO	M (hydroxy)	
Chlorpropham - code 1000000 except 1016000, 1030000 and 1040000 : Chlorpropham and 4´-hydroxychlorpropham-O-sulphonic acid (4-HSA),expressed as chlorpropham	AO	M (phenolic)	
Code 1020000: pymetrozine, 6-hydroxymethylpymetrozine and its phosphate conjugate, expressed as pymetrozine	AO	M (phenolic)	

* PO= Matrices of plant origin; AO= Matrices of animal origin

** only 6-OH-bentazone and its conjugates are included in the RD for food of animal origin (not 8-OH bentazone)

*** There is some uncertainty as to whether conjugated forms of metabolites containing the respective moieties are also included in the RD, see also text.

2. Background on acidic pesticides forming conjugates:

Many of the pesticides entailing conjugates in their residue definitions contain carboxy- or phenolic groups and act as herbicides. They are sometimes applied to clear the fields prior to the growing period of the main crop (pre-seeding/planting); sometimes during the growing period; and sometimes at the very end of the cultivation period to facilitate mechanical harvesting by drying out the plants (desiccation). Some herbicides, such as the phenoxyalcanoic acids, also exhibit an auxin hormone activity, and are thus also used as plant growth regulators at low concentrations. A special case of a growth regulator use is the post-harvest application of 2,4-D on citrus to delay the ageing process of the fruit peel.

Acidic pesticides are employed as free acids, salts or esters. Free acids and salts readily dissociate in contact with water and mostly exhibit a good water solubility, which increases with increasing pH. In formulations, the most common counter ions of acidic herbicides are ammonium derivatives, such as dimethylammonium, isopropylammonium, triisopropanolammonium and diethanolammmonium. Alkali-salts are nowadays less commonly used in formulations. The free acids (irrespective if applied as such or as salts) will typically find their way into the target plants through the roots. Within the plants the generated free acids can undergo conjugation forming a pool of interconvertible free and conjugated forms.

When applied as esters, which are more lipophilic, the compounds may also enter the interior of the plants through the leaves. Some esters of acidic pesticides are more persistent than others resulting in measurable residues in harvested crops. In most cases, however, the esters applied in the field will hydrolyse quickly with no detectable residues being found in the harvested crops. Still, esters need to be formally included in enforcement RDs in order to cover cases of late applications.

Table 2 gives an overview of the RDs of acidic herbicides that entail conjugates. The RDs applying for these compounds in 2008, are also shown. During this period the RDs and MRLs of many compounds were re-evaluated according to Article 12 of Regulation 396/2005/EC, which in many cases resulted in modifications of the RDs. Overall, we can see a trend to harmonize RDs in order to include free acids, esters and conjugates. Compounds with residue definitions entailing only acids and unspecified esters but not conjugates (e.g. the former RDs of 2,4-D, Fluroxypyr and Ioxynil) were problematic, due to the difficulty to analytically distinguish between ester-bound and conjugated residues. Currently, only the RD for 2,4,5-T shows this pattern. The residue definition of Diclofop contains a specified ester, which can be analyzed as such.

Table 2: Residue definitions of various acidic herbicides - current state and comparison with the RDs that were valid in 2008

Compound	Residue definition	Free	Bound (hydrol. needed)	
		Acids (incl. Salts)	Esters	Conj.
MCPA / MCPB	**2020**: MCPA and MCPB (MCPA, MCPB including their salts, esters and conjugates expressed as MCPA)	X	X	X
	2008: MCPA and MCPB (MCPA, MCPB including their salts, esters and conjugates expressed as MCPA)	X	X	X
Haloxyfop	**2020**: Sum of haloxyfop, its esters, salts and conjugates expressed as haloxyfop (sum of the R- and S- isomers at any ratio))	X	X	X
	2008: Haloxyfop including haloxyfop-R (Haloxyfop-R methyl ester, haloxyfop-R and conjugates of haloxyfop-R)	X	*X-Sp*	X
Fluazifop	**2020**: Sum of all the constituent isomers of fluazifop, its esters and its conjugates, expressed as fluazifop)	X	X	X
	2008: Fluazifop-P-butyl (fluazifop acid (free and conjugate))	X	*X-Sp ?*	X
2,4-D	**2020**: Sum of 2,4-D, its salts, its esters and its conjugates, expressed as 2,4-D)	X	X	X
	2008: 2,4-D (sum of 2,4-D and its esters expressed as 2,4-D)	X	X	
Fluroxypyr	**2020**: Sum of fluroxypyr, its salts, its esters, and its conjugates, expressed as fluroxypyr)	X	X	X
	2008: Fluroxypyr (fluroxypyr including its esters expressed as fluroxypyr)	X	X	
2,4-DB	**2020**: Sum of 2,4-DB, its salts, its esters and its conjugates, expressed as 2,4-DB)	X	X	X
	2008: 2,4-DB	X		
Dichlorprop	**2020**: Dichlorprop (Sum of dichlorprop (including dichlorprop-P), its salts, esters and conjugates, expressed as dichlorprop	X	X	X
	2008: Dichlorprop, incl. Dichlorprop-P	X		
Propaquizafop/ Quizalofop	**2020**: Quizalofop (sum of quizalofop, its salts, its esters (including propaquizafop) and its conjugates, expressed as quizalofop (any ratio of constituent isomers))	X	X	X
	2008: Propaquizafop		*X-Sp*	
	2008: Quizalofop, incl. quizalfop-P	X		
Acibenzolar-S-methyl	**2020**: Acibenzolar-S-methyl (sum of acibenzolar-S-methyl and acibenzolar acid (free and conjugated), expressed as acibenzolar-S-methyl)	X	*X-Sp*	X
	2008: Acibenzolar-S-methyl		*X-Sp*	
2,4,5-T	**2020**: Sum of 2,4,5-T, its salts and **esters**, expressed as 2,4,5-T)	X	X	
	2008: 2,4,5-T	X		
Diclofop	**2020**: Diclofop (sum diclofop-methyl and diclofop acid expressed as diclofop-methyl)	X	*X-Sp*	
	2008: Diclofop (sum diclofop-methyl and diclofop acid expressed as diclofop-methyl)	X	*X-Sp*	

Compound	Residue definition	Free	Bound (hydrol. needed)	
		Acids (incl. Salts)	Esters	Conj.
1-Naphthylacetamide/ 1-naphthylacetic acid	**2020**: 1-Naphthylacetamide and 1-naphthylacetic acid (sum of 1-naphthylacetamide and 1-naphthylacetic acid and its salts, expressed as 1-naphythlacetic acid	X	*X (amide)*	
	2008: 1-Naphthylacetic acid	X		
	2008: 1-Naphthylacetamide		*X (amide)*	
Bromoxynil	**2020**: Bromoxynil and its salts, expressed as bromoxynil	X (phenol)		
	2008: Bromoxynil (bromoxynil including its esters expressed as bromoxynil)	X (phenol)	X	
Ioxynil	**2020**: Ioxynil (sum of ioxynil and its salts, expressed as ioxynil)	X (phenol)		
	2008: Ioxynil, including its esters expressed as ioxynil	X (phenol)	X	
Fenoxaprop	**2020**: Fenoxaprop-P	X		
	2008: Fenoxaprop-P	X		
Mecoprop	**2020**: Mecoprop (sum of mecoprop-p and mecoprop expressed as mecoprop)	X		
	2008: Mecoprop (sum of mecoprop-p and mecoprop expressed as mecoprop)	X		
Prohexadione	**2020**: Prohexadione (acid) and its salts expressed as prohexadione-calcium)	X		
	2008: Prohexadione (prohexadione and its salts expressed as prohexadione)	X		
Clodinafop	**2020**: Clodinafop and its S-isomers and their salts, expressed as clodinafop	X		
	2008: Clodinafop and its S-isomers, expressed as clodinafop	X		
Trinexapac	**2020**: Trinexapac (sum of trinexapac (acid) and its salts, expressed as trinexapac)	X		
	2008: Trinexapac	X		
Dicamba	**2020**: Dicamba	X		
	2008: Dicamba	X		
Dalapon	**2020**: Dalapon	X		
	2008: Dalapon	X		
Triclopyr	**2020**: Triclopyr	X		
	2008: Triclopyr	X		
Cyhalofop	**2020**: Cyhalofop butyl		*X-Sp*	
	2008: Cyhalofop-butyl (sum of cyhalofop butyl and its free acids)	X	*X-Sp*	

How do laboratories deal with RDs entailing esters and conjugates?

The analysis of acidic pesticides entailing conjugates in their RDs has always been a problem to laboratories as the full RD typically cannot be covered by routine multiresidue methods (MRMs). The gradual harmonization of RDs to include both esters and conjugates, allows grouping of several acidic pesticides into one method, which is more attractive to laboratories. Nevertheless, the overall low probability of finding residues of acidic pesticides in food samples makes it difficult to justify the routine and indiscriminate application of such a method on all samples. A more favourable effort-to-benefit ratio is achieved when the automatic use of such an approach is restricted to specific sample types known to frequently contain compounds requiring the conduction of a hydrolysis step to cover the full RD (e.g. paprika powder samples which often contain 2,4-D and MCPA or citrus samples from overseas that often contain 2,4-D). For sample types where acidic pesticides are barely or never found, most labs would probably opt for applying a MRM first, and proceed with hydrolysis in case a marker compound (free acids or esters) exceeds a trigger level. To facilitate the selection of sample-types that would be analysed by a method involving hydrolysis from the beginning and on setting reasonable sample-type-specific trigger levels (that consider the typical share of conjugates to the total residue), there is a need for collecting information and spreading it to OfLs as well as monitoring program designers. The EURL-SRM is intending to pursue this task.

A true MRM involving a hydrolysis step would be desirable but its development seems very difficult as numerous labile MRM-compounds would not survive the hydrolysis step. Conducting hydrolysis on an aliquot of the final extract would also be a theoretical option. Such a procedure would cover any esters but from the conjugate-site only those would be covered that are both extractable and sufficiently lipophilic to end up in the raw extract of the MRM.

In principle, esters of acidic herbicides are amenable to MRMs, such as QuEChERS, and can be easily analysed as such both by GC- or LC-applications. Still, for laboratories the analysis of individual esters poses a dilemma for various reasons. Firstly, it is generally known, that the vast majority of esters quickly hydrolyse within plants, and that the chance of finding them in crops in intact form is low. Furthermore, considering worldwide uses, the number of different possible esters can be very large, especially for some compounds such as 2,4-D. An exemplary list of possible 2,4-D esters is given in **Table 3**. Covering this multitude of esters is impractical for the labs and it additionally complicates the setting of reasonable limits of quantification for the analysis of the full RD ("summed LOQs"). Hydrolysis of esters and conjugates to the corresponding free acids, which can be determined as single components with a defined LOQ, is thus the favoured approach. Challenging in this respect is the setting of mutually acceptable and recognized hydrolysis conditions. While hydrolysis rates of esters and available glucosides can easily be determined through experiments, there is always uncertainty as regards the release of residues that were naturally conjugated on samples. There are furthermore limitations in the validation of procedures releasing conjugates, due to the limited availability of analytical standards of conjugates and the non-availability of reference materials containing conjugated residues.

Table 3: Examples of possibly relevant 2,4-D esters

methyl~	2-ethylhexyl~	polypropoxybutyl~
ethyl~	nonyl~	tripropylene glycol~
propyl~	ethoxyethoxyethyl~	polypropylene glycol~
isopropyl~	ethoxyethoxypropyl~	propylene glycol butyl ether~
butyl~	butoxypropyl~	propylene glycol isobutyl ether~
isobutyl~	2-butoxyisopropyl~	chlorocrotyl~
octyl~	butoxy ethoxy propyl~	tetrahydrofurfuryl~.
2-octyl~	butoxy polyethoxypropyl~	

Many acidic pesticides entailing conjugates in their RDs are considered important and are thus included in the routine scope of many labs (e.g. 2,4-D, MCPA, Fluazifop, Haloxyfop and Quizalofop). At the same time, however, laboratories are reluctant to routinely cover the full RD of these compounds, as this would require parallel analysis by separate procedures involving a hydrolysis step. Therefore, most laboratories (**Group A**) only focus on components, which are amenable to MRMs, i.e. free acids and, in some cases, also specific esters explicitly mentioned in current or past RDs (e.g. Fluazifop-butyl, Propaquizafop, Haloxyfop-methyl). Individually analysed free acids or esters need to be reported separately accompanied by their proper LOQs. By skipping the hydrolysis the levels of the full RD remain unknown and in some cases even MRL-exceedances remain unnoticed. Some labs (**Group B**) go further with re-analysing samples, in which the above (MRM-amenable) marker components were found to exceed a certain trigger level. Very few, if any, labs (**Group C**) would routinely conduct a procedure covering the full RD from the very beginning. As mentioned above, this option may be restricted to selected commodities, where past experience or other background information indicates a high likelihood of finding the relevant compounds.

For risk assessors, the mixture of data derived from the abovementioned different groups of labs is difficult to handle. **Negative results**, reported by **Group A and B** labs would be largely comparable as long as the LOQs reported refer to the analysis of the individual compounds analyzed. Group B labs should be aware, that the non-detection of marker compounds by the initial procedure does not necessarily mean that the procedure covering the full RD will also lead to a negative result. Reporting "not detected" or "<LOQ" in combinations with the LOQ of the full-RD-procedure would be misleading. The most appropriate and descriptive entry for the "Full RD parameter" should thus be "Not analysed". In any case, Group B labs should aim to analysing the free components with low screening thresholds, so that re-analysis by the method covering the full RD is triggered at levels well below the LOQ of the full-RD-method. This is particularly important where conjugates form a very large share of the total residue in the sample and where the free acid concentrations are very low. For example, if the MRL of a compound is 0.02 mg/kg and from past experience it is known that the share of conjugates can be around 80 %, the trigger level should be reasonably set at 0.004 mg/kg or lower so that a numerical exceedance of the MRL can be detected and reported. Negative results from **Group C** labs would deviate, as the LOQs reported for negative findings would refer to the analysis of the full RD and not to that of the individual components. Where Group C labs analyse the free acids in parallel to the Full RD, the reported negative results should accompanied by the respective LOQ. As regards the **positive results** there are also differences between the lab groups. **Groups A** labs would only report results of individual components, whereas **Group B** labs would additionally report results for the full RD where the respective procedure was triggered. **Group C** labs

would either report data of individual components in parallel to results for the full RD, or only report results for the full RD.

Searching for a consensus approach

As conjugations can be very diverse in nature, and as conjugated target analytes are not necessarily all bioavailable, it becomes clear, that the focus of de-conjugation should not lie on the development of procedures ensuring full release of conjugated residues but rather on establishing a consensus approach. In a recent publication in which the EURL-SRM was involved, hydrolysis conditions were introduced that were proposed as a possible consensus[1]. These conditions were set considering the procedures employed in the analysis of residue trial samples by applicants of plant protection products. A drawback of **consensus extraction conditions** is that these cannot be easily transferred to approaches using different solvents, as the hydrolysis efficiency does not only depend on temperature, time and base-strength but also greatly on solvent composition.

A more universal approach is the setting of **consensus performance criteria**, which would need to be met by a method in order to be considered fit-for-purpose. The use of reference materials containing defined concentrations of conjugated residues would be an option for checking whether a method meets the criteria, but production and continuous quality control of such reference materials can be very troublesome and the logistics of distributing these materials to interested labs would also be troublesome. A more practical alternative for the labs is the selection of defined conjugates (e.g. glucosides, esters, amides) that should be readily available and that would need to be spiked onto analytical portions and sufficiently broken up during the procedure for demonstrating its fitness for the purpose. Whether this is done as a routine quality controls measure, during the routine or triggered application of the method or during initial validation should be left at the discretion of the labs. In case of a routine application, certain control conjugates that do not interfere with pesticide analysis (e.g. isotope labelled conjugates) would be required.

[1] Development of a QuEChERS-Based Method for the Simultaneous Determination of Acidic Pesticides, Their Esters, and Conjugates Following Alkaline Hydrolysis. Steinborn A, Alder L, Spitzke M, Dörk D, Anastassiades M. J Agric Food Chem. 2017 Feb 15;65(6):1296-1305.

3. Development of QuEChERS-based methods entailing a hydrolysis step

Alkaline hydrolysis preceding QuEChERS for breaking up conjugates (prior to adding acetonitrile)

One of the first analytical projects of the EURL-SRM was the development of a QuEChERS-based method covering conjugated residues of acidic pesticides. The developed method entailed an alkaline hydrolysis module that was conducted just before the actual citrate buffered QuEChERS procedure. The hydrolysis conditions chosen were relatively mild, involving addition of water (at the amounts foreseen in the QuEChERS protocol), addition of 5N NaOH solution, to reach a pH of ~12-13, and a brief incubation of the mixture for 30 min at room temperature. Before proceeding with QuEChERS, the base was neutralized by adding the same volume of 5N H_2SO_4. The amounts of base and acid varied: for high pH commodities (e.g. vegetables and cereals), 300 µL of each were used for the abovementioned pH adjustments; for acidic commodities, the added volumes increased to 500 µL for most commodities and to 1 mL for lemons.

Experiments with various samples containing conjugated phenoxyalkanoic acids, showed no further increase in the levels of the free acid when applying harsher hydrolysis conditions (see example in **Figure 1**).

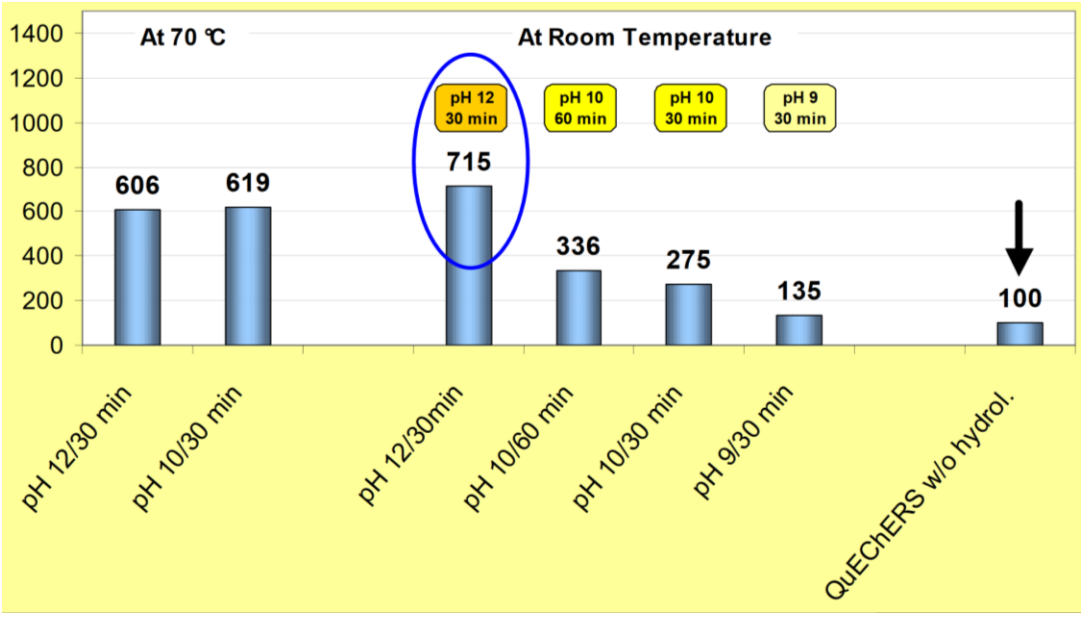

Figure 1: Impact of pH (9, 10 or 12), temperature (room temperature or 70°C) and time (20 or 60 min) on the release of conjugated residues from wheat containing incurred residues of MCPA (EUPT-SRM2 sample). Results by QuEChERS (without hydrolysis) were set at 100%

The above method[2] was distributed in 2007 to the participants of the EUPT-SRM2, with wheat as test material. The wheat had been treated with MCPA in the field, and therefore contained conjugated MCPA residues. Two years later (2009) oat containing incurred residues of Dicamba was used as test material for the EUPT-SRM4. The participants of both PTs were asked to determine both the free acids as well as the sum of acids following alkaline hydrolysis. The laboratories were free to use any method, but the vast majority sticked to the delivered method.

[2] https://www.eurl-pesticides.eu/library/docs/cf/acidicpesticides_wheat_quechers.pdf

In both PTs, a strong increment of the determined levels of MCPA / Dicamba was observed when conducting hydrolysis. When comparing the median values of the two result populations (free acid vs. sum following hydrolysis), there was a 7.1-fold increase in the case of MCPA (wheat) and a 2.5-fold increase in the case of Dicamba (oat). The PT materials contained additional acidic pesticides, which were spiked in the lab (MCPP on wheat and 2,4-D on oat). These two compounds showed a much lower share of conjugated residues and thus a more moderate concentration increment upon hydrolysis. These results are summarized in **Table 4**.

Table 4: *Overview of results for acidic pesticides analysed as free acids as well as following alkaline hydrolysis, and share of conjugates to the total residue*

	SRM2 (Wheat)				SRM4 (Oats)			
	MCPA		MCPP		Dicamba		2,4-D	
	Incurred		Spiked in lab		Incurred		Spiked in lab	
# Results	19	10	18	10	21	15	32	33
Median (mg/kg)	0.040	0.284	0.312	0.454	0.106	0.264	0.471	0.499
Increment factor	**7.1**		1.5		**2.5**		1.06	
Share of conjugated analyte on total analyte residue	**86%**		31%		**60%**		6%	

When developing the above method, the **main focus was on releasing conjugated residues** of acidic herbicides. Esters, possibly contained in the samples were not taken into account at this stage. Further experiments showed, however, that these mild conditions, which seemed effective in the case of conjugates, were not strong enough for the hydrolysis of many esters.

Enzymatic hydrolysis preceding QuEChERS

Aiming to find ways for breaking up hydrolysis-resistant ("difficult") esters without applying too harsh conditions, additional experiments were conducted involving catalysis by esterase enzymes. For example, 100µL of a suspension of porcine liver esterase (10 mg/mL; 130 U/mL) were added[3] to a QuEChERS-sized analytical portion of defrosted homogenates of fruits and vegetables (after adding water where this is foreseen in QuEChERS, e.g. cereals). Before adding the enzymes, 0.5 mL of a 1M phosphate buffer solution[4], was added to raise the pH to levels that are more favourable for esterase activity (6.5-8.5). In the case of acidic samples, additional 5N NaOH was added to the analytical portions as follows: lemons, limes, currants 900 µL; raspberries 600 µL; and most other (e.g. grapes, oranges) 200 µL. The mixture was left standing for 3 h before extracting the samples by citrate buffered QuEChERS. The effectiveness of the enzymatic hydrolysis (EH) procedure to break up the ester bonds was compared with that of alkaline hydrolysis (AH). AH was conducted either using the mild conditions described above (30 min at RT) and in parallel also using harsher conditions (30 min at 80°C or 16 h at RT). The tests were conducted on thawed sample homogenates (e.g. cucumber), which were spiked with various, including "difficult" esters. Overall, EH with porcine liver esterase proved more efficient in breaking up the ester bonds than AH at the conditions described above. The results of these studies were presented in 2010 in a poster at the EPRW in Strasbourg. **Table 5** gives an overview of some experiments conducted.

[3] Other amounts of this esterase as well as other types of esterases were also tested
[4] Prepared by diluting 20 g Na_2HPO_4-$7H_2O$ (MW: 268.07 g/mol) and 3.4 g NaH_2PO_4-H_2O (MW: 137.99 g/mol) in 80 mL water and filling up to 100 mL; ultrasonication helps to facilitate solvation.

In parallel experiments on samples with incurred residues, it was also shown, that EH is much less effective in releasing conjugated residues of acidic herbicides. This is explicable, as natural conjugation of acidic pesticides involves many types of bonds other than ester bonds. As a possibility for breaking up both conjugates and esters applying mild conditions, a combination of EH and AH was introduced (column VIII in **Table 5**).

Table 5: *Overview of experiments comparing alkaline hydrolysis (AH; columns I-III), enzymatic hydrolysis (EH; columns IV-VII) and the combination thereof (column VIII). Matrix: cucumber; Enzyme: Porcine liver esterase (1 mg enzyme = 13 U). (Source: Poster presented at the EPRW 2010 in Strasbourg)*

Matrix:	Cucumber	Redidue Definition				Experiments performed in advance to QuEChERS							
Method:	QuEChERS					I	II	III	IV	V	VI	VII	VIII
Acid	Ester	Acid	Esters	Conjug.	Residue Definition	AH RT 30 min	AH 80°C 30 min	AH RT 16 h	EH (1 mg) RT 3 h	EH (2 mg) RT 3 h	EH (5 mg) RT 3 h	EH (2 mg) RT 16 h	VII + II
2,4,5-T	2-butyl	x			2,4,5-T (F)	63	33	19	0	0	0	0	0
	butoxyethyl					7	0	0	0	0	0	0	0
	ethylhexyl					103	47	26	7	4	2	0	0
	isooctyl					104	45	26	8	5	3	0	0
	isopropyl					15	0	0	0	0	0	0	0
	methyl					1	0	1	2	2	2	1	0
	octyl					38	10	3	3	2	1	1	0
2,4-D	methyl	x	x		2,4-D (sum of 2,4-D and its esters exp. as 2,4-D)	0	0	0	0	0	0	0	0
	butyl						0			0	0	0	
	ethyl						0			0	0	0	
	isobutyl						0			0	0	0	
	isooctyl						9			2	3	0	
	isopropyl						0			0	0	0	
2,4-DP	methyl	x			Dichlorprop, incl. Dichlorprop-p	0	0	0	0	0	0	0	0
Carfentrazone	ethyl	x	x		Carfentrazone-ethyl (determined as carfentrazone and expr. as carfentrazone-ethyl)	0	0	0	0	0	0	0	0
Chlorthal	dimethyl		x		Chlorthal-dimethyl	100	61	78	93	89	78	57	24
Cinidon	ethyl		x		Cinidon-ethyl (sum of cinidon ethyl and its E-isomer)	10	0	0	2	1	0	0	0
Clodinafop	propargyl	x			Clodinafop and its S-isomers, expr. as clodinafop (F)	2	0	0	0	0	0	0	0
Cyhalofop	butyl	x	x		Cyhalofop-butyl (sum of cyhalofop butyl and its free acids)	79	2	0	0	0	0	0	0
Dicamba	methyl	x			Dicamba		52			5	1	0	
Dichlorprop	2-ethylhexyl	x			Dichlorprop, incl. Dichlorprop-p	70	58	41	8	3	1	1	0
Diclofop	methyl	x	x		Diclofop (sum diclofop-methyl and diclofop acid expr. as diclofop-methyl)	60	1	0	1	1	1	0	0
Diethatyl	ethyl					8	0	0	12	2	0	0	0
Dinoseb	acetate	x			Dinoseb	8	0	0	0	0	0	0	0
Fenoprop	isooctyl						64			11	11	0	
	methyl						0			0	0	0	
Fenoxaprop	P-ethyl					37	0	0	1	0	0	0	0
Flamprop	isopropyl					103	33	20	97	82	78	53	15
Fluazifop	butyl					77	3	1	1	0	0	0	0
	methyl					1	0	0	0	0	0	0	0
Flumiclorac	pentyl					13	0	0	0	0	0	0	0
Haloxyfop	ethoxyethyl	x	x	x	Haloxyfop incl. haloxyfop-R (Haloxyfop-R methyl ester, haloxyfop-R and conjugates of haloxyfop-R expr. as haloxyfop-R) (F) (R)	9	0	0	0	0	0	0	0
	methyl					6	0	0	1	1	1	0	0
Ioxynil	octanoat	(x)	x		Ioxynil, incl. its esters expr. as ioxynil (F)	70	16	0	3	2	1	0	0
MCPA	1-butyl	x	x	x	MCPA and MCPB (MCPA, MCPB incl. their salts, esters and conjugates expr. as MCPA) (F) (R)		1			0	0	0	
	butoxyethyl						0			0	0	0	
	ethyl						0			0	0	0	
	ethylhexyl						21			3	3	0	
	thioethyl					3	0	0	0	0	0	0	0
MCPB	ethyl	x	x	x	MCPA and MCPB (MCPA, MCPB incl. their salts, esters and conjugates expr. as MCPA) (F) (R)		3			1	1	1	
Mecoprop	methyl	x			Mecoprop (sum of mecoprop-p and mecoprop expressed as mecoprop)	0	0	0	0	0	0	0	0
	1-octylester					103	83	54	8	5	3	2	1
	2,4,4-trimethylpentyl					104	83	44	7	4	3	0	0
Mefenpyr	diethyl					2	0	0	0	0	0	0	0
Nitrothal	di-isopropyl					19	0	0	2	1	0	0	0
Picloram	isooctyl	x			Picloram		14			1	1	0	
Triclopyr	2-butoxyethyl	x			Triclopyr		0			0	0	0	
Trinexapac	ethyl	x			Trinexapac	8	0	0	82	64	29	10	0

remaining esters <5%
remaining esters 5-20%
remaining esters > 20%

AH = alkaline hydrolysis, pH 12, re-neutralized with H_2SO_4

EH = incubation of buffered samples with esterase from porcine liver (buffering at pH 6-9 with phosphate buffer

Alkaline hydrolysis integrated in QuEChERS (after adding acetonitrile)

In a collaboration project between the EURL-SRM, BfR and the German NRL-SRM the alkaline hydrolysis procedure was further elaborated to include esters, and especially those that are difficult to break up. A decisive novelty in the new procedure was the incorporation of the alkaline hydrolysis step into the first QuEChERS extraction, i.e. after the addition of acetonitrile. As shown in **Table 6**, this measure considerably accelerated the cleavage of some bulky esters spiked on cucumber even within 30 min at 40°C. When adding the base directly to the sample the temperature had to be raised to 80°C for achieving satisfactory cleavage of bulky esters.

The added acetonitrile facilitates the distribution of the base throughout the sample and mediates the hydrolysis of lipophilic esters. In the previous procedure, where the base was added directly to the analytical portion, cumbersome stirring with a spatula was needed for some commodities (e.g. citrus), to distribute the base.

Table 6: Efficiency of alkaline hydrolysis when NaOH is added before or after addition of acetonitrile. In the latter case AH was integrated into the first QuEChERS extraction step. Matrix: cucumber, spiking level 0.2 mg/kg (Table also published in[5])

Matrix: cucumber	30 min, 40°C	60 min, 40°C	30 min, 80°C	**30 min, 40°C**	60 min, 40°C	30 min, 80°C
	AH **before** ACN-addition			AH **after** ACN-addition		
	Remaining esters after alkaline hydrolysis (AH) (%)					
2,4-DP-ethyl-hexyl	104	65	11	**0**	**6**	**4**
Cyhalofop-butyl	20	10	0	**0**	**0**	**0**
Diclofop methyl	15	7	2	**2**	**1**	**1**
Fluazifop-(P)-butyl	22	12	0	**0**	**0**	**0**
Fluroxypyr-1-meptyl	70	29	3	**0**	**0**	**0**
Haloxyfop-ethoxyethyl	11	6	0	**0**	**0**	**0**
MCPA butoxyethyl	3	2	0	**0**	**0**	**0**
Mecoprop-1-octyl	110	70	12	**0**	**0**	**0**

Another advantage derived from the presence of acetonitrile during hydrolysis, is the reduced tendency of certain commodities (e.g. cereals and pulses) to coagulate into clumps. **Clumping** can be very critical as it entraps large areas of the sample surface making it inaccessible to the extraction solvent. This means, that parts of the analytes, conjugated or not, will not have the chance to be taken up by the extraction solvent and will remain unavailable for hydrolysis and/or measurement. Parts of the extract are also entrapped, which prevents the distribution of the internal standard (IS) throughout the sample (if added after neutralization[6]) and affects IS-based quantifications. The negative impact of clumping, partly due to limited accessibility and partly due the limited distribution of the internal standard within the sample is demonstrated in **Figure 2** and **Figure 3**. As can be seen in **Figure 3**, the measured concentrations of Dicamba (incurred and extensively conjugated) and 2,4-D (lab-spiked and only marginally conjugated) were considerably underestimated due to the clumping effect.

Following several tests on various esters and considering the conditions applied by applicants for the release of conjugated residues, it was finally decided to fix hydrolysis at 40°C for 30 min. The procedure integrating alkaline hydrolysis into the first QuEChERS step was published in 2017[5]. The elaborated experimental conditions (hydrolysis: 1 ml 5N NaOH, 40°C, 30 min; neutralization with 1 mL 5N H_2SO_4) were also taken up in the CEN standard of the QuEChERS method (modular approach),

[5] Development of a QuEChERS-Based Method for the Simultaneous Determination of Acidic Pesticides, Their Esters, and Conjugates Following Alkaline Hydrolysis. Steinborn A, Alder L, Spitzke M, Dörk D, Anastassiades M. J Agric Food Chem. 2017 Feb 15;65(6):1296-1305.

[6] Adding the IS after neutralization is recommended where the IS sensitive to hydrolysis (BNPU)

with some deviations for highly acidic commodities (lemon, lime), where 2 mL NaOH were used for hydrolysis and neutralization was achieved by 1.4 mL NaOH or 1.8 mL NaOH (raspberry, blackberry).

At 40°C, where clumping did not play a role, alkaline hydrolysis for 30 min was effective in de-conjugating MCPA and Dicamba irrespective of the absence or presence of acetonitrile. Performing hydrolysis in presence of acetonitrile at more harsh (60°C/30 min) or more mild conditions (RT/30 min) did not significantly alter the de-conjugation rate in the case of MCPA in wheat. This confirms previous observations that alkaline de-conjugation in cereals takes place under relatively mild conditions.

Heating up the sample for 30 min at 40°C in absence of base but presence of acetonitrile (i.e. during the first QuEChERS extraction step) resulted in no notable de-conjugation of MCPA in wheat (2nd column in Figure 2) and Dicamba in oat (1st column in **Figure 3**). This indicates a certain stability of the conjugates in cereals under neutral conditions. The addition of base is decisive for de-conjugation.

Treatment with porcine liver esterase resulted in only marginal de-conjugation (**Figure 2** and **Figure 3**; columns to the right), which suggests that acidic pesticides do not form many ester bonds within cereals.

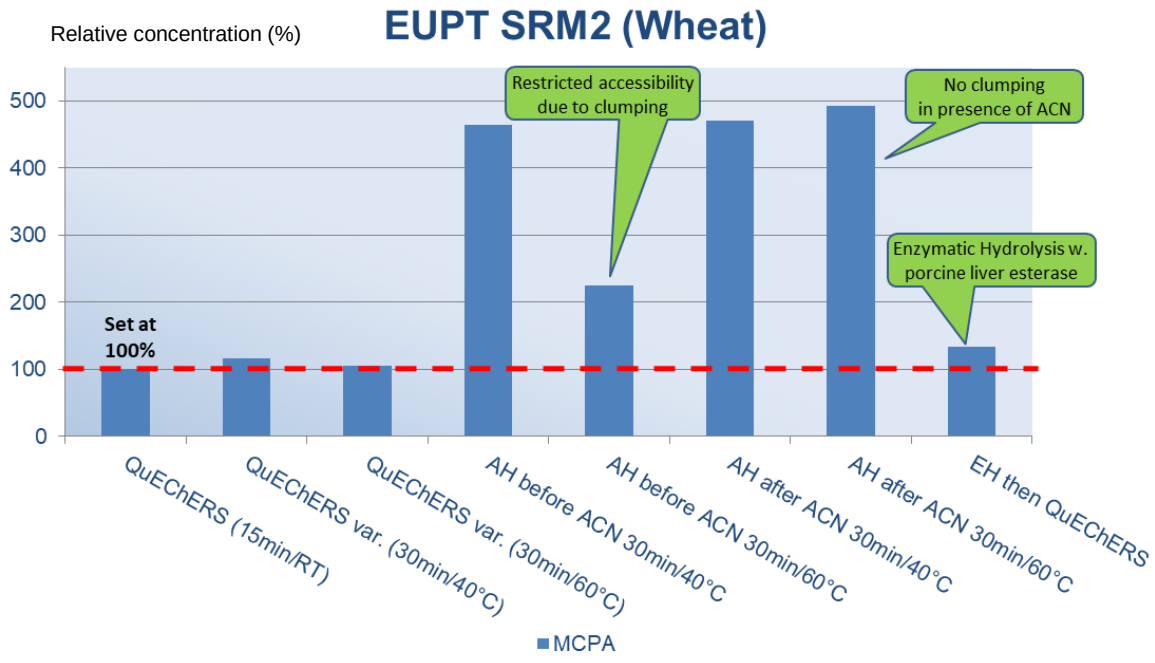

Figure 2: Comparison of different hydrolysis procedures to release naturally conjugated MCPA-residues from wheat treated with MCPA in the field. Notes: The values obtained by CEN-QuEChERS were set at 100%. These experiments were conducted 7 years after EUPT-SRM2, so the share of conjugated residues may have changed. Alkaline hydrolysis (AH) was conducted by adding 1 mL 5N NaOH. Enzymatic hydrolysis (EH) included addition of water, addition of porcine liver esterase 26 U and gentle shaking for 3 h at RT).

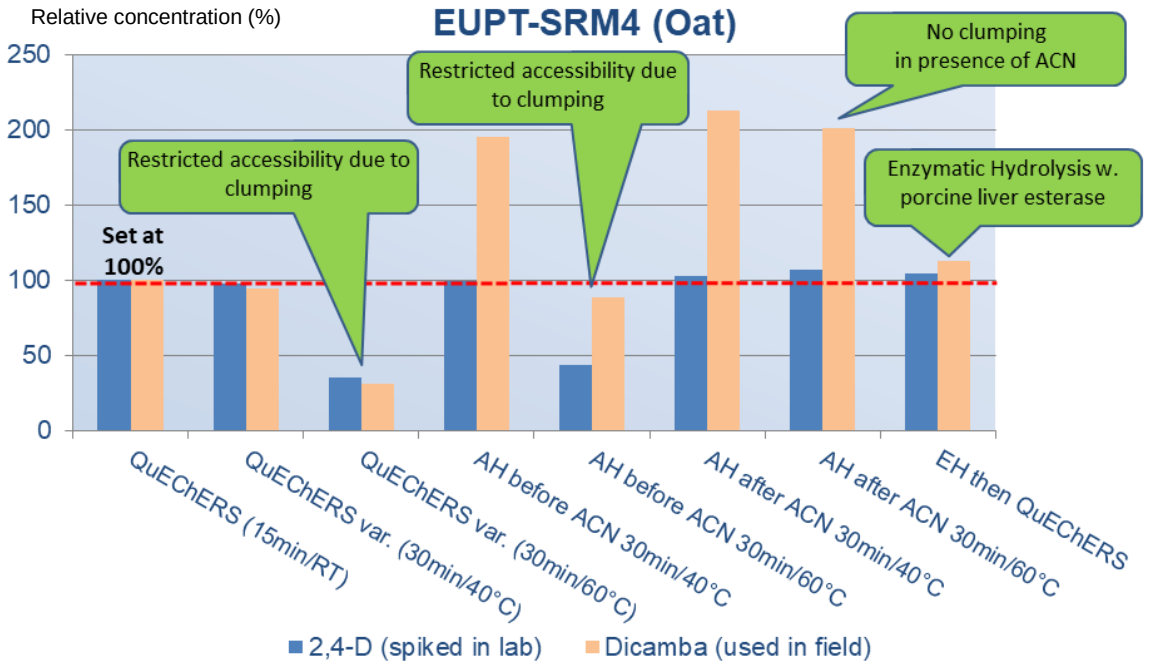

Figure 3: Comparison of different hydrolysis procedures to release naturally conjugated Dicamba-residues from oat treated with Dicamba in the field. Notes: The values obtained by CEN-QuEChERS were set at 100%. These experiments were conducted 5 years after EUPT-SRM4, so the share of conjugated residues may have changed. Alkaline hydrolysis (AH) was conducted by adding 1 mL 5N NaOH. Enzymatic hydrolysis (EH) included addition of water, addition of porcine liver esterase 26 U and gentle shaking for 3 h at RT).

Alkaline hydrolysis of resistant esters in complex commodities

The conditions described above for the alkaline hydrolysis of an analytical portion of QuEChERS (1 ml 5N NaOH, 40°C, 30 min) were successful for the cleavage of "difficult" esters in simple commodities, such as most fruits and vegetables. When dealing with more complex commodities, however, these conditions proved too weak for hydrolyzing "difficult" esters. This fact had already been highlighted in[5] giving wheat, lentils, tea and grapefruit as examples for "difficult" commodities. As mentioned above, the reasonable coverage of the full theoretical range of esters has several important advantages. It was thus attempted to further develop the method to cover "difficult" esters. Following pre-experiments with various esters (not shown), a number of esters were selected that include "intermediately difficult" as well as "difficult" ones. A standard mixture was then prepared and experiments were run applying different hydrolysis conditions.

In the case of **grapefruits** 10 g matrix were spiked with the ester mix, followed by the addition of acetonitrile and NaOH. After heating, the sample was neutralized with H_2SO_4, internal standard was added, and the normal CEN-QuEChERS procedure was conducted. At 40°C none of these esters could be satisfactorily hydrolyzed within 30 min when 1 mL 5N NaOH was added. Even when the amount of base was doubled to 2 mL 5N NaOH and the hydrolysis time prolonged to 120 min only 6 of these esters were cleaved at a satisfactory rate. A satisfactory hydrolysis rate for all 10 esters was only achieved when adding 2 mL 5N NaOH and conducting hydrolysis for 60 min at 60°C. The results of this experiment are shown in **Figure 4**.

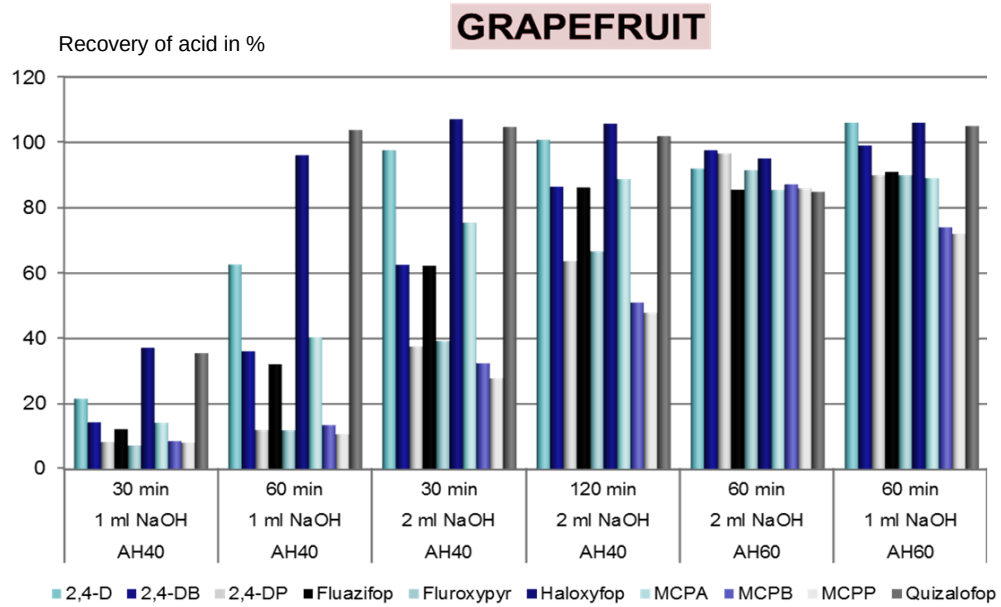

Figure 4: Hydrolysis of various "difficult" esters in grapefruit using different conditions (AH40 at 40°C; AH60 at 60°C). The esters spiked were: 2,4-D ethylhexyl; 2,4-DB methyl; 2,4-DP ethylhexyl; Fluazifop butyl; Fluroxypyr meptyl; Haloxyfop methyl; MCPA ethylhexyl; MCPB ethyl; MCPP trimethylpentyl and Propaquizafop.

In the case of **lentils** 5 g sample were used and 8 mL of water were added. The procedure was then continued as described for grapefruit. Satisfactory hydrolysis rates for all spiked esters were achieved when hydrolysis was conducted following addition of 2 mL 5N NaOH both at 60°C for 60 min (as in the case of grapefruit) as well as at 40°C for 120 min. Successful hydrolysis rates were also achieved when conducting enzymatic treatment with porcine liver esterase (13 U; 2h at RT) followed by mild alkaline hydrolysis (1 mL 5N NaOH, 40°C, 30 min), the former addressing esters and the latter the conjugates. These results are shown in **Figure 5**.

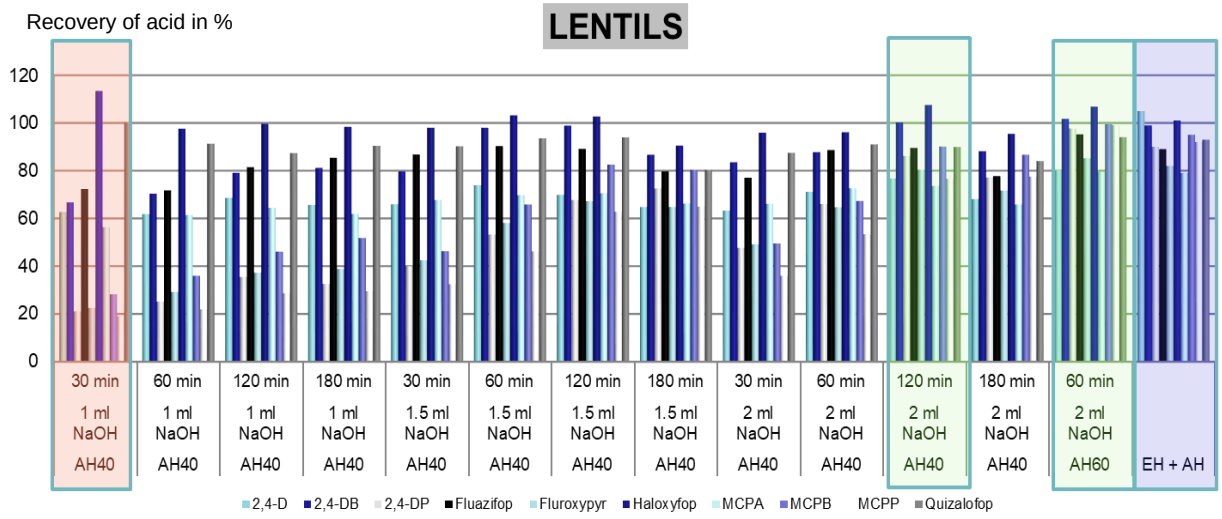

Figure 5: Hydrolysis of various "difficult" esters in lentils using different conditions of alkaline hydrolysis (AH40 at 40°C; AH60 at 60°C), and a combination of alklaine and enzymatic hydrolysis. The esters spiked were: 2,4-D ethylhexyl; 2,4-DB methyl; 2,4-DP ethylhexyl; Fluazifop butyl; Fluroxypyr meptyl; Haloxyfop methyl; MCPA ethylhexyl; MCPB ethyl; MCPP trimethylpentyl and Propaquizafop

In the case of **wheat flour** 5 g the same procedure as for lentils was used. Satisfactory hydrolysis rates for all spiked esters were achieved following addition of 2 mL 5N NaOH both at 60°C for 60 min as well as at 40°C for 120 min. See **Figure 6**.

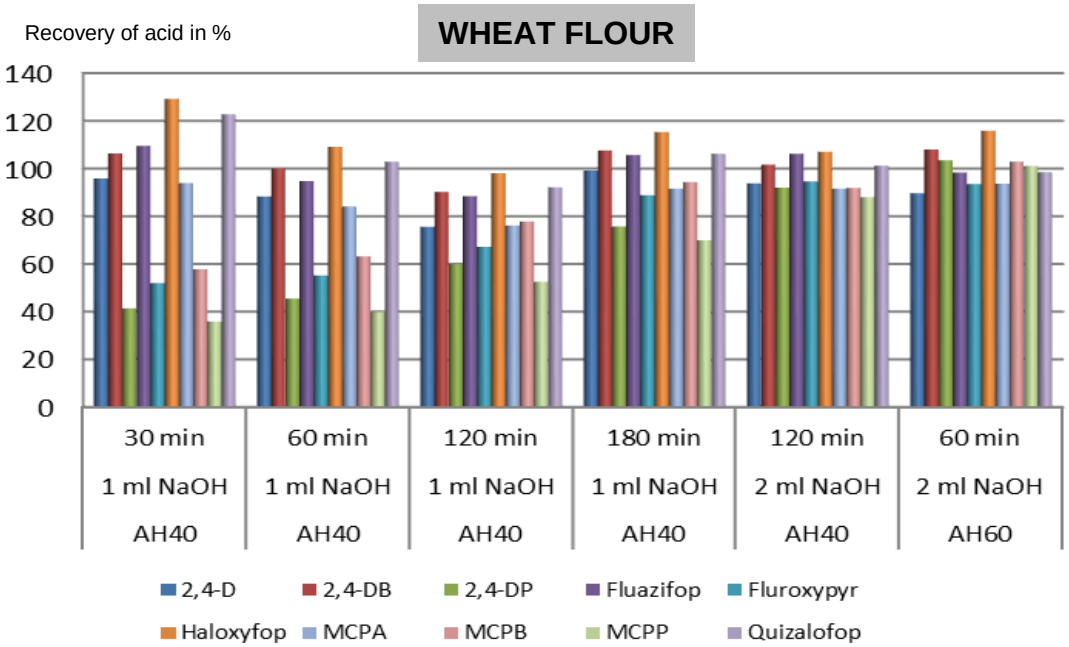

Figure 6: Hydrolysis of various "difficult" esters in wheat flour using different conditions of alkaline hydrolysis (AH40 at 40°C; AH60 at 60°C). The esters spiked were: 2,4-D ethylhexyl; 2,4-DB methyl; 2,4-DP ethylhexyl; Fluazifop butyl; Fluroxypyr meptyl; Haloxyfop methyl; MCPA ethylhexyl; MCPB ethyl; MCPP trimethylpentyl and Propaquizafop

In the case of **potatoes** 10 g sample were used and the procedure was continued as described for grapefruit. Fewer conditions were tested here. Satisfactory hydrolysis rates for all spiked esters were achieved when hydrolysis was conducted at 40°C for 120 min following addition of 2 mL 5N NaOH. The results of this experiment are shown in **Figure 7**.

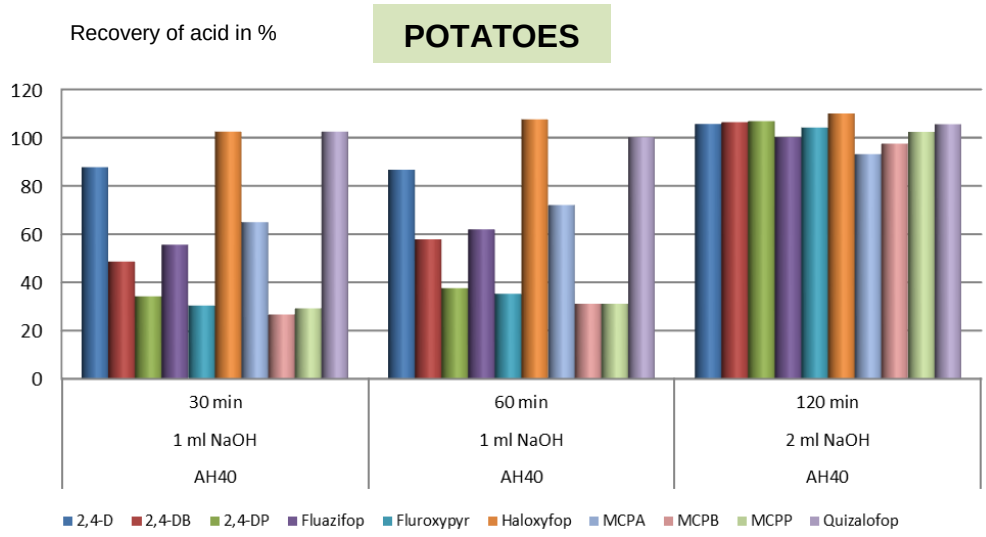

Figure 7: Hydrolysis of various "difficult" esters in potato using different conditions of alkaline hydrolysis (AH40 at 40°C; AH60 at 60°C). The esters spiked were: 2,4-D ethylhexyl; 2,4-DB methyl; 2,4-DP ethylhexyl; Fluazifop butyl; Fluroxypyr meptyl; Haloxyfop methyl; MCPA ethylhexyl; MCPB ethyl; MCPP trimethylpentyl and Propaquizafop

In the experiments Mecoprop-trimethylpentyl (=MCPP trimethylpentyl) and MCPB-ethyl proved to be the most challenging to break up with 2,4-DP-ethylhexyl and Fluazifop-butyl following. On the other side Haloxyfop-methyl ester and Propaquizafop (the propyl ester of Quizalofop) were found to be the most labile ones.

In the case of **rice and rye flour**, which was treated the same way as wheat flour, an extended set of esters was tested. Here the hydrolysis rate of the esters using 1 mL of 5N NaOH was not successful for all esters neither at 40°C over 30 min nor at 60°C over 1 h. Low break-up rates were noted for esters that identified earlier as "difficult" (MCPP-trimethylpentyl; MCPB-ethyl and 2,4-DP-ethylhexyl) as well as for Fluroxypyr-meptyl. Successful hydrolysis rates were achieved when the amount of added base was doubled (2 mL 5N NaOH) and the reaction time at 40°C was prolonged to 60 or 120 min. Also successful, when using 2 mL 5N NaOH, were the tests at 60°C for 30 or 60 min. It should be noted however, that in another experiment with a different type of rice hydrolysis at 60°C led to coagulation of the rice into an elastic opaque clump despite the presence of acetonitrile. **For rice, and cereals in general it is thus recommended to keep temperature at 40°C and prolong hydrolysis time to 120 min**. The results of these experiments are shown in **Table 7** and **Table 8**.

The poor recoveries in the case of Fenoxaprop can be explained by the lability of this compound under alkaline conditions, with 6-chloro-1,3-benzoxazolone being probably formed. This phenomenon was also observed in an experiment where the hydrolysis procedure was conducted on a sample spiked with a mixture of free acids (not shown here).

Table 7: Recoveries of free acids (or phenols in the case of bromoxynil and ioxynil) following spiking of esters on **rice flour** and alkaline hydrolysis under different conditions (n=3)

Esters	AH40 (40°C)			AH60 (60°C)		
	1 mL 5N NaOH	2 mL 5N NaOH		1 mL 5N NaOH	2 mL 5N NaOH	
	30 min	60 min	120 min	30 min	30 min	60 min
2,4,5-T-isooctyl	91	87	92	90	87	102
2,4-D ethylhexyl	90	88	92	90	88	101
2,4-DB methyl	84	86	89	86	87	103
2,4-DP ethylhexyl	**64**	93	101	**78**	94	116
Bromoxynil-heptanoate	98	93	100	91	94	110
Cyhalofop-butyl	91	89	89	84	83	87
Diclofop-methyl	95	89	98	90	93	106
Fenoxaprop-ethyl	**40**	**16**	**n.d.**	**2**	**n.d.**	**n.d.**
Fluazifop butyl	93	89	98	92	91	110
Fluroxypyr meptyl	**70**	88	91	80	84	105
Haloxyfop methyl	93	83	96	82	85	103
Ioxynil-octanoate	**76**	88	96	89	93	109
MCPA ethylhexyl	82	85	85	87	84	102
MCPB ethyl	**64**	81	87	**77**	81	102
MCPP trimethylpentyl	**51**	90	96	**77**	97	112
Propaquizafop	95	89	97	89	85	97
Triclopyr-2-butoxyethyl	95	89	96	88	90	104

Table 8: Recoveries of free acids (or phenols in the case of bromoxynil and ioxynil) following spiking of esters on **rye flour** and alkaline hydrolysis under different conditions (n=3)

Esters	AH40 (40°C)			AH60 (60°C)		
	1 mL 5N NaOH	2 mL 5N NaOH		1 mL 5N NaOH	2 mL 5N NaOH	
	30 min	60 min	120 min	30 min	30 min	60 min
2,4,5-T-isooctyl	89	96	90	91	97	101
2,4-D ethylhexyl	88	92	92	96	94	99
2,4-DB methyl	83	98	104	97	99	93
2,4-DP ethylhexyl	49	104	102	70	107	108
Bromoxynil-heptanoate	97	111	101	107	106	118
Cyhalofop-butyl	89	103	98	97	99	89
Diclofop-methyl	102	112	101	102	103	108
Fenoxaprop-ethyl	58	8	2	4	1	0
Fluazifop butyl	86	102	102	99	100	106
Fluroxypyr meptyl	45	85	90	72	94	92
Haloxyfop methyl	91	104	105	96	99	95
Ioxynil-octanoate	65	104	100	98	103	115
MCPA ethylhexyl	80	94	92	93	97	96
MCPB ethyl	49	94	98	71	90	93
MCPP trimethylpentyl	36	94	107	66	108	108
Propaquizafop	100	105	106	98	104	94
Triclopyr-2-butoxyethyl	92	97	96	95	98	99

In the case of **liver** homogenate, 10 g were used and hydrolysis was conducted after adding 1 or 2 mL of NaOH. The ester-hydrolysis rate using 1 mL of 5N NaOH wasn't fully successful at 40°C over 30 min but was successful at 60°C over 1 h. Difficulties were noticed for the same esters as for rice and rye. At 2 mL 5N NaOH hydrolysis was successful but for some reason the recovery of Cyhalofop acid and Quizalofop acid dropped. This effect needs to be re-checked. The breakdown of Fenoxaprop was not surprising as it was observed several times. These results bare shown in **Table 9**.

Table 9: Recoveries of free acids (or phenols in the case of bromoxynil and ioxynil) following spiking of esters on **liver** and alkaline hydrolysis under different conditions (n=5)

Esters	AH40 / 30 min 1mL 5 N NaOH	AH60 / 60 min 1mL 5 N NaOH	AH60 / 60 2mL 5 N NaOH
2,4,5-T-isooctyl	84	91	92
2,4-D ethylhexyl	83	94	94
2,4-DB methyl	96	118	115
2,4-DP ethylhexyl	77	98	107
Bromoxynil-heptanoate	98	104	99
Cyhalofop-butyl	100	90	48 (?)
Diclofop-methyl	86	104	101
Fenoxaprop-ethyl	23	n.d.	n.d.
Fluazifop butyl	104	110	108
Fluroxypyr meptyl	70	89	89
Haloxyfop methyl	96	105	101
Ioxynil-octanoate	92	102	106
MCPA ethylhexyl	85	94	96
MCPB ethyl	69	105	104
MCPP trimethylpentyl	56	90	108
Propaquizafop	97	104	79 (?)
Triclopyr-2-butoxyethyl	85	95	102

4. Analytical procedures

Based on the above experiments, three different hydrolysis procedures are proposed for commodities of plant origin. Here are the conditions in brief:

a) For **most fruits and vegetables** **1 mL 5N NaOH** is added (corresponds to ~0.25 mmol/mL*) and the reaction takes place at **40°C for 30 min** (as in CEN procedure)
b) For **cereals, pulses and starchy vegetables** **2 mL 5N NaOH** are added (= ~0.5 mmol/mL*) and the reaction takes place at **40°C for 120 min**
c) For **citrus fruits** **2 mL 5N NaOH** are added (= ~0.5 mmol/mL*) and the reaction takes place at **60°C for 60 min**

** calculated on the basis of ~20 mL total volume after adding acetonitrile*

Further tests on additional commodities of plant origin are being conducted to find optimal hydrolysis conditions for nuts and oily seeds; spices, dry herbs tea and other. Further experiments on commodities of animal origin are also planned.

Where acidic pesticides showing considerable losses during the partitioning step of the citrate-buffered QuEChERS are to be analyzed, a lower partitioning pH helps to increase recovery rates. Here, the alkaline hydrolysis step may be combined with FA-QuEChERS, which involves addition of formic acid and no buffering.

A flow chart showing the citrate-buffered QuEChERS procedure involving alkaline hydrolysis (AH) is given in **Figure 8** (proposed acronym: ***AH-CB-QuEChERS***) and of the acidified QuEChERS procedure involving alkaline hydrolysis in **Figure 9** (proposed acronym: ***AH-FA-QuEChERS***). All reagents required for these procedures are also used in EN-15662 with exception of the 5N H_2SO_4 and the conc. formic acid.

AH-CB-QuEChERS

Weigh 10 g of sample homogenate in 50 mL centrifuge tube

Fresh fruit and vegetables: 10 g ± 0,1 g,
Previously rehydrated dry fruit: 13.5 g ± 0,1 g (containing 5 g of original dry fruit),
Cereals, pulses: 5 g ± 0,05 g
Spices, herbs: 2 g± 0,02 g

↓

Stable ISs (that would not degrade during hydrolysis) may be added at this stage
e.g. MCPA-D6 oder MCPP-D6 (e.g. 100 µL of a solution)

↓

Add water in the case of dry commodities
Cereal, pulses, spices, herbs: 8 g water

↓

Add 10 mL acetonitril and **5N NaOH; shake vigurously**

Most fruits and vegetables: 1 mL
Citrus, currants, blackberries, raspberries: 2 mL
Pulses, cereals, potatoes: 2 mL

↓

Place in a shaking water bath

Most fruits and vegetables: for 30 min at 40°C
Citrus fruit: for 60 min at 60°C;
Cereals, pulses, potatoes: for 120 min at 40°C

↓

Allow 60°C vials to cool down to e.g. 30 °C (e.g. cool water bath)
Add 5N H_2SO_4 to neutralize base (same volume as 5N NaOH added above);
Shake vigorously

↓

Unstable ISs (that would degrade during hydrolysis) may be added at this stage
e.g. BNPU (e.g. 100 µL of a solution)

↓

Add 4 g $MgSO_4$, 1 g NaCl, 1 g Na_3Citrate x $2H_2O$, 0.5 g Na_2H-Citrate-Sesquihydrate

↓

Shake for 1 min, **allow vials to cool down** and **centrifuge**
(e.g. at 3500 g for 5 min)

↓

OPTIONAL (but effective for some commodities; e.g. of high lipid content)

a) dSPE (6 mL extract with 0.9 g $MgSO_4$ + 150 mg **C_{18}-sorbent**)
OR
b) freeze-out

↓

LC-MS/MS analysis of free acids or phenols in ESI-Neg. mode

Figure 8: Method at a glance: Citrate-buffered QuEChERS involving alkaline hydrolysis (AH-CB-QuEChERS).

AH-FA-QuEChERS

Weigh 10 g of sample homogenate in 50 mL centrifuge tube

Fresh fruit and vegetables: 10 g ± 0,1 g,
Previously rehydrated dry fruit: 13.5 g ± 0,1 g (containing 5 g of original dry fruit),
Cereals, pulses: 5 g ± 0,05 g
Spices, herbs: 2 g± 0,02 g

↓

Stable ISs (that would not degrade during hydrolysis) may be added at this stage
e.g. MCPA-D6 oder MCPP-D6 (e.g. 100 µL of a solution)

↓

Add water in the case of dry commodities
Cereal, pulses, spices, herbs: 8 g water

↓

Add 10 mL acetonitril and **5N NaOH; shake vigurously**

Most fruits and vegetables: 1 mL
Citrus, currants, blackberries, raspberries: 2 mL
Pulses, cereals, potatoes: 2 mL

↓

Place in a shaking water bath

Most fruits and vegetables: for 30 min at 40°C
Citrus fruit: for 60 min at 60°C;
Cereals, pulses, potatoes: for 120 min at 40°C

↓

Allow 60°C vials to cool down to e.g. 30 °C (e.g. cool water bath)
Add 5N H_2SO_4 to neutralize base (same volume as 5N NaOH added above);
Add 100 µL Formic acid (98%) (not essential for acidic commodities);
Shake vigorously

↓

Unstable ISs (that would degrade during hydrolysis) may be added at this stage
e.g. BNPU (e.g. 100 µL of a solution)

↓

Add 4 g $MgSO_4$, 1 g NaCl

↓

Shake for 1 min, **allow vials to cool down** and **centrifuge**
(e.g. at 3500 g for 5 min)

↓

OPTIONAL (but effective for some commodities; e.g. of high lipid content)

a) dSPE (6 mL extract with 0.9 g $MgSO_4$ + 150 mg **C_{18}-sorbent**)
OR
b) freeze-out

↓

LC-MS/MS analysis of free acids or phenols in ESI-Neg. mode

Figure 9: Method at a glance: A-QuEChERS involving alkaline hydrolysis (AH-FA-QuEChERS)

5. Hydrolysis of esters during storage of sample homogenates

Out of interest, it was also checked, whether esters would also hydrolyse in the homogenates of commodities, possibly facilitated by the sample-own esterase enzymes. This scenario would be common for labs that homogenize their samples at ambient conditions or that leave frozen homogenates to defrost over many hours. For the experiment, fresh homogenates of cucumber and apple that were milled under ambient conditions were used for this purpose. The analytical portions of the homogenates were spiked with a mix of esters at 0.2 mg/kg and the esters were distributed within the homogenate by a short vortexing step. The portions were left standing for at RT for different time intervals (0 min, 30 min, 120 min) and the degradation of the esters during this periods was studied by measuring both the esters and the free acids. **Figure 10** and **Figure 11** show the results of the esters. The results at immediate analysis (0 min) were set at 100%.

Many of the esters degraded during the storage forming the acids. The results for the acids (not shown here) suited very well with the degradation of the esters. Where the residue definitions included both acids and esters, this degradation in the homogenate was considered non-critical. Critical at that time was the degradation of esters the residue definitions of which did not include the free acids (previously Propaquizafop[7] and nowadays Cyhalofop-butyl) as well as acids (or phenols) the residue definitions of which do not include the esters (e.g. Bromoxynil, Clodinafop, Fenoxaprop, Mecoprop (MCPP), Trinexapac and previously also 2,4-DB; Dichlorprop and Quizalofop).

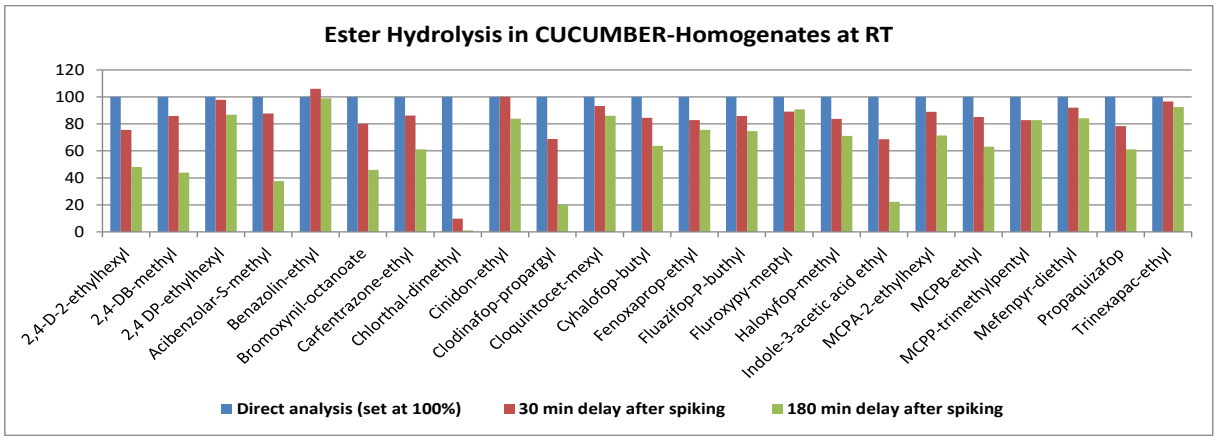

Figure 10: Hydrolysis of various esters during the storage of cucumber homogenates at room temperature

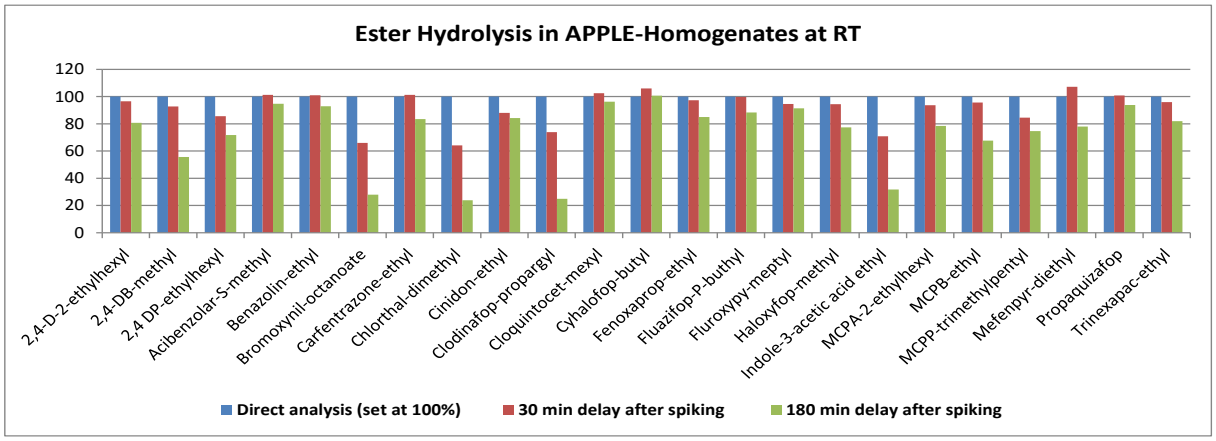

Figure 11: Hydrolysis of various esters during the storage of apple homogenates at room temperature

[7] Propaquizafop was recently included in the residue definition of quizalofop

6. Analysis of samples with incurred residues

Knowledge about commodities typically containing acidic pesticides and on the increment of determined concentrations upon hydrolysis enables labs to selectively apply methods involving a hydrolysis step. **Tables 10-12** give an overview on results from the analysis of 2,4-D, Fluazifop and Haloxyfop in real samples.

Table 10: Examples of commodities with incurred residues of 2,4-D

Group	Product	2,4-D	2,4-D, (sum)	Factor	Conjugated residue in %
Cereals	**Wheat**	0,075	0,068	0,91	-10%
		0,164	0,212	1,29	23%
	Wheat Average			**1,10**	**6%**
Cereals Average				**1,10**	**6%**
Citrus	**Grapefruit**	0,018	0,037	2,06	51%
		0,018	0,063	3,50	71%
		0,025	0,036	1,44	31%
		0,027	0,12	4,44	78%
		0,03	0,094	3,13	68%
		0,036	0,098	2,72	63%
		0,06	0,41	6,83	85%
		0,062	0,13	2,10	52%
		0,069	0,16	2,32	57%
		0,075	0,26	3,47	71%
		0,09	0,28	3,11	68%
	Grapefruit Average			**3,19**	**63%**
	Lemon	0,012	0,045	3,75	73%
		0,032	0,12	3,75	73%
		0,085	0,12	1,41	29%
	Lemon Average			**2,97**	**59%**
	Lime	0,05	0,055	1,10	9%
		0,398	0,657	1,65	39%
	Lime Average			**1,38**	**24%**
	Mandarine/Clementine	0,016	0,085	5,31	81%
		0,025	0,086	3,44	71%
	Mandarine/Clementine Average			**4,38**	**76%**
	Orange	0,009	0,063	7,00	86%
		0,01	0,063	6,30	84%
		0,013	0,082	6,31	84%
		0,013	0,13	10,00	90%
		0,015	0,094	6,27	84%
		0,018	0,16	8,89	89%
		0,02	0,12	6,00	83%
		0,021	0,1	4,76	79%
		0,031	0,21	6,77	85%
		0,043	0,29	6,74	85%
		0,047	0,26	5,53	82%
		0,074	0,092	1,24	20%
		0,088	0,135	1,53	35%
		0,11	0,24	2,18	54%
		0,11	0,37	3,36	70%
		0,14	0,28	2,00	50%
		0,158	0,393	2,49	60%
	Orange Average			**5,14**	**72%**
Citrus Average				**4,08**	**65%**

Group	Product	2,4-D	2,4-D, (sum)	Factor	Conjugated residue in %
Fruits, dry	Raisins	0,015	0,018	1,20	17%
	Raisins Average			**1,20**	**17%**
Fruits, dry Average				**1,20**	**17%**
Oily seeds	Rapeseed	0,006	0,02	3,33	70%
	Rapeseed Average			**3,33**	**70%**
Oily seeds Average				**3,33**	**70%**
Pulses	Lentil	0,011	0,012	1,09	8%
			0,016	1,45	31%
			0,018	1,64	39%
		0,013	0,02	1,54	35%
		0,019	0,02	1,05	5%
		0,022	0,03	1,36	27%
		0,078	0,077	0,99	-1%
	Lentil Average			**1,30**	**21%**
	Peas	0,107	0,104	0,97	-3%
	Peas Average			**0,97**	**-3%**
Pulses Average				**1,26**	**18%**
Spices/Infusions	Caraway	0,012	0,021	1,75	43%
	Caraway Average			**1,75**	**43%**
	Chilli	0,013	0,017	1,31	24%
		0,03	0,072	2,40	58%
	Chilli Average			**1,85**	**41%**
	Cumin	0,013	0,038	2,92	66%
	Cumin Average			**2,92**	**66%**
	Fennel-infusion	0,01	0,011	1,10	9%
		0,012	0,024	2,00	50%
		0,017	0,023	1,35	26%
	Fennel-infusion Average			**1,48**	**28%**
	Oregano	0,01	0,011	2,63	62%
	Oregano Average			**2,63**	**62%**
	Paprika spice	0,008	0,032	4,00	75%
		0,009	0,017	1,89	47%
		0,011	0,018	1,64	39%
		0,014	0,022	1,57	36%
		0,019	0,023	1,21	17%
		0,031	0,051	1,65	39%
		0,042	0,061	1,45	31%
		0,044	0,086	1,95	49%
		0,051	0,08	1,57	36%
		0,052	0,067	1,29	22%
		0,076	0,093	1,22	18%
		0,11	0,12	1,09	8%
	Paprika spice Average			**1,71**	**35%**
	Pepper spice	0,015	0,014	0,93	-7%
	Pepper spice Average			**0,93**	**-7%**
	Tea	0,015	0,014	0,93	-7%
	Tea Average			**0,93**	**-7%**
Spices/Infusions Average				**1,72**	**34%**
Vegetables	Spinach	0,012	0,011	0,92	-9%
		0,039	0,037	0,95	-5%
	Spinach Average			**0,93**	**-7%**
Vegetables Average				**0,93**	**-7%**
Overall Average				**2,81**	**46%**

Table 11: Examples of commodities with incurred residues of Fluazifop

Group	Product	Fluazifop	Fluazifop (sum)	Factor	Conjugated residue in %
Oily seeds	Rapeseed	0,49	0,49	1,00	0%
	Rapeseed Average			**1,00**	**0%**
	Soja	0,16	0,34	2,13	53%
		0,43	0,52	1,21	17%
	Soja Average			**1,67**	**35%**
Oily seeds Average				**1,45**	**24%**
Pulses	Bean; dry	0,053	0,067	1,26	21%
		0,084	0,22	2,62	62%
	Bean; dry Average			**1,94**	**41%**
	Peas	0,362	0,48	1,33	25%
	Peas Average			**1,33**	**25%**
Pulses Average				**1,74**	**36%**
Spices/Infusions	Oregano	0,01	0,03	3,00	67%
		0,014	0,029	2,07	52%
		0,015	0,077	5,13	81%
		0,047	0,2	4,26	77%
		0,095	0,25	2,63	62%
		0,096	0,55	5,74	83%
		0,5	0,48	0,96	-4%
		0,62	2,7	4,35	77%
	Oregano Average			**3,52**	**62%**
	Peppermint-infusion	0,017	0,027	1,59	37%
	Peppermint-infusion Average			**1,59**	**37%**
Spices/Infusions Average				**3,30**	**59%**
Vegetables (Root+Tuber)	Beetroots	0,013	0,027	2,08	52%
		0,022	0,044	2,00	50%
		0,023	0,045	1,96	49%
	Beetroots Average			**2,01**	**50%**
	Carrot	0,011	0,034	3,09	68%
	Carrot Average			**3,09**	**68%**
Vegetables (Root+Tuber) Average				**2,28**	**55%**
Vegetables (Sprouting)	Cauliflower	0,016	0,026	1,63	38%
	Cauliflower Average			**1,63**	**38%**
Vegetables (Sprouting) Average				**1,63**	**38%**
Vegetables (Legumes)	Bean, fresh	0,038	0,078	2,05	51%
		0,359	0,4	1,12	10%
	Bean, fresh Average			**1,58**	**31%**
Vegetables (Legumes) Average				**1,58**	**31%**
Vegetables (Fruiting)	Tomatoes, dry	0,036	0,041	1,14	12%
	Tomatoes, dry Average			**1,14**	**12%**
Vegetables (Fruiting) Average				**1,14**	**12%**
Overal Average				**2,36**	**45%**

Table 12: Examples of commodities with incurred residues of Haloxyfop

Group	Product	Haloxyfop	Haloxyfop (sum)	Factor	Conjugated residue in %
Oily seeds	**Chia seeds**	0,009	0,11	12,22	92%
	Chia seeds Average			**12,22**	**92%**
Oily seeds Average				**12,22**	**92%**
Pulses	**Bean; dry**	0,011	0,012	1,09	8%
		0,014	0,02	1,43	30%
		1,133	1,12	0,99	-1%
	Bean; dry Average			**1,17**	**12%**
	Lentil	0,128	0,149	1,16	14%
	Lentil Average			**1,16**	**14%**
Pulses Average				**1,17**	**13%**
Vegetables (Root+Tuber)	**Potatoes**	0,011	0,016	1,45	31%
		0,026	0,038	1,46	32%
	Potatoes Average			**1,46**	**31%**
Vegetables (Root+Tuber) Average				**1,46**	**31%**
Overall Average				**2,83**	**29%**

7. Instrumental Analysis Conditions

Exemplary LC conditions and MS/MS settings can be found in **Table 13** and **Table 14**, respectively.

Table 13: LC details for acidic pesticides (exemplary)

Instrument	**Waters Acquity, ABSciex API 4000 QTrap**			
Ionisation mode	ESI-Neg.			
Column	Waters Acquity UPLC BEH C18,1.7 µm; 2.1 x 100 mm			
Pre-column	Van Guard BEH C18, 1.7 um			
Eluent A	0.01 % acetic acid in water (with 5% acetonitrile)			
Eluent B	0.01 % acetic acid in acetonitrile			
Gradient	Time [min]	Flow [µL/min]	A [%]	B [%]
	0	400	80	20
	4	400	70	30
	7	400	10	90
	8.5	400	10	90
	8.6	400	80	20

Table 14: MS/MS details for acidic pesticides (ESI-negative mode, Tune-data ABSciex 4000Q) (exemplary)

Compound	Sensitivity Ranking (1= best)	Parent Mass	Daughter Mass	DP	CE	CXP
2,4,5-T	3	253	159	-50	-40	-7
	1	253	195	-50	-18	-9
	2	255	197	-55	-18	-11
2,4,5-TP (Fenoprop)	3	267	159	-50	-40	-9
	1	267	195	-50	-16	-9
	2	269	197	-50	-18	-9
2.4-D	3	219	125	-50	-38	-7
	1	219	161	-50	-18	-9
	2	221	163	-50	-18	-9
2.4-DB	3	247	125	-50	-38	-5
	1	247	161	-50	-12	-9
	2	249	163	-35	-14	-9
2.4-DP (Dichlorprop)	3	233	125	-50	-38	-5
	1	233	161	-50	-18	-9
	2	235	163	-50	-18	-7
4-CPA	1	185	127	-55	-20	-7
	2	187	129	-55	-20	-7
Bentazon	1	239	132	-75	-38	-7
	2	239	175	-75	-28	-9
	3	239	197	-75	-28	-11
Bromoxynil	1	274	79	-60	-48	-1
	2	276	81	-70	-42	-3
	3	278	81	-60	-50	-3
Dicamba	1	219	175	-25	-8	-8
	2	221	177	-25	-8	-11
Fenoxaprop-P	3	334	262	-70	-18	-1
	2	332	152	-70	-32	-7
	1	332	260	-70	-18	-13
Fluazifop	3	326	108	-65	-56	-5
	2	326	226	-65	-38	-11
	1	326	254	-65	-22	-5
Fluroxypyr	1	253	195	-50	-20	-9
	3	253	233	-50	-10	-1
	2	255	197	-55	-20	-11
Haloxyfop	3	360	196	-70	-52	-9
	1	360	288	-70	-20	-15
	2	362	290	-75	-20	-15
Ioxynil	1	370	127	-60	-44	-7
	2	370	215	-60	-50	-13
	3	370	243	-60	-32	-13
MCPA	1	199	141	-55	-20	-7
	2	201	143	-55	-20	-7
MCPB	1	227	141	-50	-18	-7
	2	229	143	-55	-16	-7
MCPP	1	213	141	-55	-20	-7
	3	213	71	-55	-14	-1
	2	215	143	-55	-18	-7
Quizalofop	1	343	271	-36	-22	-15
	2	345	273	-41	-22	-13

Compound	Sensitivity Ranking (1= best)	Parent Mass	Daughter Mass	DP	CE	CXP
Internal standards						
MCPP-D6		219	147	-26	-20	-7
MCPA-D6		205	147	-56	-20	-9
BNPU		301	137	-45	-16	-11
Propyzamide D3		257	231	-70	-20	-1

8. Summary

This document deals with the analysis of pesticides requiring the conduction of a hydrolysis step to cover the full residue definition. A short general overview on conjugates is given but the focus is on pesticides with carboxylic groups and the possibilities to break up conjugated and ester-bound residues. Both alkaline hydrolysis and enzymatic hydrolysis (with porcine liver esterase) are discussed. Three different QuEChERS-integrated hydrolysis procedures are applied to hydrolyze resistant esters:

- For **"simple" commodities** (like most fruits and vegetables) the hydrolysis conditions remain as described in CEN-QuEChERS (0.25 mmol/mL* / 40°C / 30 min).
- For **cereals, pulses and potatoes** harsher conditions are needed. The base amount is doubled but the temperature is kept at 40°C to avoid clumping, therefore the reaction time is extended
  (0.5 mmol* /mL / 40°C / 120 min).
- For **"complex" commodities, such as citrus fruits**, the harshest conditions are employed
  (0.5 mmol* /mL / 60°C / 60 min).

*calculated on the basis of 20 mL volume after addition of acetonitrile.

To give a hint on the extend of conjugation within real samples and the impact of hydrolysis on the release of acidic pesticides, a compilation of results from the analysis of incurred 2,4-D, Fluazifop and Haloxyfop, with and without applying hydrolysis, is presented.

History

Action	When	Document Version
Experiments	2006-2020	
Observation document placed on-line	05.03.2019	V1