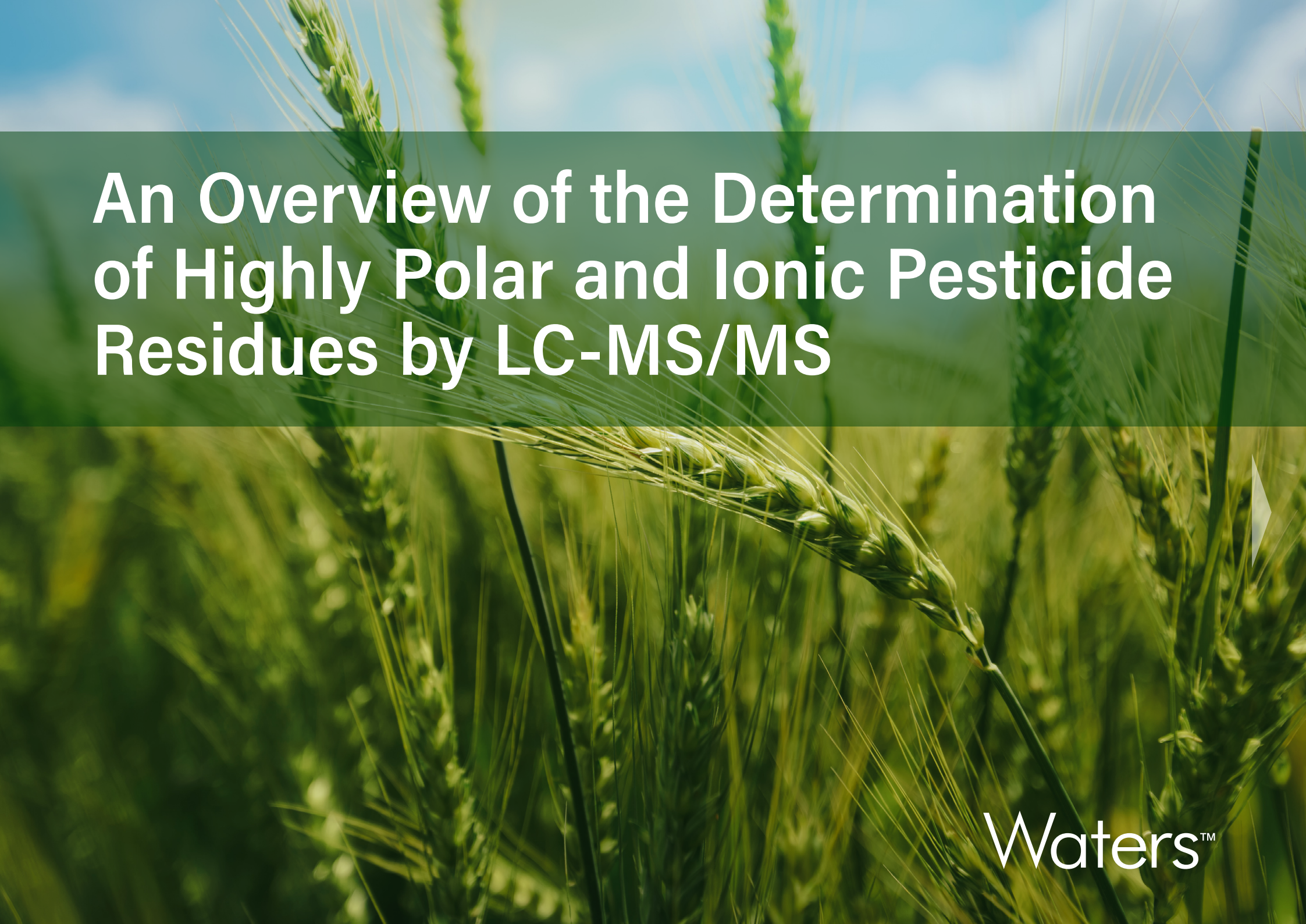


An Overview of the Determination of Highly Polar and Ionic Pesticide Residues by LC-MS/MS

Waters™

Introduction

Implementing effective methods for the determination of residues of highly polar and ionic pesticides has been an important objective for food testing laboratories over the last two decades. This has partly been driven by potential safety concerns and increased public interest in the herbicide glyphosate, but also issues associated with residues of other highly polar pesticides, such as ethephon, and contaminants, such as chlorate. As a result of these intensive activities, many of these compounds have been included in the scope of official and food industry monitoring programs and many food testing laboratories have sought to achieve an efficient and reliable multi-analyte methodology, to meet the demands of increased surveillance and brand protection.

The direct analysis of highly polar pesticides is possible but various aspects of the workflow need to be considered, such as:

- Extraction and clean-up
- Chromatography
- Determination

This eBook aims to provide a short background on the direct determination of highly polar pesticides by liquid chromatography, coupled to tandem quadrupole mass spectrometry (LC-MS/MS).

INCREASING NEED FOR MORE EFFECTIVE AND EFFICIENT METHODOLOGIES

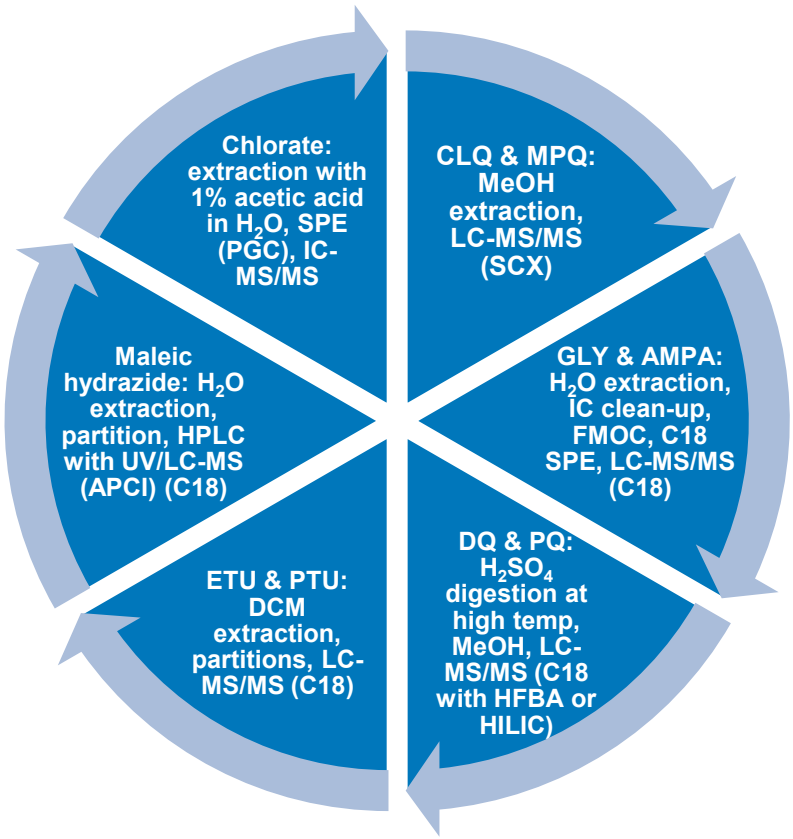
The monitoring of highly polar and ionic pesticides in foodstuffs has noticeably increased over the last decade. Where usage is approved, maximum residue limits (MRLs), or tolerances in the USA, are often set relatively high. Some countries (*e.g.* European Union [EU] and Japan) operate a system of "default MRL", equal to the limit of quantification (LOQ), which is applicable for pesticides not explicitly mentioned in the MRL legislation. The value of the default EU MRL is typically 0.01 mg/kg, but for these more challenging analytes, they are often set higher (*e.g.* glyphosate at 0.1 mg/kg).

However, laboratories often target lower LOQs, in part driven by public concern over some of these pesticides, the need to generate data for risk assessment, industry protecting their brands and laboratories preparing for the impact of possible bans in the future. Monitoring of the residues of these pesticides is now mandatory as a part of national residue control programs in many parts of the world and are of significant interest to the food industry. In part, this is due to the advances made in developing suitable methods and the availability of analytical standards.

For MRL compliance testing, metabolites are sometimes included in the residue definition (*e.g.* EU glufosinate: sum of glufosinate and its salts, 3-methyl-phosphinico-propionic acid (MPPA), and N-acetyl-glufosinate (NAG expressed as glufosinate).

	Glyphosate in wheat (mg/kg)	Chlormequat chloride in oat (mg/kg)
EU	10	15
USA	30	40*
Canada	5	40
China	5	10
Japan	30	6

**When a registration does not exist, interested persons may submit a petition requesting that the competent authority establish an import MRL/tolerance for a pesticide residue on a food commodity, which will allow the food treated with the pesticide in foreign countries to be lawfully imported.*



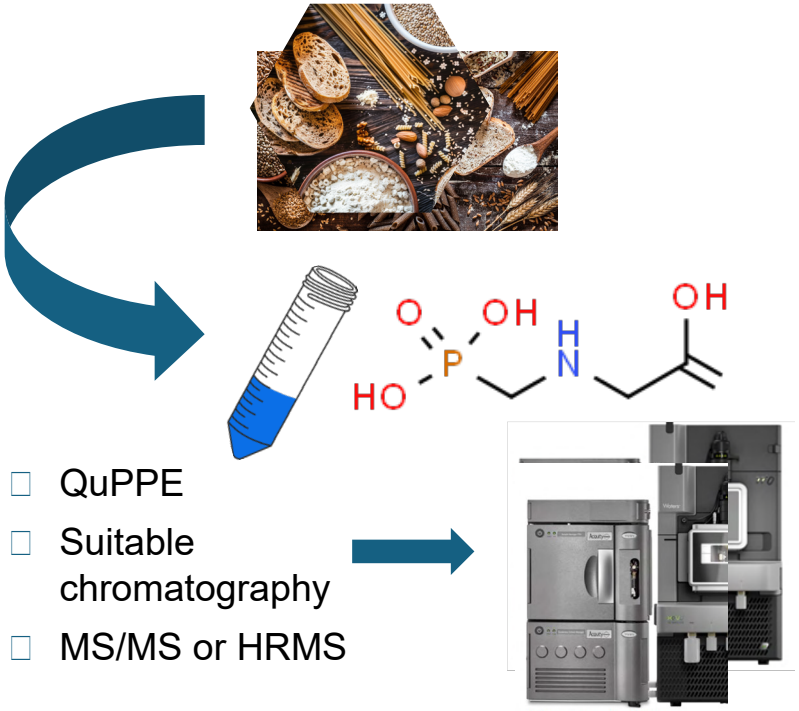
TRADITIONAL APPROACHES

THE ANALYTICAL CHALLENGE

Although various multi-residue LC-MS/MS methods are available to analyze food for pesticide residues, residues of highly polar and ionic pesticides remain a considerable challenge. These pesticides, and their metabolites, are not "amenable" to common multi-residue methods. They need alternative conditions for extraction and are not sufficiently retained on the typical reversed phase (RP) liquid chromatography (LC) columns using conditions used for multi-residue analyses.

SINGLE RESIDUE METHODS

Historically, the analysis of these analytes has been achieved using a series of selective single residue methods, resulting in multiple workflows. For example, workflows for glyphosate often employed time consuming derivatization steps to allow retention and separation by gas chromatography (GC) or LC using traditional RP column chemistries. Diquat and paraquat required the use of ion pairing reagents with RP LC. As this all added significantly to the overall costs of monitoring, highly polar and ionic pesticides were often omitted from monitoring or methods were employed judiciously, focusing just on commodities known to potentially contain the analytes in question. Consequently, knowledge about the prevalence and hence the risk from residues of these highly polar and ionic pesticides in food was limited.



Link out to QuPPe website

QuPPe (QUICK POLAR PESTICIDES) METHOD

Some earlier methods targeting glyphosate only were based on an extraction with acidified water, which often contained ethylenediaminetetraacetic acid (EDTA), sometimes acidified. Some of the analytes, such as cyromazine, are included in multi-residue methods but have been shown to give low but consistent recoveries.

A SIGNIFICANT STEP FORWARD

Although alternative approaches remain in use, one major influence has been the introduction and continued development of the QuPPe (Quick Polar Pesticides) method, from the EU Reference Laboratory for Pesticides requiring Single Residue Methods (EURL-SRM).[1] QuPPe entails a simple one-step/single-phase extraction with an acidified methanol/water mixture. This method allows the simultaneous extraction of many highly polar/ionic pesticides, their metabolites, and other contaminants of interest in foods. The final extract is suitable for determination by various LC or ion chromatography (IC) methods to determine different combinations of highly polar and ionic pesticides. These chromatographic methods are usually coupled to tandem quadrupole mass spectrometry (MS/MS) but High Resolution Mass Spectrometry (HRMS) has also been utilized. QuPPe has been successfully validated by the EURL for more than 50 highly polar pesticides and their metabolites in various food matrices.

DETAILS OF THE QuPPe METHOD

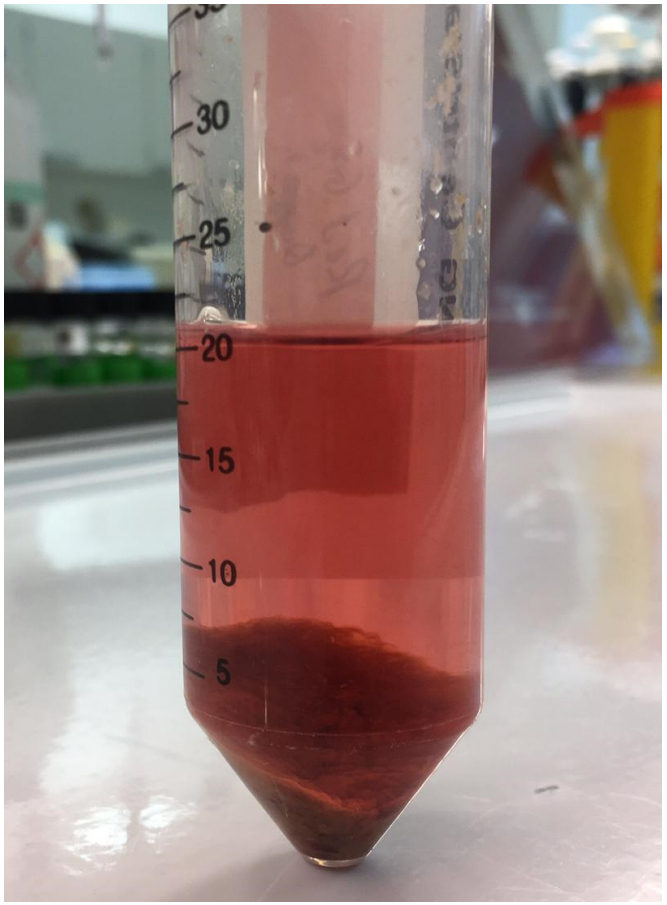
GENERIC EXTRACTION CONDITIONS

The QuPPe method employs generic extraction conditions using acidified methanol as the extraction solvent. There is one version of the method for food of plant origin (QuPPe-PO) and one for food of animal origin (QuPPe-AO), currently focusing on animal tissues, milk and eggs but excluding honey. Unlike the Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) extraction, commonly used for multi-residue methods, there is no partitioning step in the QuPPe extraction method which can result in a significant level of co-extractives in the final extract. For example, fruit and vegetables are rich in sugars and pigments and maize and soybean contain starch and oil. The extraction procedure is modified for better extraction efficiency of paraquat and diquat with the addition of 0.1M hydrochloric acid (HCl) and heating in a water bath.

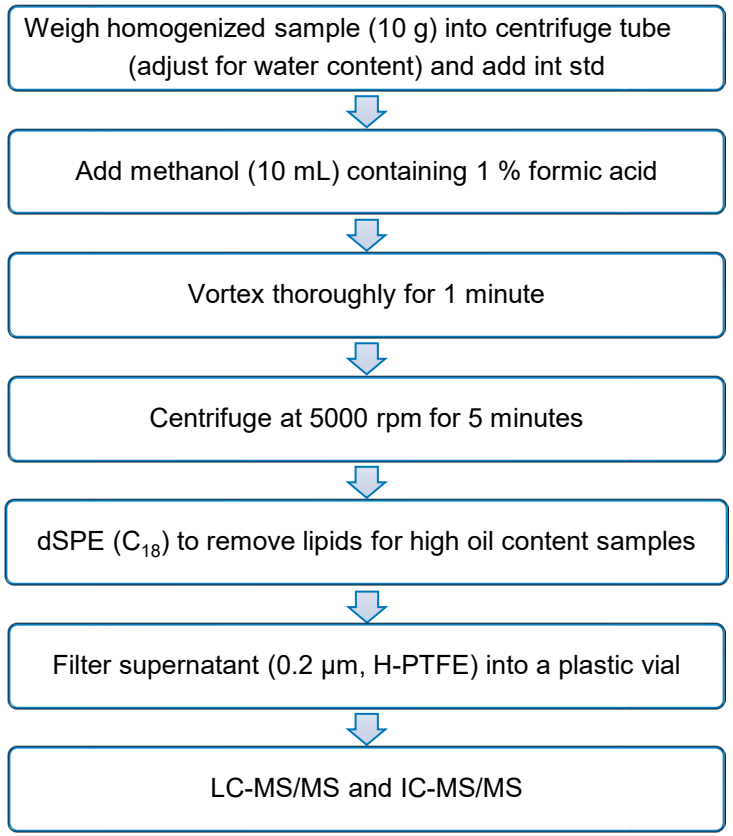
The use of plastic material for standard preparation, storage and sample extraction is recommended. Plastic vials are recommended for sample analysis.

LIMITED CLEAN-UP OPTIONS

Currently, there are limited clean-up options described in the QuPPe method, which can lead to issues with matrix effects and isobaric interferences unless measures are taken to mitigate these effects. Many laboratories employing this method utilize stable isotope analogues as internal standards to adjust for any losses during the method and the impact of matrix effects. Dilution of the extracts is also a popular option but dependent on fundamental instrument sensitivity to be successful.



An example of a red grape QuPPe extract .



Generic version of the QuPPe-PO method procedure.

EXTRACTION

The current version of QuPPe-PO has three separate procedures; the original for most commodities, separate ones for cereals, pulses, nuts, and oily seeds and a third for honey, reflecting the added complexity of these commodities. Some additional steps when working with low water content and/or oily commodities include:

- Freeze-out, prior to centrifugation for the precipitation of poorly soluble co-extractives
- Addition of EDTA solution for the complexation of metal cations, especially when analysing glyphosate and AMPA
- Precipitation with acetonitrile for the removal of proteins
- Precipitation with acetonitrile combined with dSPE using C_{18} sorbent for the removal of proteins and fat
- Ultrafiltration using cut-off filters of 5 or 10 kDa (optional)
- Dilution to reduce matrix load

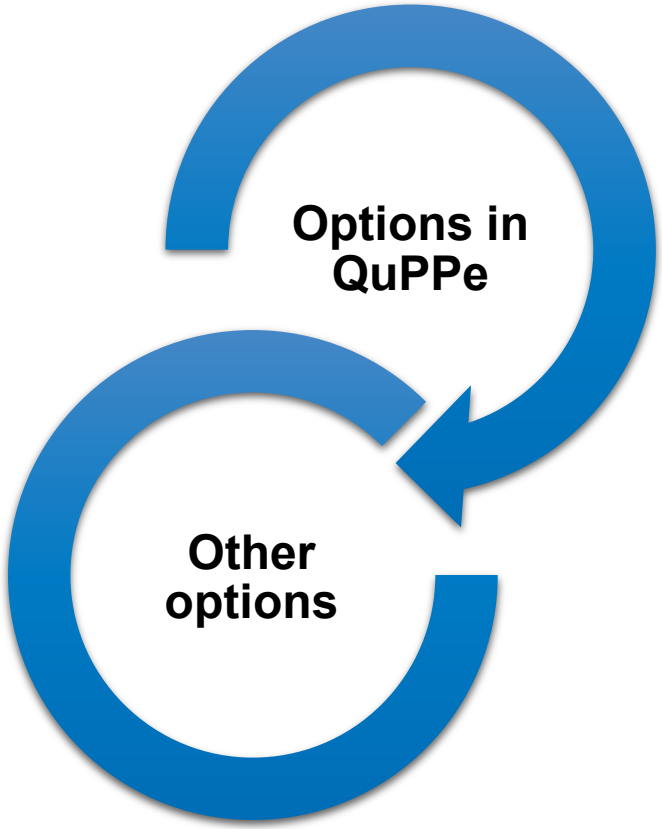
CLEAN-UP

Clean-up options in QuPPe are limited as must be suitable for the range of highly polar, ionic analytes listed in the method, but recommendations include:

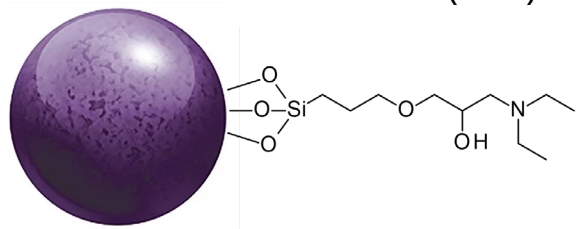
- Freeze-out step to precipitate out co-extracted nonpolar components
- Cryogenic centrifugation (*e.g.* at -10 °C).
- Dispersive solid phase extraction (dSPE) using C_{18} to remove lipids
- Protein precipitation via the addition of acetonitrile
- Centrifugation assisted ultrafiltration through a 5 kDa cut-off filter.

OTHER OPTIONS

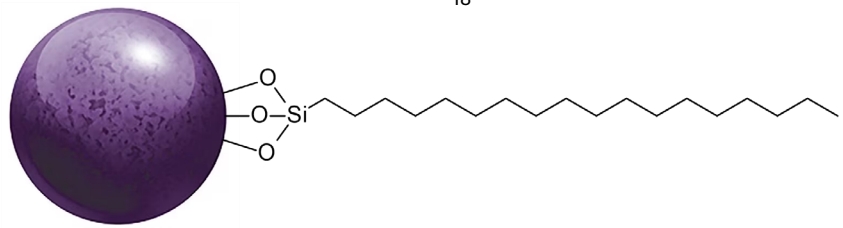
- Pass-through SPE using reverse-phase mechanisms (*e.g.* C_{18}, Oasis HLB)
- Molecular imprinted polymers (MIPS)
- Anion exchange resin packed into a glass column
- Strong or weak anion and cation exchange SPE, alone or in combination with the pass-through approach described above



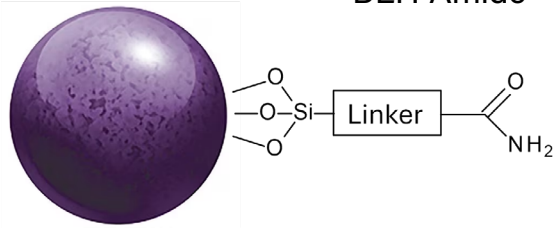
Anionic Polar Pesticide Column (DEA)



BEH C_{18} AX



BEH Amide



CHROMATOGRAPHIC CONSIDERATIONS

When faced with the analysis of a large range of highly polar pesticides, there are now plenty of chromatographic options to choose from.

CHOOSING A COLUMN

Use of reserved-phase with ion pair reagent or pre-column derivatization.

There are several column stationary phases which have been promoted for the direct analysis of highly polar pesticides. These include:

- Hydrophilic Interaction Liquid Chromatography (HILIC)
- Mixed Mode Chromatography
- Porous Graphitic Carbon (PGC)
- Ion Exchange or Ion Chromatography (IC)

SOME KEY CONSIDERATIONS

- Complexity of method setup
- Retention
- Separation
- Peak shape

CHROMATOGRAPHIC CONSIDERATIONS

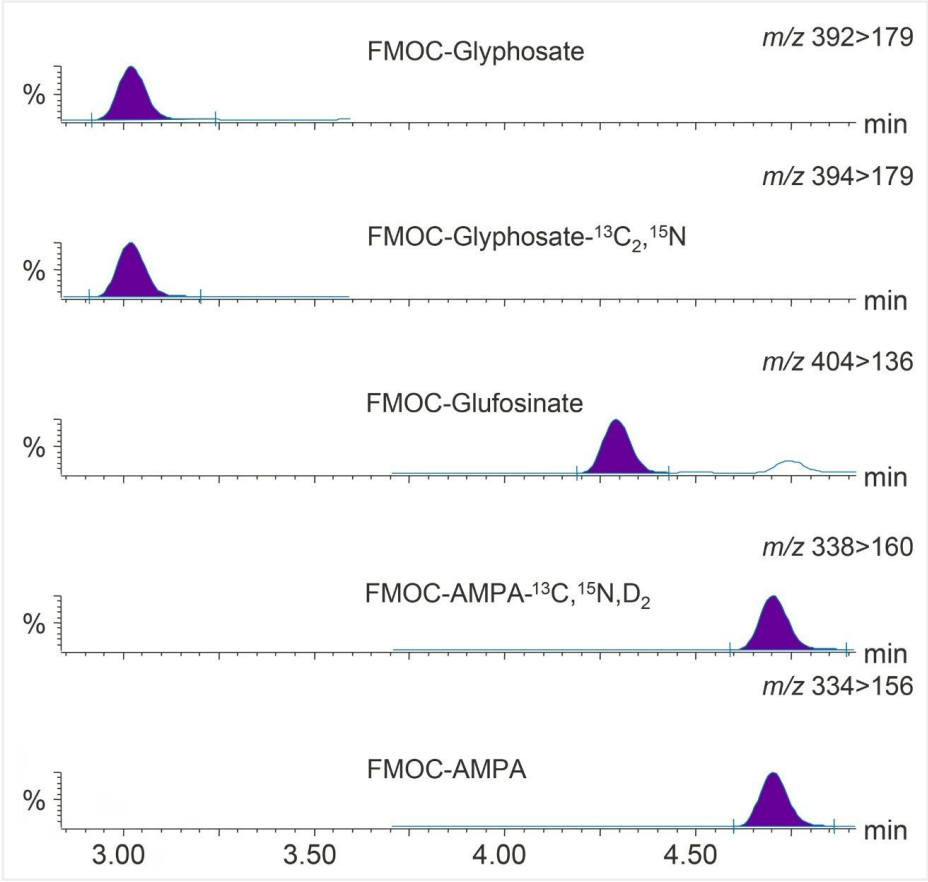
USE OF ION PAIRING REAGENT OR PRE-COLUMN DERIVATIZATION

Highly polar and ionic compounds have little or no retention on RP columns when using MS-friendly mobile phases. To overcome this problem, ion pairing reagent or pre-column derivatization have been used in some cases.

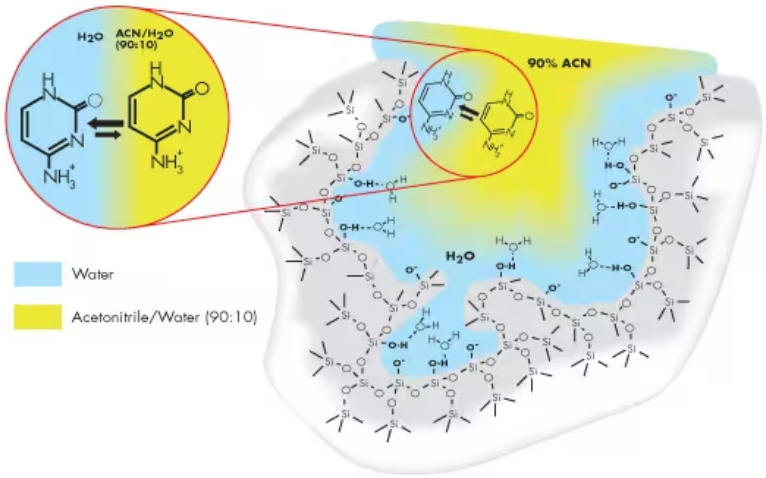
The US FDA employed 4 mM tetrabutylammonium hydroxide as an ion pairing regent to successfully determine glyphosate, N-acetyl glyphosate, and another herbicide, glufosinate, in a range of foodstuffs.[2]

Glyphosate, its metabolite aminomethylphosphonic acid (AMPA) and glufosinate, are amenable to reverse phase chromatography after derivatization with fluorenylmethoxycarbonyl chloride (FMOC).[3]

However, many laboratories have sought methodology that not only simplifies the analysis, but also extend the scope to the determination of the N-acetyl metabolites of glyphosate and glufosinate, as well as other highly polar and ionic pesticides, with very different structures. These will not be amenable to FMOC derivatization unless the compounds are primary or secondary amines.



Chromatograms showing glyphosate, glufosinate, and AMPA after derivatization with FMOC.



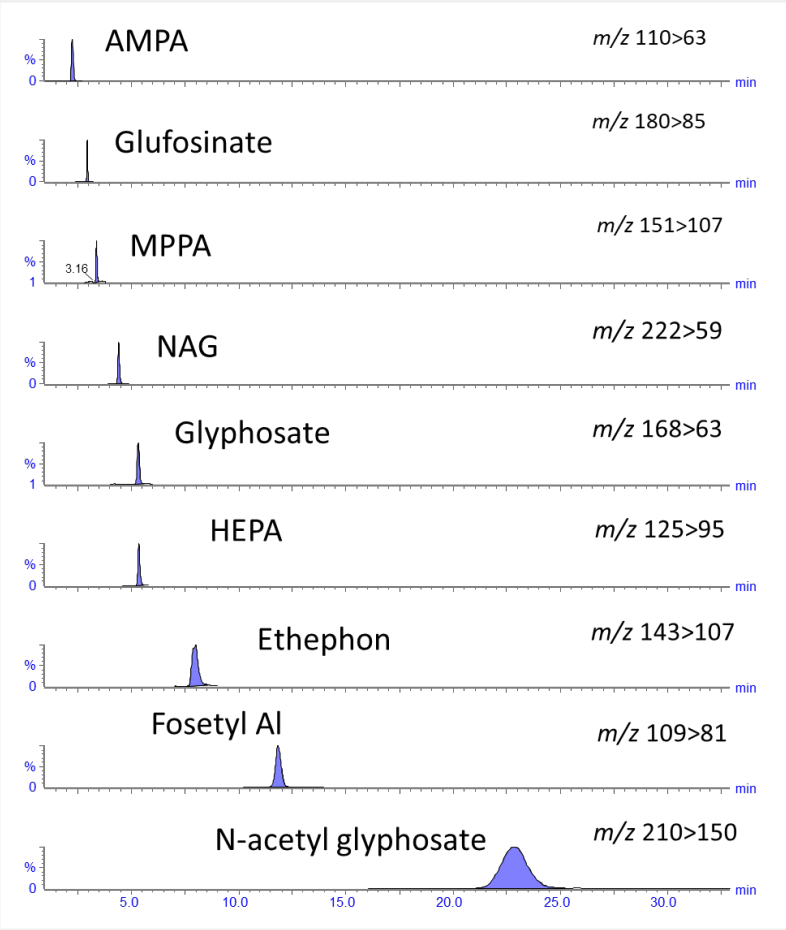
HILIC mechanism image.

CHROMATOGRAPHIC CONSIDERATIONS

HYDROPHILIC INTERACTION CHROMATOGRAPHY (HILIC) OR MIXED MODE?

HILIC is a technique in which the separation mechanism is primarily due to the partitioning of the analyte between a water-rich layer near the hydrophilic surface of the stationary phase and a hydrophobic-rich mobile phase. Polar stationary phases retain water strongly on their surface, and in these conditions, a partitioning phenomenon is formed, in which the pesticides will move from the water rich layer near the hydrophilic surface of the stationary phase to the acetonitrile rich solvent, based on their hydrophilicity. The more hydrophilic the pesticide, the more the partitioning equilibrium is shifted toward the immobilized water layer on the stationary phase, and thus, the more the pesticide is retained. However, the overall retention is more complex and typically comprises several processes, which is why it is often described as a mixed-mode retention mechanism. Several interactions/ mechanisms (partitioning between an aqueous-rich layer at the stationary phase and the rest of the mobile phase; hydrogen bonding between polar functional groups and aqueous layer and/or stationary phase; electrostatic interactions of ionized functional groups; and ion exchange) can all contribute to retention of the pesticides.

Read this whitepaper to learn how to compare different HILIC stationary phases for the determination of highly polar pesticides.



Chromatograms of highly polar, anionic pesticides and metabolites in rice extract using the Waters APP column.

CHROMATOGRAPHIC CONSIDERATIONS

HIGHLY POLAR, ANIONIC PESTICIDES AND THEIR METABOLITES

The Waters™ Anionic Polar Pesticide (APP) Column, is made up of ethylene bridged hybrid (BEH™) particles with tri-functionally bonded diethylamine (DEA) ligands. The combination of the hydrophilic surface and the anion-exchange properties of the ligand provides chromatographic characteristics well suited to the retention and separation of highly polar and anionic compounds. The column is best used with an acidified mobile phase for optimum sensitivity. Two methods (M 1.6 and M 1.7) using the APP column are published in the QuPPe document.

Chlorate and perchlorate have been previously determined using the APP column, but the method relied on using a high concentration of ammonium formate buffer, which can result in signal suppression. The BEH C_{18} AX stationary phase contains both C_{18} and tertiary alkylamine groups, the latter creating a positive surface charge below approximately pH 9. The combination of the hydrophobic and anion-exchange properties provide chromatographic characteristics that facilitate separation and retention of these oxyanions with a reduced concentration of ammonium formate.

Read the application notes for methods using the Anionic Polar Pesticide Column and the Atlantis™ Premier BEH C_{18} AX Column

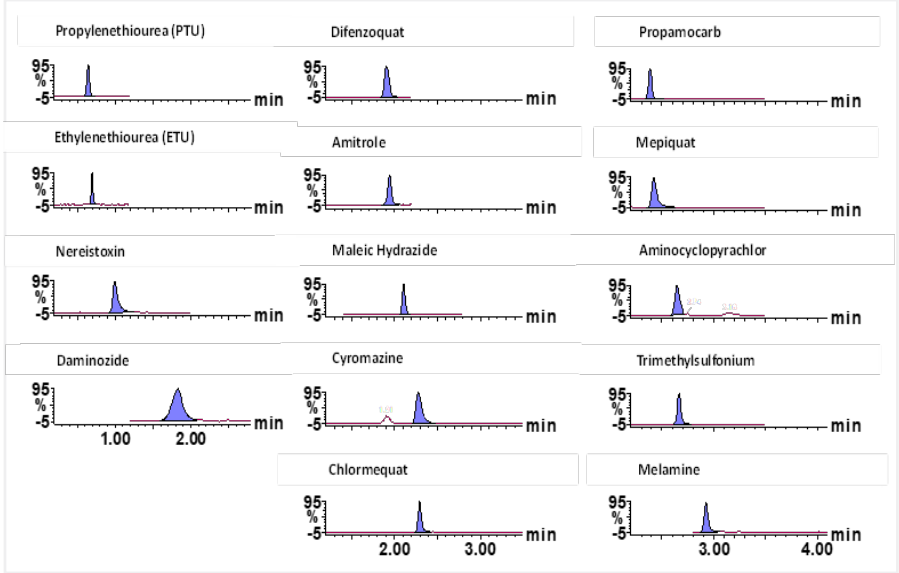
CHROMATOGRAPHIC CONSIDERATIONS

HIGHLY POLAR, CATIONIC AND BASIC PESTICIDES AND THEIR METABOLITES

Residues of some of the highly polar, cationic and basic analytes included in the QuPPe method have been determined using RP LC. However, in most cases the retention is poor or insufficient.

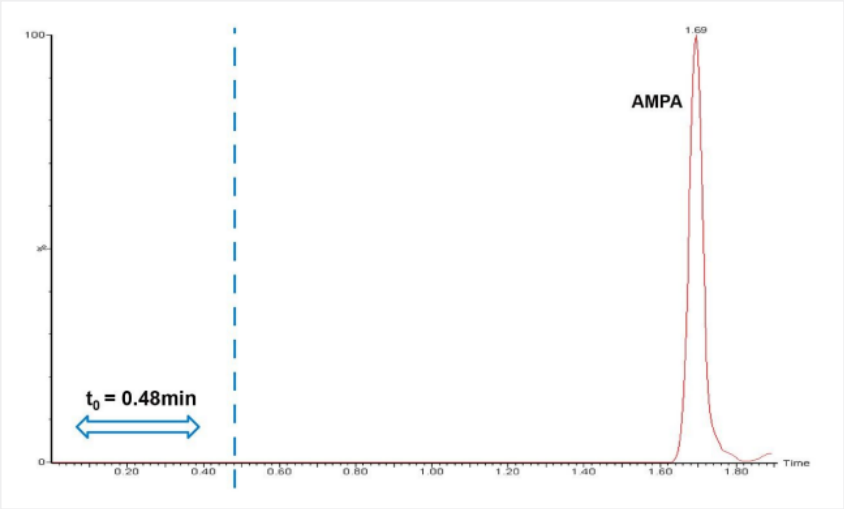
The main benefits of the use of HILIC are the better retention of very polar compounds that are usually difficult to retain in reversed phase chromatography. The separation mechanism can depend on many factors, such as the physicochemical properties of the stationary phase, the mobile phase, and the structures of the samples investigated. The ACQUITY™ BEH Amide Column is packed with ethylene-bridged hybrid particles covalently attached by trifunctionally bonded amide groups. Here, retention is driven by both partitioning and ionic interactions, so the method is capable of determination of a wide range of basic/cationic pesticides in one run.

A method (M 4.2) based upon the BEH Amide Column is published in the QuPPe document.



Chromatograms of highly polar, cationic and basic pesticides and metabolites in wheat extract using the Waters BEH Amide Column.

Read the application note on the determination of cationic polar pesticides and plant growth regulators



Chromatograms showing the retention of AMPA on the APP column.

RETENTION OF HIGHLY POLAR PESTICIDES

ANALYTE RETENTION

The retention time (RT) is the time taken for the solute to interact with the stationary phase and pass through the column. Retaining analytes beyond the retention time corresponding to the void volume is important to avoid ion suppression and isobaric interference from unretained co-extractives. Retention time should be stable independent of commodity

GUIDELINES FOR ANALYTE RETENTION

Analytical quality control, performance and method validation guidelines such as SANTE/11312/2021v2[4] state "the minimum acceptable retention time for the analyte(s) should be at least twice the retention time corresponding to the void volume of the column (t_0)." The column void volume (v) is a measure of the internal volume inside the column packed with the stationary phase particles and can be estimated from a column's length (L) and internal diameter (ID). Mobile phase flow rate should also be considered when assessing if an analyte is retained or not. The use of a void marker compound is the most accurate way to assess t_0 but rarely used in practice.

Read our blog titled, out to blog "Retained or Not Retained? How Much is Enough Retention?"

ESSENTIAL SEPARATIONS

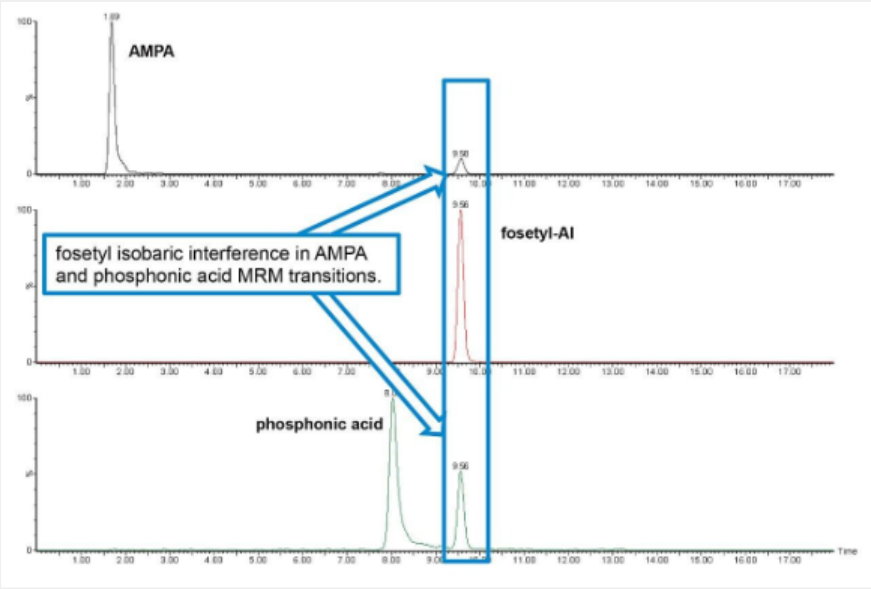
Optimization of the gradient program is essential to enhance the selectivity provided by the interaction between the analytes, mobile phase and column chemistry.

CRITICAL SEPARATIONS TO AVOID ISOBARIC INTERFERENCE FROM TARGETED ANALYTES LEADING TO FALSE DETECTIONS

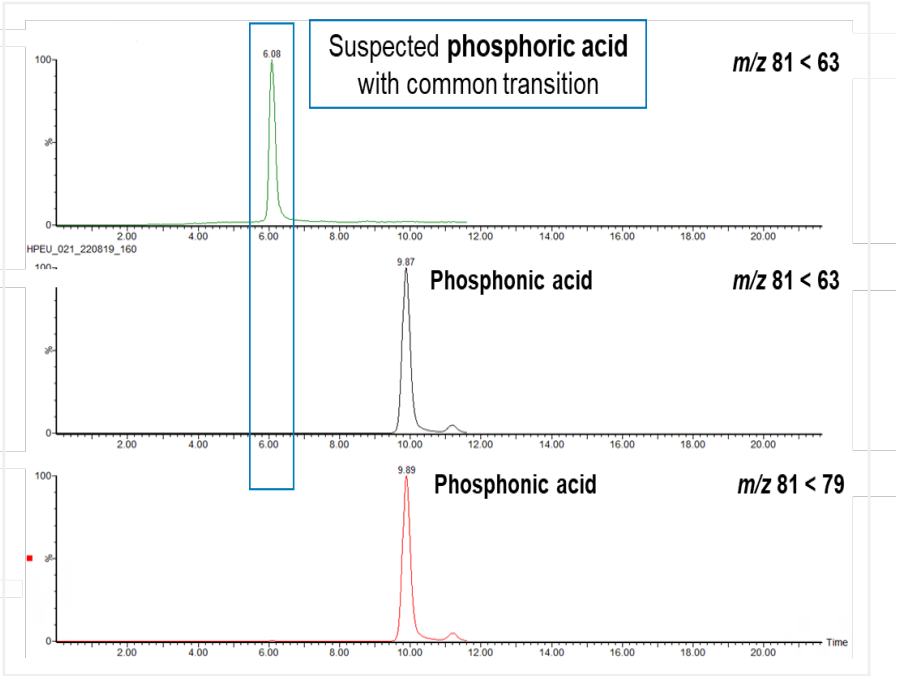
The QuPPe document describes some key chromatographic separations that are required for any method which has been developed for the analysis of highly polar pesticides.

These separations include those between AMPA, fosetyl, and phosphonic acid. Fosetyl Al and AMPA both share the same multiple reaction monitoring (MRM) transition, *m/z* 110>81, so chromatographic separation is required to avoid false identification. Fosetyl also degrades to phosphonic acid in LC-MS/MS sources so good separation is also required between these two compounds.

Another set of critical pairs that needs chromatographic separation due to in source fragmentation issues are AMPA/N-acetyl AMPA and chlorate/perchlorate.



Using the Waters APP Column, AMPA, fosetyl Al, and phosphonic acid are completely separated.



Using the Waters APP Column, phosphonic acid and phosphoric acid are completely separated.

SEPARATION IS CRITICAL

CRITICAL SEPARATIONS TO AVOID ISOBARIC INTERFERENCE FROM MATRIX CO-EXTRACTIVES LEADING TO FALSE DETECTIONS

During the determination of pesticide residues in crude extracts, the presence of co-extracted components, typically at much higher concentrations than the analytes, can cause problems with ion suppression and isobaric interference.

Many samples naturally contain high concentrations of phosphoric acid, which is coextracted during the sample preparation process. Although *m/z* 81>63 is a very minor transition for phosphoric acid, its potential interference on phosphonic acid can be very significant due to the typically high levels of phosphoric acid in samples. In addition, if the chromatographic separation is insufficient, the response of the phosphonic acid can be suppressed and, in some cases, even leads to false negative results. High levels of phosphoric acid can also affect the determination of bromide if not separated chromatographically.

MRMs based upon the singly charged, protonated precursor ion for paraquat, at *m/z* 185>170 and 185>169, can interfere with typical MRMs used for diquat. The same applies to the respective MRM for paraquat D8 using precursor ion at *m/z* 193 which is interfered by diquat D8. Although more selective transitions are available, adequate chromatographic separation of diquat and paraquat is recommended.

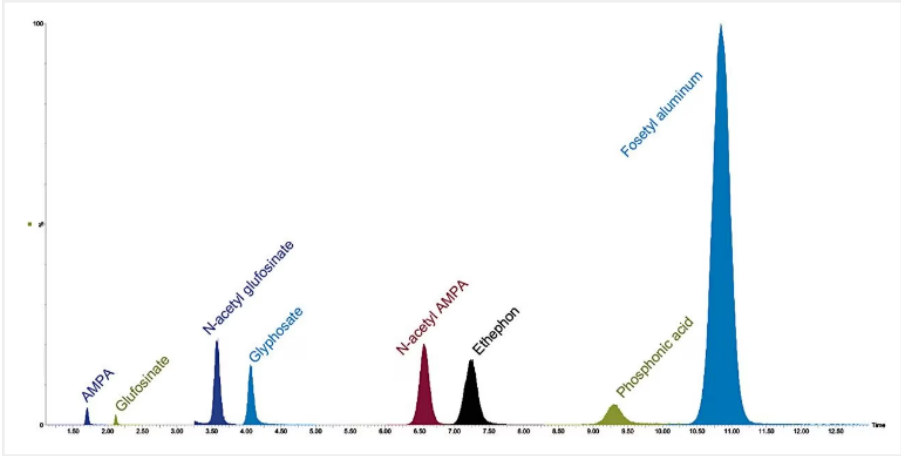
IMPORTANCE OF PEAK SHAPE

POOR AND GOOD PEAKS SHAPES

Analytes that give “poor”, asymmetrical peak shapes tend to have reduced sensitivity (S/N), are more difficult to reliably detect and integrate, and are more prone to interferences than stable analytes that give narrow peaks. Consistent “good”, Gaussian peaks shapes improve detection, quantification, and allow fully automated peak integration avoiding lengthy and inconsistent manual intervention.

Obtaining “good” peak shape for all highly polar pesticides and metabolites of interest in a single run, using MS-compatible mobile phases, can be challenging due to the large variation in chemical structure and physiochemical parameters such as pKa. Analytes tend to be split into subgroups based upon their chemistry as it is currently not feasible to combine anionic and cationic/basic compounds in a single run.

Peak shapes and retention times for all the analytes need to be stable for all the commodities analyzed across lengthy periods of time to avoid having to adjust peak assignment and integration parameters. Consistent retention times are also critical for the successful identification of the analytes.



Chromatograms showing the peak shapes on the Waters APP Column.

ACQUITY Premier and Xevo™ TQ Absolute System

Read this application note: Detection of Anionic Polar Pesticides in Food Samples Using the Xevo TQ Absolute With Sub µg/kg Limits of Quantification

Determination of Highly Polar Pesticides

LC-MS/MS is an essential part of the multi-residue approach to the analysis of these challenging highly polar and ionic analytes

These pesticides rarely have chromophore or fluorophore groups so fluorescence or photometric detectors can only be used with derivatization procedures, which often involve long and complex steps, and limit the analytical scope of any developed method.

Chromatographic methods for highly polar and ionic pesticides can be implemented on a range of modern LC-MS/MS systems. Electrospray is the preferred mode of ionisation; negative ion for the anionic compounds and positive for the cationic and basic analytes. Good negative ion performance is essential to meet the required detection limits for anionic compounds such as AMPA. Improving negative ion detection for MS/MS is described in this whitepaper. LC-MS/MS provides enhanced selectivity and excellent sensitivity when using targeted MRM acquisition.

The determination of highly polar and ionic pesticides is also hampered in some cases by their low molecular weight and hence low mass product ions in MS/MS. At least 2 MRM transitions are required for each analyte for identification using the ion ratio. Whilst this is achievable using MS/MS, HRMS has been applied as an alternative. In cases where the number of available fragments might be limited, HRMS relies on accurate mass measurements for identification; of the molecular ion and one fragment.

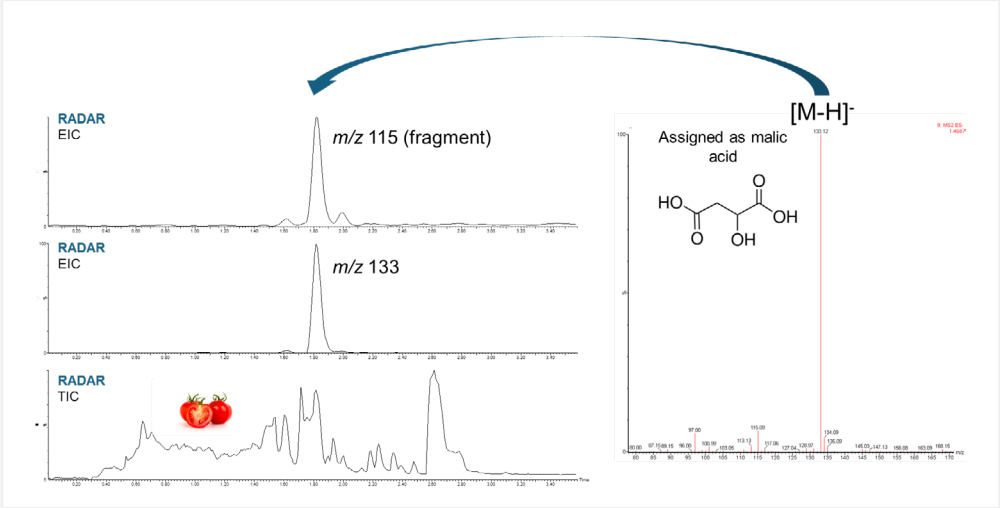
INVESTIGATION OF MATRIX INTERFERENCES

INVESTIGATE MATRIX COMPLEXITY DURING METHOD DEVELOPMENT

During the analysis of food extracts, the presence of co-extracted components, typically in excess of the analytes, can cause problems with suppression and isobaric interference. For example, two major organic acids, citric acid and malic acid, are present in tomatoes at significant levels. Without chromatographic separation, the presence of these organic acids will suppress the signal from compounds such as AMPA and glufosinate, significantly reducing the sensitivity of the method.

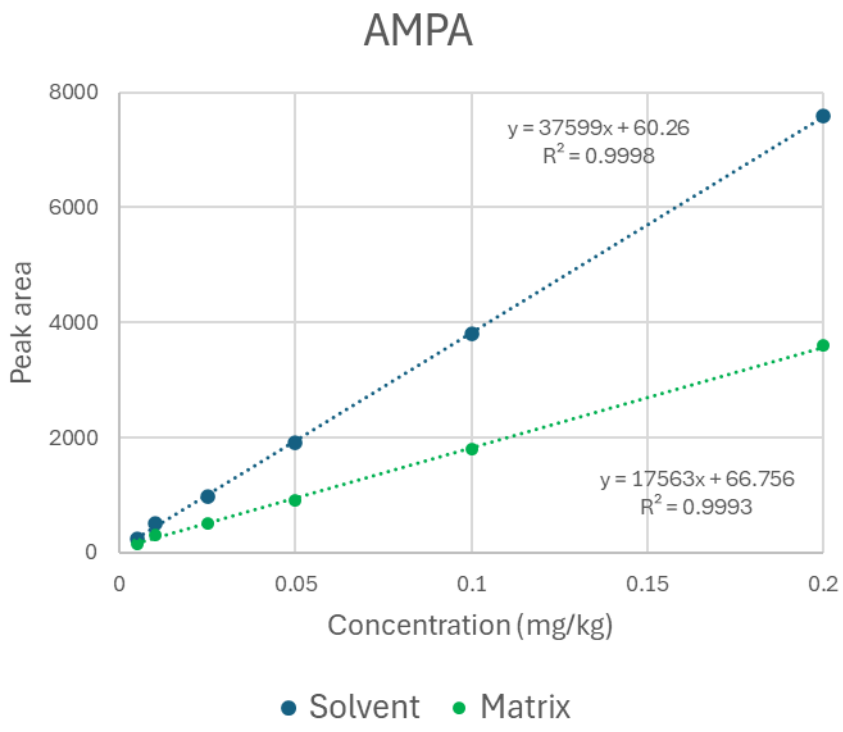
CRITICAL SEPARATIONS TO AVOID MATRIX EFFECTS LEADING TO FALSE DETECTIONS

Collecting full scan data, simultaneously with targeted MRMs, can be useful to investigate what is likely to co-elute with compounds of interest. We can look for likely candidates in the full scan data and monitor how changes to the methods alter the retention of the analytes relative to the endogenous peaks. By better understanding the complexity of the data, we can ensure that there is sufficient separation between the first two eluting polar anionic pesticides, AMPA and glufosinate, and the malic acid from the tomato.



Chromatograms showing the detection of malic acid using RADAR.

Read this whitepaper on RADAR technology



Watch this webinar on compensating for matrix effects in complex samples to learn more.

UNDERSTANDING MATRIX EFFECTS

MATRIX EFFECTS

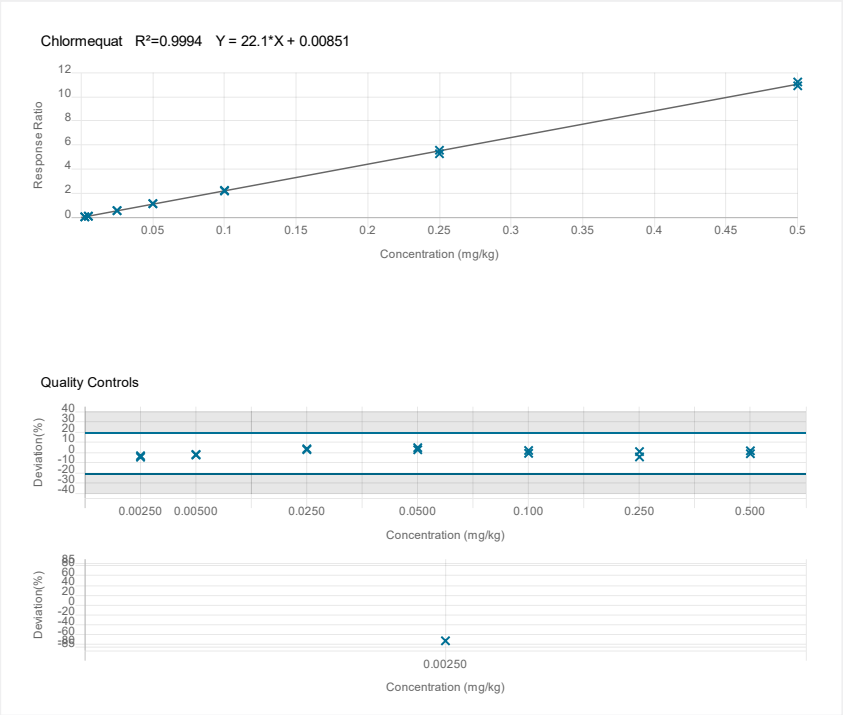
The use of LC-MS/MS has revolutionized the determination of highly polar and ionic pesticides in food testing laboratories. However, one major drawback of limited or no sample clean-up is the potential for the phenomenon of matrix effects. The influence of matrix on the reliability of your method should be assessed during method development and recorded as part of validation procedures.

COMPENSATING FOR MATRIX EFFECTS

Ionization efficiency in the source is impacted by the co-elution of matrix co-extractives with analytes, resulting in ion suppression or enhancement. Significant variations in the magnitude of matrix effects have been observed between different commodities. The use of matrix-matched calibration and stable isotope analogies as internal standards is recommended to mitigate for matrix effects. In cases where obtaining true blanks is an issue, standard addition is an accurate quantitative option for confirmations.

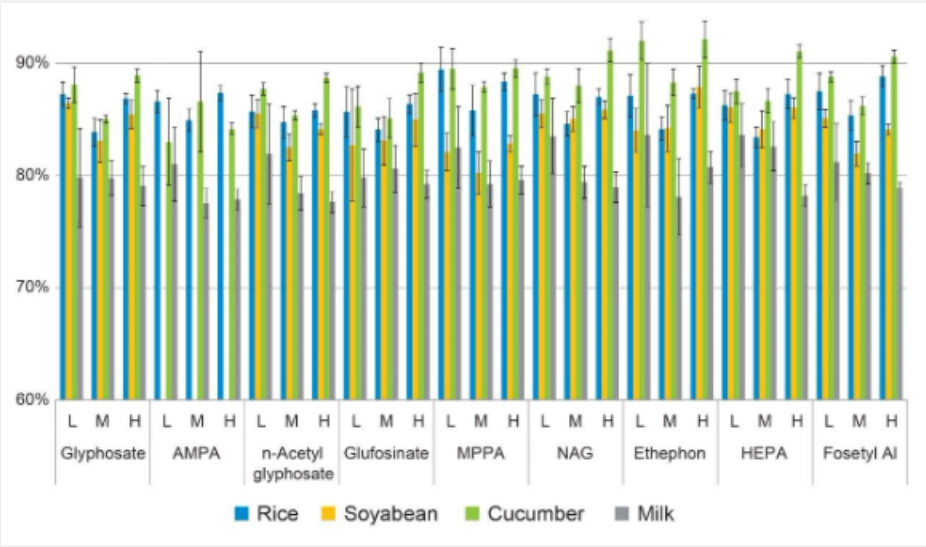
DATA ACQUISITION, PROCESSING, AND REVIEW

The determination of multiple highly polar and ionic pesticides, each with two MRM transitions, creates a significant amount of data for review. Matters can be complicated further when batches comprise samples from a wide range of different commodities or finished food products, all with differing isobaric interferences. Along with the automatic but accurate integration of the peaks of interest, various characteristics and acceptance criteria associated with detection, quantitation and identification must be adhered to. Effective automation of data processing and review can help avoid bottlenecks and provide a more standardised approach by reducing manual intervention. Software with review by exception and the use of flagging functionality can increase the quality and efficiency of the data review process. This can enable analysts to quickly identify exceptions which fall out-side of the rule sets, such as suspect peak integrations, failure to meet identification criteria or calibration graphs that do not meet the minimal acceptance criteria. In addition, setting a suitable minimum reporting limit for each analyte allows the user to concentrate on key samples, for example those that have concentrations of pesticides at concentrations exceeding regulatory limits.



Viewing calibration graph and QC sample in the MS Quan app.

Read this whitepaper The benefits of waters_connect™ MRM processing application, MS Quan



Summary of the measured recoveries and repeatability from the analysis of spikes from cucumber, rice, soyabean, and milk.

Read this application note **on the reliable determination of polar pesticides in a range of foods**

Enabling Technologies and Services from Waters

Glyphosate is the most known of the "highly polar pesticides", due to the controversy about its toxicity in recent years. However, besides glyphosate, there are many other relevant, highly polar pesticides and metabolites, which form an extremely challenging group to be analyzed due to their physical-chemical properties.

When approved on certain crops, MRLs have been established, sometimes with metabolites included in the residue definition. In the absence of approved use in the EU, the default MRL applies. Monitoring of residues, either by competent national authorities for official control or by the food industry for due diligence, need flexible, fast, reliable and efficient testing solutions.

With an extensive portfolio of instruments, services and support we provide quality, knowledge and confidence for optimum productivity in your laboratory. We partner with you to ensure a successful purchase outcome, employing our global team of application experts to assist in instrument setup and user training. Provision of reference guides for customers is an important part of this relationship. For example, Waters provide a Startup Guide, which includes an analytical method and troubleshooting for the analysis of anionic polar pesticides and its related compounds.

Resources

1. Anastassiades, M et al. (2023). Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement. Food of Plant Origin (QuPPe-PO-Method), Version 12.2.
2. Chang E et al (2021). Glyphosate and Related Residues in Food – Harmonized Method for Detection and Quantitation. FDA Method C-013.01.
3. Ehling S and Reddy T (2015). Analysis of Glyphosate and Aminomethylphosphonic Acid in Nutritional Ingredients and Milk by Derivatization with Fluorenylmethyloxycarbonyl Chloride and Liquid Chromatography–Mass Spectrometry. J. Agric. Food Chem. 63(48):10562–10568.
4. SANTE/11312/2021v2. Analytical Quality Control and Method Validation Procedures for Pesticide Residue Analysis in Food and Feed.

Waters™

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
waters.com