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METHODOLOGY ANALYSIS

QuEChERS

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The term QuEChERS was first coined in 2002, and subsequently published in 2003, as an acronym of **Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged, and **S**afe and describes a method of extracting multiple analytes from biological samples (1). While the scientific concepts that underpin the technique were not particularly new, the success of QuEChERS came from combining these into one relatively simple sample analysis approach coupled with improvements in instrumentation.

Despite its benefits, QuEChERS is not always seen as a go-to methodology outside of residue analysis. The throughput benefits of QuEChERS are not always required, notably for those studies conducted in a research setting.

This review will explore the application of QuEChERS beyond residue analysis and offer discussion on the pros and cons in each case, and review some of the available variants and their applications.

ENVIRONMENTAL SCIENCES

OVERVIEW

History of QuEChERS

QuEChERS has been embraced by the residue monitoring industry, since fast and reliable high-throughput analysis is of paramount importance based on the need to monitor the levels of, or contamination by, pesticides in food commodities. QuEChERS methods (there are multiple adaptations) can accommodate multiple compounds in one analysis making them a suitable extraction technique for multiresidue methods (MRMs). Before the advent of QuEChERS, MRMs were labor intensive, complex, time-consuming, and used a significant quantity of reagents, and as such, were expensive, error prone and environmentally problematic.

Extraction and Analytical Principles

The full history and detailed theory of QuEChERS is described in detail elsewhere (2). The process of extraction is to separate the analyte from the matrix as much as possible, followed by clean-up of potentially interfering matrix components to allow accurate and precise quantification. In simplest terms the goal is to enhance the signal (analyte) to noise (matrix) ratio (S/N) by removing enough of the matrix. Sufficient matrix removal is of key importance since it is not always necessary in terms of time and resources to over-optimize a method when it is not needed.

A sufficiently precise and accurate method (extraction + instrument) where there is a high degree of confidence that the correct analyte(s) are being quantitated and that matrix components are not introducing bias can be said to be suitably selective. The selectivity of a method refers to the extent to which it can determine analyte(s) in a complex mixture without interference from other components in the mixture (3).



QUECHERS AT SMITHERS

Smithers labs perform a broad array of methods that require sample extraction and analysis. The number of samples to analyze in each study can vary greatly from a handful to thousands. QuEChERS methods prove to be especially valuable tools when there are many samples to analyze. Crop residue packages routinely consist of several hundreds of samples, with field dissipation and livestock feeding studies having a significant but lower sample load. Other tests where QuEChERS has been employed at Smithers include ecotoxicology studies,fish bioconcentration, and field and lab pollinator studies.

BACKGROUND OF USE

Applications

Metabolism and Triggered MOR Studies

Due to the low sample number, and limited requirement for extensive sample clean-up, QuEChERS is not usually considered for metabolism studies since there are limited economic or analytical benefits. As a consequence of the requirement for total radioactive recovery (TTR), tissue samples are extensively extracted with various solvents and techniques so that every significant radioactive component (parent + metabolites) is identified. The extraction must consider the efficiency of the solvent and the energy that can be delivered to the sample without destroying it. Vigorous shaking or grinding (e.g. with a Geno/Grinder), Ultra-Turrax® blending, microwave-assisted extraction (MAE) and accelerated solvent extraction (ASE) are additional tools employed to enhance the energy delivered and therefore the extraction efficiency in metabolism studies.

Where QuEChERS is beneficial is when metabolism study results trigger further magnitude of residue (MOR) non-radiolabeled studies. Such studies can generate high numbers of samples and involve independent laboratory validations to facilitate use as a post-approval monitoring method. Care must be taken to ensure that analyte levels are not underestimated through incomplete extraction, and typically, regulators require that the MOR samples undergo the same extensive extraction regime as the metabolism samples. Such extensive extractions can be impractical at the expanded scale of MOR studies. Many of the metabolites identified in the metabolism studies will not form part of the residue definition for the MOR studies due to their levels being below the required thresholds. Due to these potentially reduced identification requirements, the MOR studies might not require such extensive extraction.

To overcome this apparent contradiction (same method of extraction used as the metabolism study, while being not necessarily required or scalable), radio- or cross-validations using a QuEChERS technique can be performed. QuEChERS extraction followed by quantitation is compared to a selection of incurred tissue samples where the concentrations of the significant components are known. If the results are comparable the QuEChERS technique is appropriate to analyze the incurred samples from the MOR study.

If the initial cross-validation is unsuccessful using a QuEChERS technique, the approach can be modified, while retaining the elements of time and cost effectiveness. For example, if it is determined that sample extraction needs to be more extensive than the standard QuEChERS, higher energy extraction can be carried out before the “salting out” (a hydrophobic analyte’s partition into the acetonitrile phase by eliminating the water) step. An aliquot of the extract would be manipulated to have the right composition of organic solvent and water so that partitioning would occur after the salt mixture is added for “salting out.” At that point a normal QuEChERS workflow would continue. If planned and implemented correctly, this considerably reduces the effort and cost expended on these types of studies.

Residue Chemistry Validation Studies

With reference to development of the correct extraction conditions as referenced above, Smithers scientists have employed QuEChERS approaches to validate pesticide methods in a variety of matrices such as animal tissues/blood, soils, and plant commodities. While these are usually smaller-scale studies across multiple matrices, the use of QuEChERS does provide a standardized approach to analysis across a variety of studies, and therefore benefits in terms of laboratory standardization for method development.



QuEChERS in Terrestrial and Aquatic Ecotoxicology Studies

Ecotoxicology sample analysis presents probably some of the most varied challenges in terms of the combination of analyte and matrix. Diverse analytes can be pesticides, biocides, industrial chemicals, human/veterinary pharmaceuticals and unknown or variable composition, complex reaction products or biological materials (UVCBs). Matrices include various water-based media, sediment, soils, numerous tissues, honey, royal jelly and plant commodities. Many of the studies are of small scale in terms of sample analysis requirements, so QuEChERS may not immediately present an economic or scientific benefit to a single study. However, a uniform approach employed across the various analytes and matrices can still bring long term value based on a standard workflow for method development and application.

There are some ecotoxicology studies with a significant sample analysis requirement: higher tier, long-term fish and amphibian tests, pollinator field and semi-field studies and non-radiolabeled fish bioconcentration studies.

Even using QuEChERS protocols for water analysis is practical in some cases. Water is commonly analyzed by liquid/liquid partitioning (LLP), solid phase extraction, direct injection and dilution before injection (dilute and shoot). In cases where liquid/liquid partitioning is required a QuEChERS adaptation can be made where the water to be analyzed is mixed with the QuEChERS salt mixture and acetonitrile in the right proportions for the water and acetonitrile to separate. This is actually a scaled down version of a common LLP technique called “salting out” that has been used for many decades before QuEChERS was first described. If the matrix load in the water sample is particularly high, a a further dispersive solid phase extraction (dSPE) step could clean up the sample before analysis.

Another example of adapting QuEChERS methodologies at Smithers has been in the regular validation and analysis of bee diet samples during terrestrial ecotoxicology studies. The adapted workflow consists of shaking royal jelly (or sucrose solution) with a mix of water and acetonitrile. A QuEChERS salt mixture is then added, and an aliquot of the upper acetonitrile phase is subjected to dSPE before LC-MS/MS analysis.



QuEChERS Workflow

The simplified sample workup of QuEChERS will often be less selective compared to traditional methods, however improvements in analytical instruments have focused on refining the ability to select the analyte above background noise – elevating the applicability of the method. As a consequence, analysis methods often do not require as extensive workup as was the norm with older instruments. This is where QuEChERS comes into its own.

A typical QuEChERS method workflow comprises the following steps:

Mix sample with acetonitrile (mixing / grinding / blending)

Add water if dry matrix. Isopropyl alcohol, ethyl acetate, or toluene can also be used but with reduced extraction efficiency.

Add salts, mix

"Salting out." Normally acetonitrile and water are miscible, but by dissolving enough salt (buffered or non-buffered) in the aqueous phase the two solvents will partition.

Centrifuge

Retain acetonitrile (top layer). Matrix components partition to aqueous layer (1).

dSPE of ACN layer, spin, keep supernatant for analysis

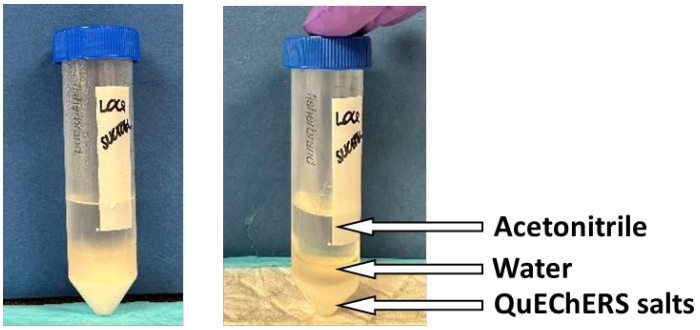
dSPE: usually magnesium sulphate with a combination of primary-secondary amine (PSA), octadecylsilane (C18) and graphitized carbon black (GCB)(1)

Analysis

Typically LC-MS/MS, GC/MS, or GC-MS/MS

The QuEChERS approach provides many benefits to analytical methods. The simplified and standardized approach, while also being flexible enough to provide options to maximize extraction efficiency, provides a regular starting point for extractions. This can save time and resources during method development and validation. QuEChERS uses lower quantities of materials, a significant resource saving compared to other commonly used methods. Its streamlined workflow also offers high sample throughput in less time and at reduced cost. The methodology is very fast, enabling a quick turnaround of results. Based on its simplicity and uniformity, QuEChERS extractions are less prone to systematic and random errors. Finally, the whole extraction process is amenable to automation, providing further opportunities for cost savings and increased throughput.

While QuEChERS has many benefits, there can be some downsides to the approach, mostly as consequences of the uniform approach. As might be expected, selectivity of the extraction is not always as effective as traditional, more iterative methods. The more advanced instrumentation that are usually required for QuEChERS to be successful can be expensive to purchase and maintain. As the extraction solvent volume is limited, there can be reductions in extraction efficiency and total residue recovery methods can be challenging to transfer using QuEChERS analysis.



Pre-and post-centrifugation after addition of QuEChERS salts – notice the fine demarcation between layers after centrifugation.

The use of isotopically labeled internal standards (IL-IS) can be required to compensate for analytical errors. These are not always readily available depending on the analyte(s) and can be expensive to obtain. QuEChERS methodology does not cover the entire range of pesticides that are currently typically tested. Highly polar compounds such as chlormequat, glyphosate and fosetyl are difficult to analyze with a traditional QuEChERS approach as they fail to partition effectively into the organic phase.

In addition, lipophilic analytes can be difficult to extract from fatty matrices – especially important to consider for animal residue feeding studies or high oil content commodities.

These issues, while certainly not trivial, can be mitigated or worked around:

ISSUE	MITIGATION
Reduced selectivity vs. other methods	Selectivity using QuEChERS is often fit for purpose, not perfect. QuEChERS sample extracts can be further manipulated if additional cleanup is required.
Expensive instrumentation	The method is over 20 years old and modern instruments typically found in analytical laboratories are quite capable of running QuEChERS extracts.
Limitations for some types of pesticides	Modified methods such as the QuEChERSER “Mega-Method” and QuPPe are available.

The core QuEChERS method is a simultaneously standardized and flexible approach to the efficient extraction of analytes from a range of biological samples.



GOING BEYOND

QuEChERSER (Mega-Method)

Traditional QuEChERS has already been around for 20 years. The instrument advancements at the time allowed these methods to be implemented but since then, many QuEChERS methods have remained largely the same while instrument performance has advanced further. Using a QuEChERS method designed 20 years previously with the sensitivity of today's QqQ, Q-TOF or Orbitrap™ mass spectrometers, greatly reduces the shortcomings of the approach.

Before QuEChERS, typical sample weight to solvent ratios could range from 1:2 to 1:10 and it was common for 50 g of material to be extracted with 100 mL or more of solvent, followed by various labor-intensive shaking, evaporation, partition and SPE steps. With QuEChERS, the typical sample weight to solvent ratio is 1:1 (g/mL) and typically 10 g of commodity was extracted with 10 mL of acetonitrile. This is convenient for a few reasons:

1. This setup easily fits into ubiquitous and inexpensive 50 mL polypropylene centrifuge tubes.
2. The lower solvent volume allows for more concentrated extracts (and thus lower detection limits) often negating the need to include an evaporation step to concentrate the sample.
3. The weight to solvent ratio delivers sufficient extraction efficiency for most applications but note this is problematic for lipophilic analytes in fatty or oily commodities.

The limitation of QuEChERS for lipophilic analytes is solved by the QuEChERSER (Quick, Easy, Cheap, Effective, Rugged, Safe, Efficient and Robust) variant, or "Mega-Method."

The aim of the Mega-Method is to encompass more analytes and more matrices while at the same time reducing even further the use of consumables and chemicals and with fewer method iterations. As with QuEChERS 20+ years ago, advancements in instrument performance allow laboratories to simplify sample workup even more while still retaining method robustness.

One advantage of QuEChERS can also be seen as a disadvantage depending on the context. The single extraction method can include both liquid chromatography (LC) and gas chromatography (GC) amenable analytes. While this is certainly efficient, it can force compromises in analytical performance. An acetonitrile extract can be directly injected onto a GC without too much difficulty but injecting a "strongly organic" sample into a reverse phase LC system can introduce peak shape and ultimately signal to noise issues for mostly polar analytes.

The LC peak shape problem can be overcome by additional steps:

ISSUE

"Strong organic" sample peak shape

FIX	CAUTION
Dilution with aqueous solvent	Increases detection limits
Solvent exchange by evaporation	Less polar analytes excluded
Reducing injection volume	Detection limit issues

QUECHERS VS. MEGA-METHOD

In contrast to the standard QuEChERS method, the "salting out" step is omitted from the Mega-Method workflow, allowing retention and therefore analysis of highly polar analytes in the organic extract.

Mega-Method Workflow

Mega-Method for GC of hydrophobic analytes

Mix sample with acetonitrile (mixing / grinding / blending)

4:1 samples / ACN ratio - no additional water needed.

Reserve portion for LC. Add salts, mix

"Salting out." Normally acetonitrile and water are miscible, but by dissolving enough salt in the aqueous phase the two solvents will partition.

Centrifuge

Retain acetonitrile (top layer). Matrix components partition to aqueous layer.

µSPE clean up

Automated or manual clean up $MgSO_4$, PSA, C18 and CarbonX containing cartridge.

Analysis

Hydrophobic analytes by GC.

Mega-Method for LC of hydrophilic analytes

Mix sample with acetonitrile (mixing / grinding / blending)

4:1 samples / ACN ratio - no additional water needed.

Centrifuge

200 µL ACN supernatant.

Evaporation, reconsitution

200 µL aliquot transferred to an ultra-centrifuge tube evaporated by Nitrogen and the sample is reconstituted with the initial mobile phase composition of the LC method.

Analysis

Multi Residue Method

Hydrophobic analytes: by direct LC or see GC process flow.

Hydrophilic analytes: remove insolubles by ultra-centrifugation.

While the LC-focused method can be used for the analysis of hydrophobic analytes, a more suitable design is to reserve the hydrophobic analytes for GC analysis.

Those familiar with GC analysis will see the problem with injecting a raw 20% aqueous extract sample: contamination of the injection liner, the front of the GC column and the inlet gas lines. Such a raw extract would leave behind non-volatile matrix components which would significantly increase the number of maintenance cycles and consumable usage of the instrument. Additionally, when water is injected, the liquid expands approximately 1200-fold as it vaporizes, which can overwhelm an injection port liner and cause backflash where the gas lines leading into the inlet are contaminated. This then causes injection carryover and interference with subsequent injections. To reduce the water in the sample, a "salting out" step is included here where the remaining 10 mL of extract is mixed with 2 g of salt mixture. As with QuEChERS, this serves to separate the water and the acetonitrile and eliminates most of the hydrophilic components.

For the analysis step, the reconstituted extract will at this point have hydrophobic matrix and analyte precipitation. For an MRM, the hydrophobic analytes can be analyzed by GC (see next section), or a separate aliquot of the bulk extract could be directly injected with an LC method. Although the extract would be mostly organic solvent the hydrophobic analytes are not as greatly affected by poor peak shape in reverse phase LC. For hydrophilic analytes, the precipitate must be eliminated to prevent LC column deterioration. The most effective method is ultra-centrifugation to obtain a clean enough extract (filtration can compromise at least a selection of analytes for MRM by either eliminating them or introducing other unwanted contaminants). Using a strongly aqueous reconstitution solvent will eliminate a substantial proportion of the hydrophobic components that would have otherwise fouled the column (4). After centrifugation, the extract is ready for injection.

Mega Method Considerations

An important consideration of the Mega-Method is the smaller sample weight of 2 g. Care should be taken to ensure that samples are homogenous and representative of the whole sample to be tested. The quality of sample processing is an area that merits greater scrutiny (5,6). Quality equates to achieving smaller particle size (comminution) of the homogenate. The most common method to obtain a homogenous sample is to blend pre-frozen portions of the sample with dry ice using a heavy-duty blender. After the dry ice has sublimated, a frozen, powdery mixture remains which is generally adequate for the kind of sample weights typically used for QuEChERS (10 g). With 2 g sub-samples, it is recommended to blend non-frozen portions of the samples with liquid nitrogen to achieve improved comminution (5).

When developing extraction methods of solids, complete extraction of the analyte into the solvent (near 100% recovery) is important. Incomplete extraction can lead to underestimation of the levels of chemicals present in the sample and influence the downstream risk assessment. With the Mega-Method, the improved sample comminution increases the surface area of the particles encountering the extraction solvent. Additionally, the combination of the higher solvent to sample weight ratio and the extraction solvent of choice (acetonitrile: water 4:1 (v/v)) has been shown to be efficient across a variety of analytes and matrices (6).



QuPPe (Quick Polar Pesticides)

As already discussed, QuEChERS is not especially effective for the analysis of polar pesticides. The Mega-Method addresses this issue to an extent, but QuPPe goes even further for highly polar pesticides such as paraquat, diquat and perchlorate. Additionally, these compounds are problematic to analyze by reverse phase chromatography and may require ion chromatography (IC) or hydrophilic interaction chromatography (HILIC) (5).

Mix sample with formic acid / methanol

Likely need adjustment for water. IL-IS commonly employed. Vigorous shaking (with shaking aids if required). EDTA can be added to remove complexing metals.

Freezing out

Sample mixture is frozen for at least several hours. This serves to precipate lipids, sugars, and proteins. Fat samples heated to 80°C to fully melt prior to freezing step.

Centrifuge

Lipids, sugars, and proteins form a precipitate.

dSPE spin, keep supernatant for analysis

dSPE: usually magnesium sulphate with a combination of primary-secondary amine (PSA), octadecylsilane (C18) and graphitized carbon black (GCB).

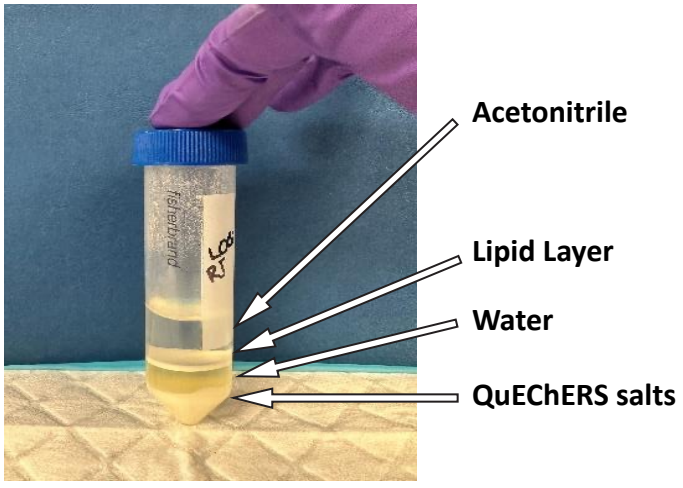
Analysis

Final extract in plastic vessels. Typically LC-MS/MS.

"Salting out" is replaced by freeze-precipitation to remove lipids, sugars and proteins (with pre-heating, if necessary, to melt and separate fats). These matrix components are then removed by centrifugation, performed in a refrigerated centrifuge, while retaining the polar pesticides in the acidified methanol. Due to the simplicity of the method, matrix effects are more likely to be encountered, and so this method can often rely on isotopically labelled internal standards (IL-IS) being introduced at the beginning of the method in order to compensate for matrix effects. This also has the benefit of addressing volume deviations resulting from the varying water content present in samples and the volume adjustment step, and of addressing analyte loss through binding to vessels. Where internal standard is not available, quantification by standard addition is likely to be required to achieve acceptable accuracy and repeatability.

Due to the relevant recency of QuPPe, the literature database is still small in comparison with QuEChERS publications. What appears to be an accompanying development with QuPPe methodology is the employment of accurate mass spectrometry detectors such as quadrupole time of flight (Q-TOF) and Orbitrap MS.

QuOil methodology also deserves a mention here. As the name suggests this modified procedure is used to extract oily samples, especially seeds such as rapeseed, sesame seeds and poppy seeds. Sample proportions are usually scaled down, the initial "salting out" step is skipped, and the acetonitrile extract is mixed with water and subjected to direct dSPE using high amounts of C18 sorbent to absorb the lipids. Conventional QuEChERS can still be used for oily samples, but it is likely a tertiary middle lipid layer will form between the aqueous and organic layer. If the analyte is lipophilic, then losses could occur with underestimation of the levels present. An effect that would quickly manifest itself when analyzing quality control samples.



Post-centrifugation oily sample that has been extracted using conventional QuEChERS – the tertiary lipid layer is evident and can cause losses of lipophilic analytes.

FINAL NOTES

Conclusion

QuEChERS, its derivatives, and its iterations are are already in widespread use in the food monitoring industry. Its application in environmental testing for the development of pesticides, chemical and pharmaceuticals studies has been more limited, mainly due to the scale (sample number) of the studies involved in GLP testing of new compounds and those undergoing testing for registration renewal or extension. Smithers uses QuEChERS as part of the analytical toolkit available for all study types, with the knowledge that its use should always be an option in the arsenal of analytical extraction techniques. While it is often the economic benefits that are discussed when it comes to QuEChERS, these benefits also translate to better quality and reproducible science.



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