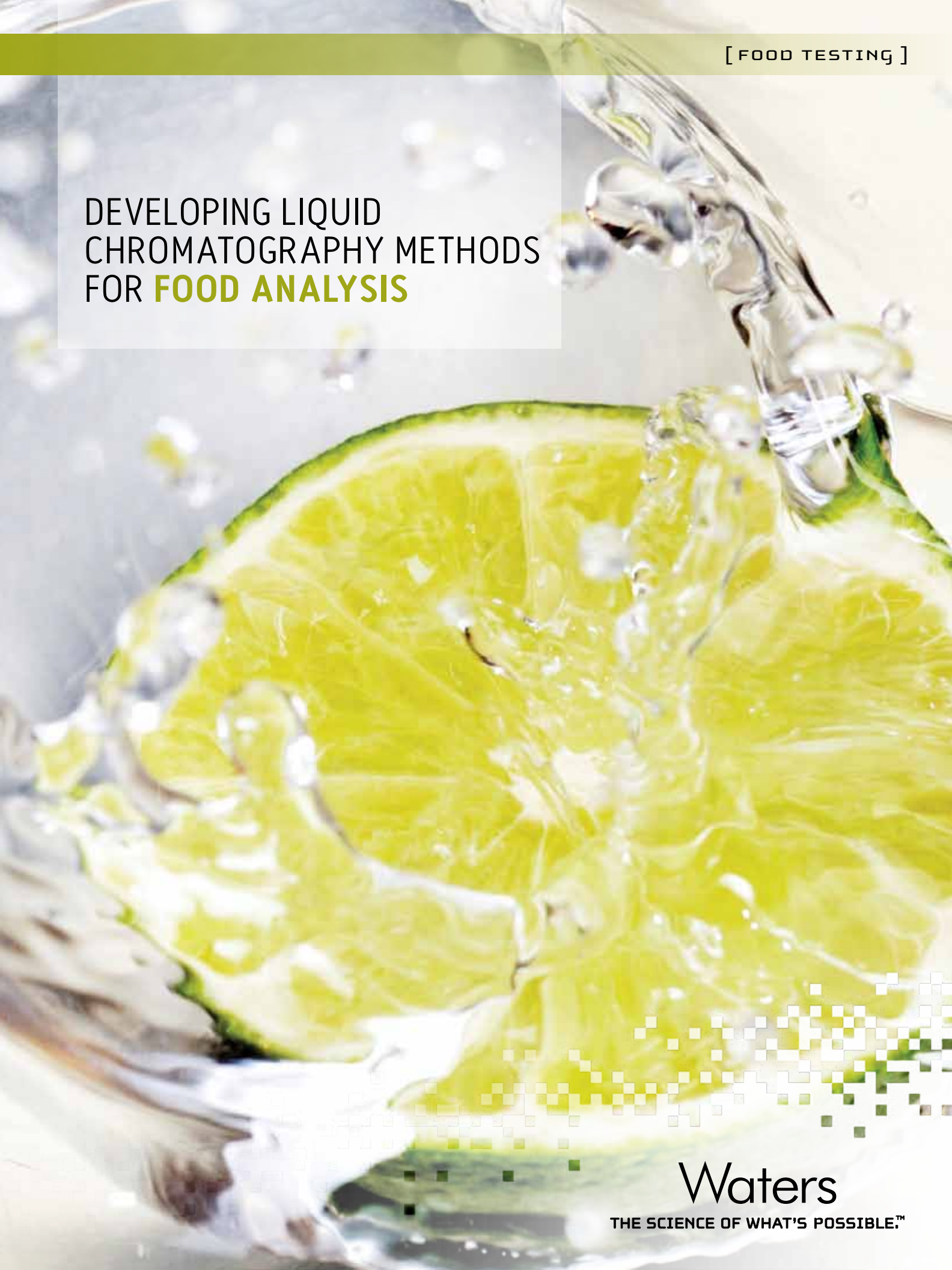
DEVELOPING LIQUID CHROMATOGRAPHY METHODS FOR **FOOD ANALYSIS**



Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

With the expansion of the food chain to now include the entire globe, the challenges facing food companies and scientists have grown exponentially. Consumers worldwide are becoming more aware of the quality and origin of the food they consume and are demanding stricter regulations to protect their safety. Governments are enacting new laws in an effort to protect their populations as well as local businesses. Global food corporations are searching for strategic ways to grow their businesses while also ensuring that products are safe and their brands are protected.

Working towards achieving these goals, it is critical that new analytical methods fulfill their intended purpose. A clear and concise understanding of how to develop methods can enhance a laboratory's operation and maximize both the analytical and business impacts of the information which is generated.

Waters Corporation has been working for decades to develop tools and techniques to streamline the method development process. Partnering with governments, research organizations, and industry partners has equipped Waters with the keen understanding necessary to produce accurate, fast, and cost-effective solutions to help ensure a safe food supply.

CRITICAL DRIVERS FOR FOOD TESTING

REGULATIONS

- Surveillance, national monitoring programs, import/export

ECONOMIC

- Brand differentiation/marketing
- Increased consumer prosperity, greater choice, greater competition
- Green lobby—organic and sustainability

PUBLIC AWARENESS/ PUBLICITY

- Brand protection
- Food scares—Bovine Spongiform Encephalopathy (BSE), dioxins, antibiotics, mycotoxins, melamine
- Adulteration/fraud—wine, orange juice, olive oil

SCIENTIFIC CHALLENGES IN FOOD TESTING

Sensitivity is required to accurately identify contaminants and meet lower allowable regulatory limits

- Regulated methods

High throughput is a necessity

- Hundreds of samples
- Fast turnaround time

Method **ruggedness** and **reliability** is essential

- Co-eluting endogenous materials can results in reduced assay accuracy

Data quality must be maintained

- Better, more informed decisions

Understanding the goals (or drivers) of our desired analytical method, as well as the challenges in undertaking this type of analysis, allows us to formulate a method development plan. In almost all instances, we will be pursuing either a multi-residue analysis or a compound- or class-specific analysis. This basic understanding provides us with a starting point for our method development.

	Multi-Residue/Multi-Class	Compound or Class Specific
Entire Procedure (sample preparation & analytical method)	Generic to a diverse set of analytes	Specific for one compound or class of compounds
Sample Preparation Protocol	Simple (1 or 2 steps)	Multi-step
Goal of Sample Cleanup	Speed Recovery and cleanup are compromised for a large number of analytes	Maximizing recovery & matrix cleanup Minimizing interference/ion suppression
Level of Sample Cleanup	Minimum/moderate	Maximum
Liquid Chromatography (LC) Column Consideration	Retain wide variety of unrelated compounds; acceptable separations of large number of species	Closely-related compounds—higher degree of separation required; can be optimized for class or compound needs
Detection Techniques	Tandem mass spectrometry (MS), time-of-flight (Tof)	Single quad MS, ultraviolet (UV), fluorometer (FLR), Evaporative Light Scattering (ELS), (flame ionization detector [FID] or MS)

MULTI-RESIDUE ANALYSIS

In recent years, maximum residue limits (MRLs) have become much more stringent. Lowering these limits has placed additional pressure on analytical instrumentation to provide increased sensitivity. In particular, liquid chromatography combined with tandem-mass spectrometry (LC/MS/MS) has become more widely used than LC/UV. The need to analyze for an ever increasing number of compounds has led to the increased adoption of multi-residue screening methods. Thanks to these methods, it is now possible to quickly screen commodities for hundreds of compounds which provides the food scientist with much more information about their samples. The development and deployment of these types of analytical techniques greatly enhances the quality and scope of food safety testing programs.

MULTI-RESIDUE TEST METHODS

- Why are they attractive?
  - More information per analytical run
  - Streamlined (fewer) workflows
  - Better asset utilization
- What are the technical challenges?
  - "Universal" sample extraction for a large group of compounds
  - Performance demands on separation and detection for selectivity and sensitivity (rather than on sample preparation)

METHOD DEVELOPMENT GOALS

- Rapid, cost-effective sample preparation
  - Wide variety of analyte/commodity combinations
- Rapid and efficient separation
  - Complex extracts, many residues
- Highly selective, sensitive detection
  - Demanding regulatory limits (low parts-per-billion [ppb])
  - Demanding quality control (QC) criteria
- Simple set up and operation of complex methods

SAMPLE PREPARATION FOR MULTI-RESIDUE METHODS

There are several challenges to developing multi-residue methods. They must be quick and easy to implement. The methods must prepare and transform samples for analysis while not removing any compounds of interest (which many times may be unknown). They must be suitable for an extremely large number of compounds.

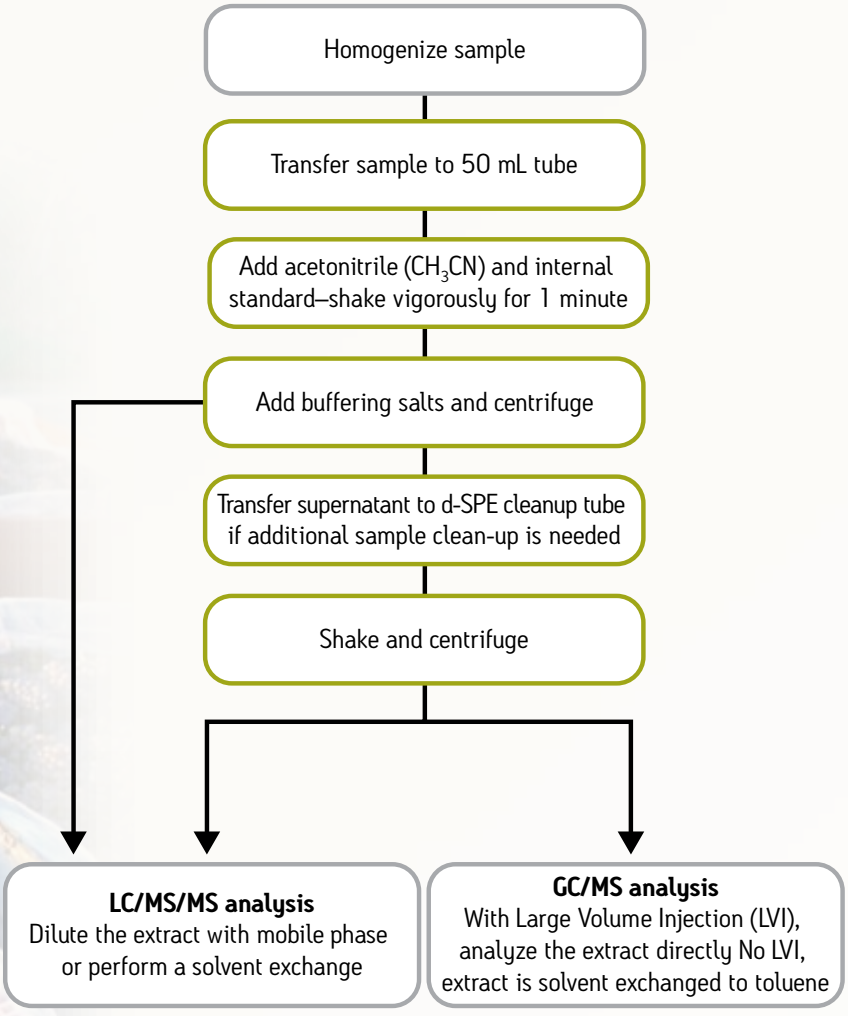
The preparation of samples for this type of analysis is of particular importance. Food commodities must be placed into a format that can be analyzed while not removing any compounds requiring identification. Two popular and effective forms of sample preparation which have been successfully employed for multi-residue analysis are solid-phase extraction (SPE) and dispersive SPE (d-SPE), or "QuEChERS".

QuEChERS Dispersive SPE

QuEChERS methods (an acronym for **Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged and **S**afe) were originally developed to extract pesticides from fruits and vegetables. Combining an initial salting out liquid-liquid extraction with dispersive SPE cleanup, these methods have been demonstrated to be very effective for multi-residue analysis of pesticides, veterinary drugs, and mycotoxins in a wide variety of food and agricultural products.

Standard Method

Simplified QuEChERS Method Sample Preparation Protocol



APPLICATION EXAMPLE—QuEChERS FOR MULTI-RESIDUE EXTRACTION

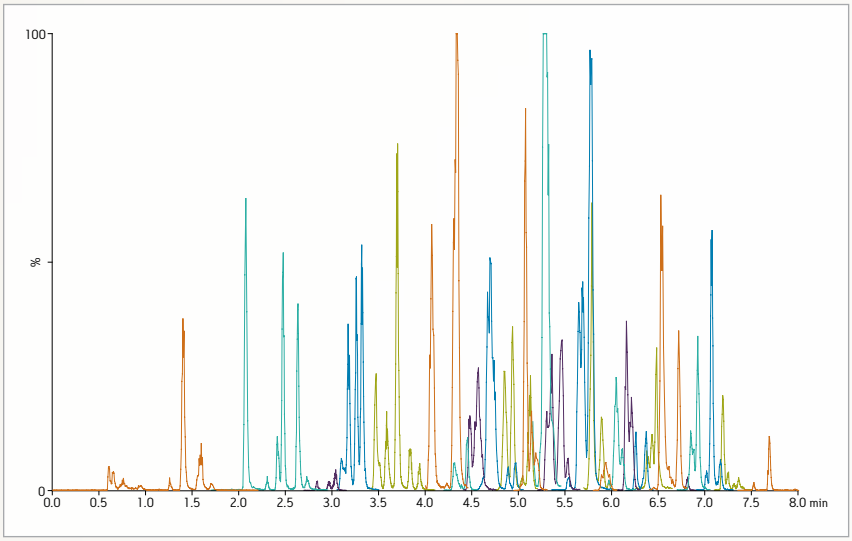
Pesticides in Mango, Avocado, and Baby Food

In this example we were challenged to extract and identify over 400 pesticides from samples of mango, avocado, and fruit-based baby food. In order to achieve the sensitivity and separation which were required from the method, we used an ACQUITY UPLC® instrument with a tandem quadrupole (TQD) mass spectrometer. Minimal sample cleanup was needed but we were required to remove fats and color from the samples which might inhibit the analysis. The Association of Official Analytical Chemists (AOAC) QuEChERS Method was used to achieve this desired result.

Extraction Procedure

1. Add 15 g homogenized sample to a 50-mL DisQuE extraction tube containing 1.5 g sodium acetate (NaOAc) and 6 g magnesium sulfate ($MgSO_4$). Add 15 mL 1% acetic acid (AcOH) in CH_3CN.
2. Add any pre-extraction internal standards.
3. Shake vigorously for one minute and centrifuge > 1500 rcf for one minute.
4. Transfer 1 mL of the CH_3CN extract in to the 2-mL DisQuE extraction tube containing 50 mg primary and secondary amine exchange material (PSA) and 150 mg of $MgSO_4$.
5. Shake for 30 seconds and centrifuge >1500 rcf for one minute.
6. Transfer 100 µL of final extract into an autosampler vial. Add any post-extraction internal standards. Dilute with 900 µL water (H_2O).

Results



Chromatogram showing all 402 pesticide residues in one 10 minute run in injection solvent.

UPLC/MS Conditions

LC system:	ACQUITY UPLC System
Column:	ACQUITY UPLC BEH C_{18}, 2.1 x 100 mm, 1.7 µm
Column temp.:	40 °C
Sample temp.:	4 °C
Flow rate:	0.45 mL/min
Mobile phase A:	98:2 H_2O: methanol (CH_3OH) + 0.1% formic acid (HCOOH)
Mobile phase B:	CH_3OH + 0.1% HCOOH
Weak needle wash:	98:2 H_2O: CH_3OH + 0.1% HCOOH
Strong needle wash:	CH_3OH + 0.1% HCOOH
Total run time:	10 min
Injection volume:	20 µL, full loop injection
MS system:	Waters ACQUITY® TQ Detector
Ionization mode:	ESI positive polarity
Capillary voltage:	1 kV
Desolvation gas:	Nitrogen, 800 L/Hr, 400 °C
Cone gas:	Nitrogen, 5 L/Hr
Source temp:	120 °C
Acquisition:	Multiple Reaction Monitoring (MRM)
Collision gas:	Argon at 3.5 x 10^{-3} mBar
Gradient:	See full application note, P/N: 720002628EN

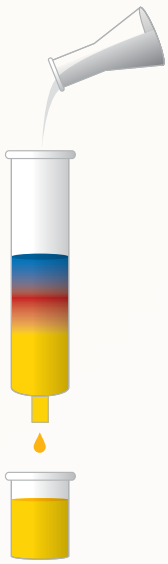
SOLID-PHASE EXTRACTION

SPE methods can be used for multi-residue sample preparation and are particularly helpful if your matrix of interest is complex. Using an SPE sorbent which is adept at removing fats and proteins, for instance, and can enhance your sample preparation and reduce potential interferences with your MS analysis.

Basic Pass-Through Sample Cleanup Using SPE Sorbent and Cartridge

Pass-through cleanup methods optimize matrix retention while the analytes of interest pass-through the cartridge unretained. No sample enrichment occurs during the SPE step.

1. Sample is passed through sorbent and collected.
   - No sample enrichment.
2. Matrix interferences are retained on sorbent.



Pass-Through

SEP-PAK SPE CARTRIDGES & PLATES

Sep-Pak® bonded-silica devices are recognized throughout the world and remain the most referenced solid-phase extraction product for sample preparation. A diverse selection of formats and sorbents make Sep-Pak SPE products ideally suited for all types of samples for GC, HPLC, and UPLC® analysis methods.

Formats:

- Cartridges
- 96-well plates

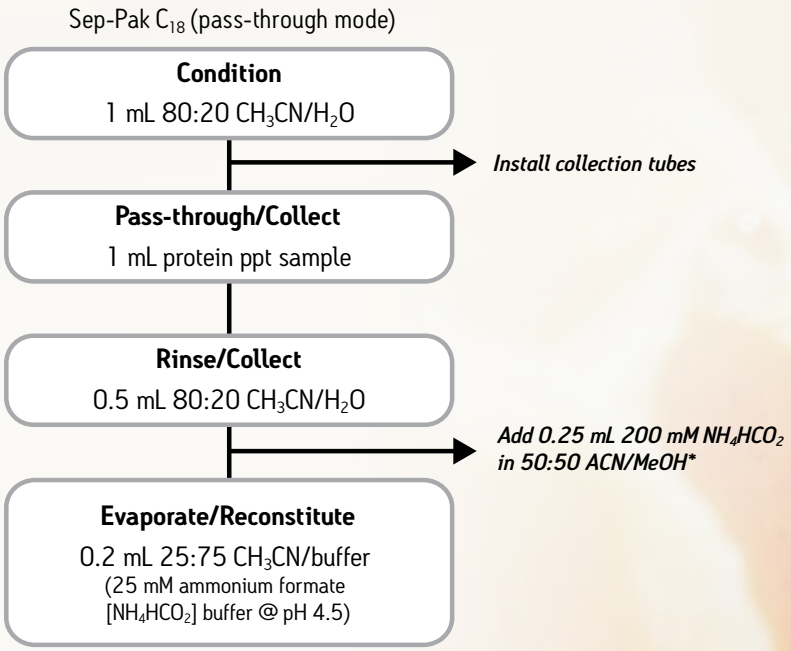
Sep-Pak®
Sample Extraction Products

APPLICATION EXAMPLE—SPE FOR MULTI-RESIDUE EXTRACTION

Veterinary Drugs in Milk

Veterinary drugs are a group of compounds that are widely used in the food industry and are subject to regulation and testing. The presence of these compounds in milk is particularly challenging to analyze because of the high percentage of fats and proteins that need to be removed. Using a C_{18} SPE sorbent will remove the fats and, in conjunction with a protein precipitation for protein removal, will provide a clean extract for further analysis.

SPE Cleanup



** Buffers sample to protect acid labile analytes*

Excellent Recovery and Repeatability for a Wide Variety of Veterinary Drugs in Milk

Compound	Spike Level (ng/g)	%REC (%RSD) (n=3)	%Suppression**
Carbadox	67.0	27 (27)	-43.0
Chloramphenicol (P)	67.0	94 (16)	10.0
Chlorotetracyline (T)	67.0	22 (20)	7.0
Ciprofloxacin (F)	67.0	67 (20)	32.0
Dexamethasone (St)	67.0	87 (6)	-8.0
Enrofloxacin (F)	134.0	76 (11)	26.0
Erythromycin (M)	6.7	59 (10)	5.0
Lincomycin (M)	33.0	102 (9)	25.0
Oxacillin (B-L)	67.0	79 (12)	-9.0
Oxytetracycline (T)	67.0	24 (16)	-9.0
Penicillin (B-L)	33.0	73 (8)	-8.0
Phenylbutazone (NSAID)	67.0	67 (18)	20.0
Ractopamine	200.0	65 (14)	0.0
Salbutamol	67.0	80.4 (3)	96.0
Sulfamerazine (S)	67.0	71 (4)	-16.0
Sulfamethasine (S)	67.0	71 (6)	-74.0
Sulfanilamide (S)	67.0	110 (30)**	60.0
Tetracycline (T)	67.0	31 (18)	-21.0

*** Negative number signifies enhancement*

Note that some compounds (tetracyclines in particular) do not exhibit a particularly high recovery. Multi-residue screening does not require a high recovery in order to provide an indication of whether a particular compound is present in your sample. If you would like to quantify a particular compound of interest, beyond what is possible using this type of method, a compound- or class-specific method would have to be considered (see page 12 for a specific example involving tetracyclines).

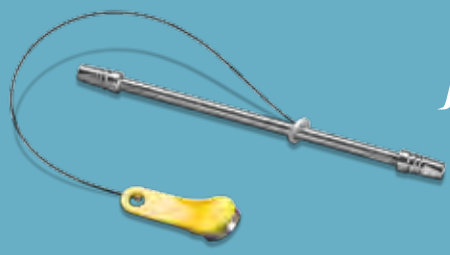
COLUMNS FOR MULTI-RESIDUE METHODS

When selecting a column for multi-residue analysis it is important that the column chemistry be able to accommodate the wide variety of compounds associated with these techniques. In the majority of instances, a standard C_{18} column will provide sufficient retention and selectivity. Repeatability and robustness are also highly sought after and a polymeric C_{18} particle is the best option for those characteristics.

ACQUITY UPLC BEH COLUMNS

The 1.7 µm BEH particle is one of the key enablers behind UPLC Technology. It is available in 4 different pore sizes and several bonded phases for reversed-phase and hydrophilic-interaction chromatography. Due to the intrinsic chemical stability of hybrid particle technology, a wider usable pH range (pH 1–12) can be employed, enabling a versatile and robust separation technology for method development.

BEH Technology™ is also available in HPLC particle sizes (2.5, 3.5, 5, and 10 µm) in the XBridge™ family of HPLC columns, enabling seamless transfer between HPLC and UPLC Technology platforms.



Acquity UPLC®

COMPOUND-SPECIFIC ANALYSIS

There are a variety of reasons why a compound- or class-specific method may be desirable. Lower detection limits may be needed than what is possible using a multi-residue method. Using more extensive sample preparation to concentrate a sample may be the only way to achieve this. A compound-specific method may be used to further quantify a specific compound or to confirm the presence of a compound which is seen using some other analytical approach. In some cases a multi-residue screening method may not work, necessitating a compound-specific approach. Finally, compounds of interest may only be able to be analyzed using a non-MS detector (UV for instance); this situation would typically require a compound-specific analysis. Regardless of the reason, compound-specific methods are widely used for food analysis and it is extremely useful to understand the considerations required to develop these types of methods.

Major Application Areas

QC TESTING

- Raw material
- GMP
- Finished goods

REGULATORY TESTING

- Import/export
- Adulteration
- Action levels

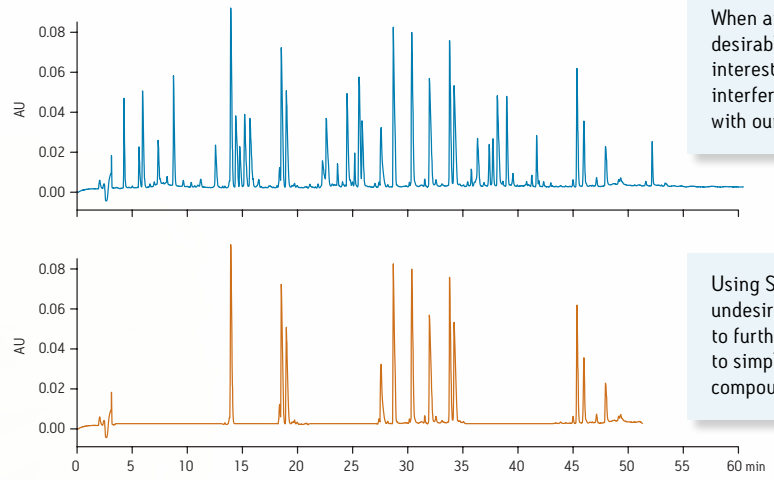
LABEL CLAIMS

- Additives
- Dietary supplements
- Health claims

SAMPLE PREPARATION FOR COMPOUND-SPECIFIC METHODS

The purpose of sample preparation for compound-specific methods is to isolate and concentrate only the compounds of interest. Achieving this goal will provide much lower detection limits than otherwise possible. In these types of methods, SPE is often the most appropriate sample preparation technique. Using very specific sorbents, SPE is able to easily isolate compounds of interest.

Using SPE to Simplify a Complex Sample Matrix



When analyzing complex samples it is sometime desirable to eliminate compounds which are not of interest. Some of these extraneous compounds could interfere with those which are of interest or interfere with our analysis in some other way.

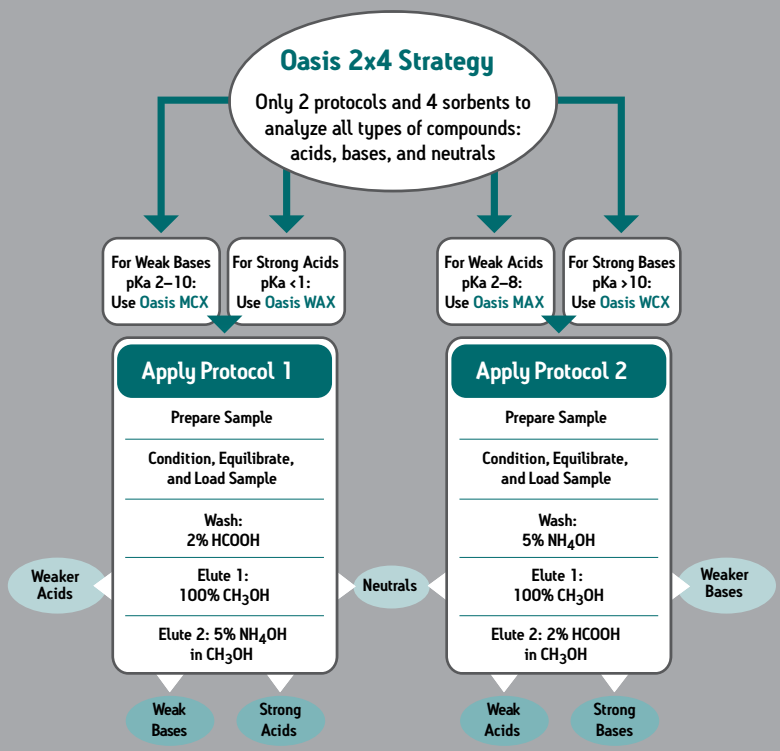
Using SPE, we can clean up our sample and eliminate undesirable compounds before subjecting our sample to further analysis. Here we can see that we are able to simplify our sample and can focus on our specific compounds of interest.

OASIS SPE PRODUCTS

The Oasis® family of SPE products is based on a polymeric sorbent which is optimal for reversed-phase SPE. Providing faster throughput, higher recovery and reproducibility, and stronger retention and selectivity, these products can be used to optimize sample cleanup and enhance sensitivity.

OASIS®
SAMPLE EXTRACTION PRODUCTS

Oasis 2 x 4 Method Development



APPLICATION EXAMPLE—SPE FOR COMPOUND-SPECIFIC ANALYSIS

Melamine and Cyanuric Acid in Infant Formula

Melamine has many industrial uses, including the production of laminates, adhesives, and melamine resins, some of which may contact foods, leaving trace levels of detectable residues. Cyanuric acid is a structural analogue of melamine and may be found as an impurity of melamine. It has been shown to damage renal tissue in several sub-chronical oral toxicity studies. A mixture of melamine and cyanuric acid forms an insoluble precipitate in renal tubiles leading to progressive tubular blockage and degeneration. It is therefore important for scientists to have a method of detection which includes both melamine and cyanuric and melamine at very low levels.

SPE Procedure

Melamine SPE Cleanup
Oasis MCX, 6 cc/150 mg

Condition
A.5 mL 0.1 M sodium hydroxide (NaOH) in CH_3CN
B. 5 mL 0.1 M hydrogen chloride (HCl) in CH_3CN

Equilibrate
A. 5 mL CH_3CN
B. 5 mL 4% HCOOH

Load
A. 3 mL 4% HCOOH in H_2O
B. 2 mL of sample supernatant

Wash
A. 5 mL CH_3CN
B. 5 mL 0.2% Diethylamine ($CH_3CH_2NHCH_2CH_3$) in CH_3CN

Elute
4 mL 2% $CH_3CH_2NHCH_2CH_3$ in CH_3CN

Filter
Into vial using 0.2 µm PTFE syringe filters and syringe

Cyanuric Acid SPE Cleanup
Oasis MAX, 6 cc/150 mg

Condition
5 mL 0.1 M HCl in CH_3CN
5 mL 0.1 M NaOH in CH_3CN

Equilibrate
5 mL CH_3CN
5 mL 5% NH_4OH in H_2O

Load
A. 3 mL 5% NH_4OH in H_2O
B. 2 mL of sample supernatant

Wash
5 mL CH_3CN

Elute
2 mL 4% HCOOH in CH_3CN

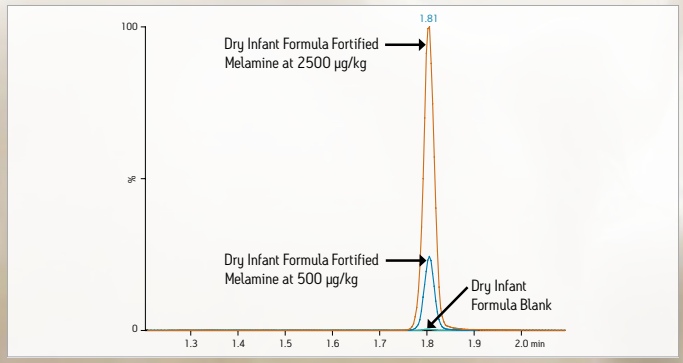
LC Conditions

Instrument:	ACQUITY UPLC System
Column:	ACQUITY UPLC BEH HILIC, 2.1 x 100 mm, 1.7 µm
Mobile phase A:	10 mM ammonium acetate (NH_4OOCH_3)
Mobile phase B:	10 mM $NH_4C_2H_3O_2$ in 95/5 CH_3CN/H_2O
Injection volume:	10 µL
Gradient table:	See full application note, P/N: 720004582EN

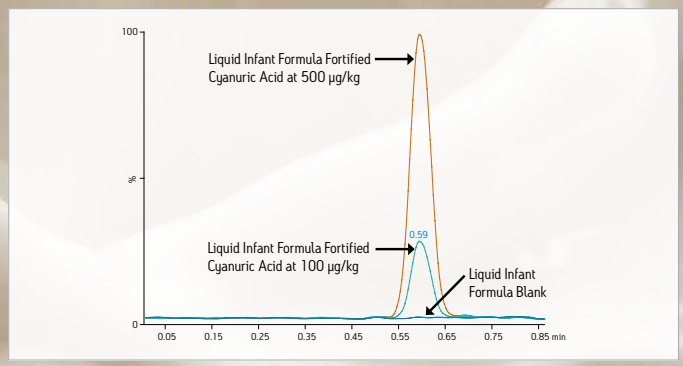
MS Conditions

MS system:	ACQUITY TQD
Ionization mode:	ESI Positive (melamine) ESI Negative (cyanuric acid)

Results



Chromatograms of Melamine in Dry Infant Formula at 500 ppb and 2500 ppb using ACQUITY BEH HILIC Column.



Chromatograms of Cyanuric Acid in Liquid Infant Formula at 100 ppb and 500 ppb using ACQUITY BEH HILIC Column.

APPLICATION EXAMPLE —SPE FOR COMPOUND-SPECIFIC ANALYSIS

Tetracyclines in Milk

Multi-residue screening methods are useful in that they provide us with the ability to very quickly examine our samples for a large number of compounds. We can rapidly determine what is present and what it not. A short coming of these methods, however, is that because we are searching for a large number of often unrelated compounds, our methods must be very general. By employing these generic methods, we are not able optimize our analysis to achieve a maximum level of sensitivity or recovery. In fact, the more diverse our compounds are, the lower our recoveries may be.

One example of this occurs when screening milk for veterinary drugs. Because the classes of compounds which fall into this category are so different, multi-residue screening is unable to provide optimal recovery for tetracyclines. We can address this by performing a confirmatory analysis using a compound specific method developed specifically for these drugs.

Sample Pretreatment

1. EDTA/McIlvaine Buffer:

In a 1 L volumetric flask, dissolve 28.41 g anhydrous dibasic sodium phosphate (Na_2HPO_4) in approximately 900 mL H_2O, dilute to volume, and mix. In a separate 1L volumetric flask, dissolve 21.01 g citric acid monohydrate ($C_6H_8O_7$) in approximately 900 mL H_2O, dilute to solution with 625 mL of phosphate solution. Add 60.5 g disodium EDTA and mix well until dissolved. Prepare fresh weekly.

2. Initial Extraction/Precipitation:

Transfer 1.5 mL milk to a 15 mL centrifuge tube. Add 6 mL to EDTA/McIlvaine buffer and vortex for 30 seconds. Centrifuge at 4000 rpm for 5 minutes. Collect the supernatant and adjust to pH 10 with 0.75 mL 1 M NaOH.

SPE Procedure

Oasis MAX, 1 cc/300 mg

Condition/Equilibrate
2 mL CH_3OH, 2 mL H_2O

Load Sample
From pre-treatment

Wash 1
0.5 mL 5% NH_2OH/H_2O

Wash 2
0.5 mL CH_3OH

Elute
0.5 mL 45.55 CH_3CN/75 mM oxalic acid ($C_2H_2O_4$).
Dilute 1:3 with H_2O for LC

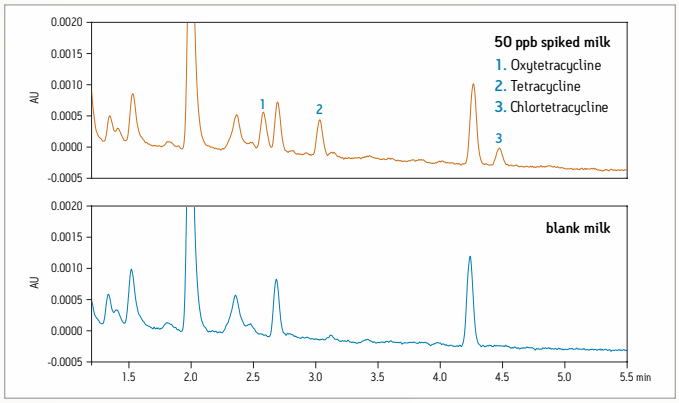
LC Conditions

System:	Alliance e2690/5 HPLC with 2998 Detector
Column:	XBridge BEH C_{18} ***XP***, 100 x 4.6 mm, 2.5 µm
Column temp.:	30 °C
Flow rate:	1.20 mL/min
Mobile phase A:	10 mM $C_2H_2O_4$ in H_2O
Mobile phase B:	10 mM $C_2H_2O_4$ in CH_3CN
Injection volume:	35 µL
UV detection:	PDA (extracted 355 min)
Gradient:	See full application note, P/N: 720002865EN

MS Conditions

Instrument:	Quattro Premier™ XE
Ionization mode:	Positive electrospray (ESI+) Multiple reaction monitoring

Results

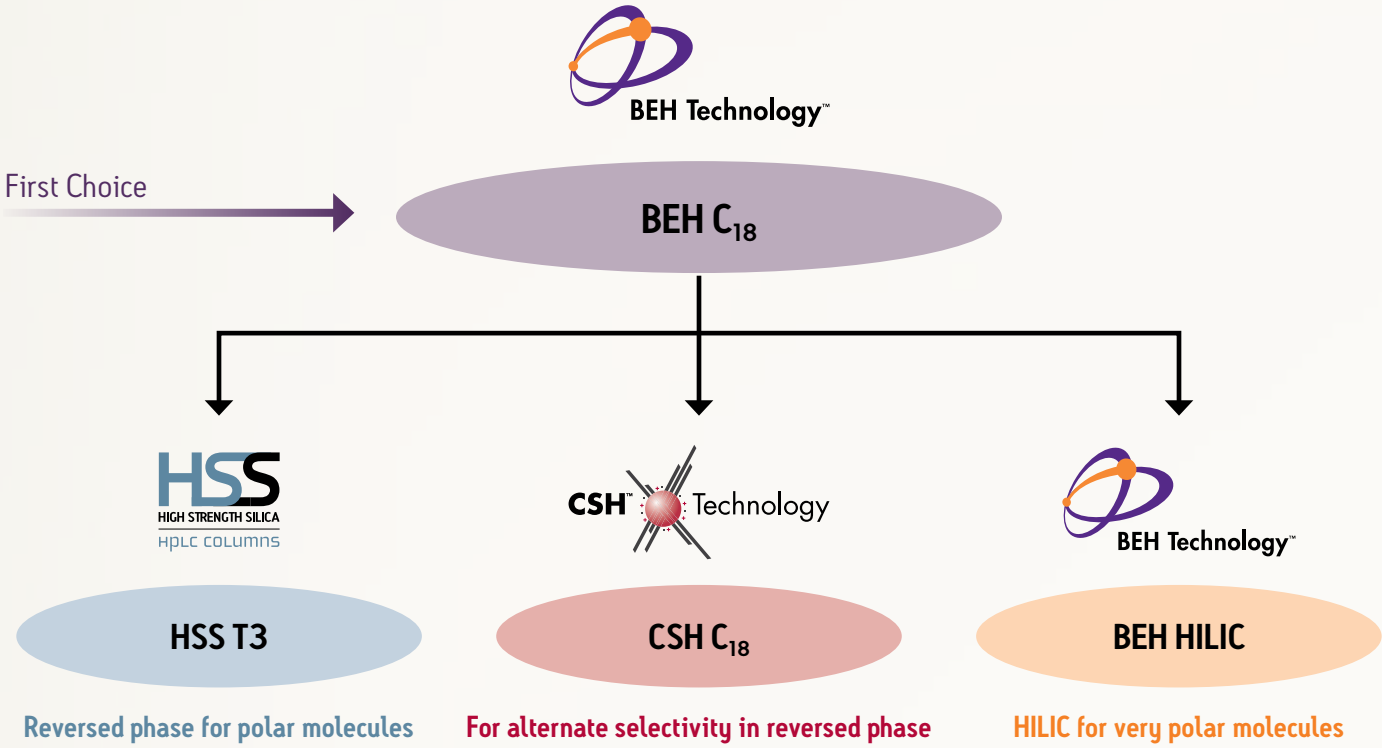


*A typical HPLC/UV chromatogram (355 nm extracted wavelength, (extracted 355 min) **XP** column) obtained from analysis of a sample spiked with 50 ppb (ng/g) of three tetracyclines.*

COLUMN FOR COMPOUND-SPECIFIC METHODS

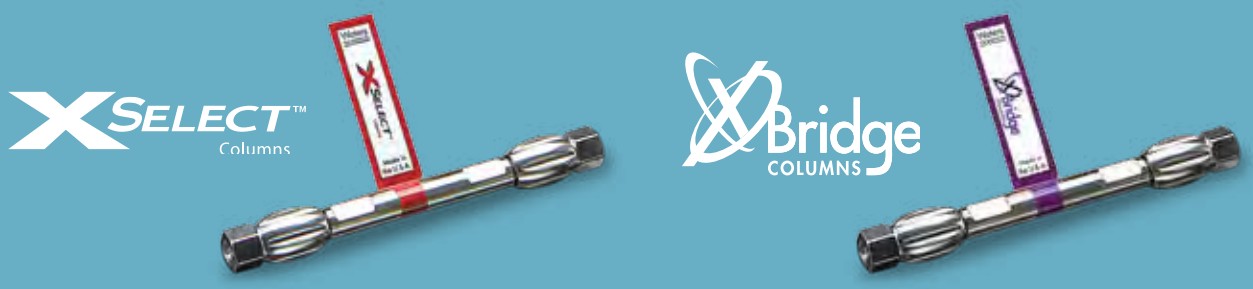
When selecting columns for class-specific method development, keep in mind the compounds being targeted and select a column chemistry that will provide optimal separation and retention of those compounds. In many cases, this may be a C_{18} particle but there are a variety of other choices that can optimize your method.

Column Choices for Compound-Specific Analysis



EXTENDED PERFORMANCE [XP] COLUMNS

XBridge and XSelect™ *XP* 2.5 µm Columns are a good starting place for analytical laboratories using HPLC instruments. These columns enable exceptional separation performance, robustness, and throughput for HPLC assays and offer a wide range of selectivity and column dimensions.



ANALYTICAL STANDARDS AND REAGENTS AND TRUVIEW CERTIFIED VIALS

There are two additional considerations which can affect your method development—analytical standards and reagents and vials. With a goal of developing methods to increase laboratory productivity, minimize waste, and increase analytical confidence, removing any variability or uncertainty in the process is critical. Ensuring that you are using certified standards, reagents, and vials will increase confidence in your methods and analytical results.

WATERS ANALYTICAL STANDARDS AND REAGENTS

TruView™ LCMS CERTIFIED

ADDITIONAL FOOD METHOD DEVELOPMENT TOOLS

BOOKS

Description	Literature Code*
Beginner's Guide to SPE [Solid-Phase Extraction]	715003405
Beginner's Guide to UPLC [Ultra Performance Liquid Chromatography]	715002099
Beginner's Guide to Liquid Chromatography	715001531

BROCHURES

Description	Literature Code*
ACQUITY UPLC Columns	720001140EN
DisQuE Dispersive Sample Preparation for QuEChERS	720003048EN
e***X***tended ***P***erformance ***[XP]*** Columns	720004195EN
Oasis Sample Extraction Products	720001692EN
Sep-Pak Solid-Phase Extraction Products	720000860EN
XBridge HPLC Columns	720001255EN
XSelect HPLC Columns	720004178EN

OTHER RESOURCES

Description	Literature Code*/Website
Waters Sorbent Selection Guide to Solid-Phase Extraction Wall Chart	720002007EN
Efficient Reversed-Phase Method Development Strategy Wall Chart	720001978EN
Oasis 2x4 Method Guide Slide Chart	720003977EN
DisQuE Sample Preparation for QuEChERS Demonstration Video	www.waters.com/disque

* Search by literature code at www.waters.com

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Australia 61 2 9933 1777

Belgium and Luxembourg 32 2 726 1000

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Canada 1 800 252 4752

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Finland 358 9 5659 6288

France 33 1 30 48 72 00

Germany 49 6196 400 600

Hong Kong 852 2964 1800

Hungary 36 1 350 5086

India 91 80 2837 1900

Ireland 353 1 448 1500

Italy 39 02 265 0983

Japan 81 3 3471 7191

Korea 82 2 6300 4800

Mexico 52 55 52 00 1860

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