

Tissue Sample Preparation — Not the Same Old Grind

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Extracting analytes from biological tissue often presents serious challenges for analytical chemists. This month's "Sample Preparation Perspectives" discusses and compares conventional extraction technologies and novel approaches to tissue sample preparation.

Preparing biological tissue samples remains an extremely tedious and time-consuming laboratory task. From the moment the tissue is excised, analysts must pay great attention to how the sample is stored, processed (whether mechanical or chemical), extracted and analysed. After this process is complete, the utility of the data obtained from the tissue and their analytical quality — accuracy, precision and reproducibility — is still debatable. Despite the somewhat thankless nature of tissue analysis, researchers have made significant progress in the past decade exploring alternative tissue sample preparation approaches and gaining better understanding of the risks and benefits of conventional methods.

This "Sample Prep Perspectives" column will provide an overview of human and animal tissue sample preparation, focusing on quantitative bioanalytical applications. However, we will not cover applications for microscopy, imaging and elemental analysis. We will also not cover the preparation of plant samples, because plant tissues are very different in structure and physical content from animal tissues. We will discuss and compare traditional extraction technologies and new approaches.

Why Tissue Analysis?

Tissue preparation is needed in several application areas, including pharmaceuticals, molecular biology, food science, forensics and toxicology. In pharmaceutical research, chemists can use quantitative analysis of tissue to assess the tissue uptake at the site of drug action, correlate drug concentration with pharmacokinetic and

pharmacodynamic response, and predict toxicity and dose. Tissue samples can also be processed to obtain proteins, peptides, purified deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) for molecular biology research. The determination of preservatives, pesticide residues, growth hormone and drug residues in feed animal tissues is important in food science. The use of antibiotics in food animals has generated growing concern that lingering traces of fluoroquinolone antibiotics could, for example, contribute to increased bacterial resistance (see <http://www.nal.usda.gov/ttic/tektran/data/000012/76/0000127692.html>).

Another important avenue for tissue analysis is forensic or toxicology purposes, whether to confirm the identity of an alleged suspect from crime scene evidence or to determine if poisoning or an overdose has occurred. Because DNA can be extracted from almost any human tissue, extracted DNA from crime scene evidence can be compared with extracted DNA from known individuals. For toxicological or DNA testing, tissues are generally used together with blood, urine and hair.

Types of Tissue Preparation Techniques

Tissue preparation techniques can be categorized into mechanical, digestion or extraction instruments. Some of the techniques used successfully for other types of solid samples, such as soil or plant material, can also be used for tissues.^{1–5} Tissue samples, although solid, should be considered highly aqueous in nature, a characteristic that can be exploited to rupture cells within the tissue matrix.

Generally, analysts snap-freeze chunks of tissue in liquid nitrogen immediately after sampling and store them at 220–270 °C before processing. As when working with any biological matrix, chemists should follow proper biohazard safety precautions. Tissue samples are best processed immediately after removal from the freezer, because thawing will produce a rubbery nugget that evades slicing or dicing. If workers need to prepare many tissue samples, they should serially process several small batches rather than allow the entire set of samples to thaw.

Table 1 compares the techniques discussed in this column. Figure 1 shows the workflows for the methods described. Each technique has both advantages and disadvantages, and some can be used in combination for optimum extraction.

Mechanical Techniques

Homogenization or grinding remains the most popular and, generally, the most practical means of preparing tissues for a range of qualitative or quantitative applications. Initially during homogenization, a small stainless steel probe-style blender with a generator and a set of blades causes vigorous mixing and turbulence as well as physically shearing the sample into small pieces. Next, workers place a weighed amount of sample, which can be 10 mg–1 g in size, in a vial with a known volume of buffer solution. The pH of the buffer can be tailored to the desired extraction conditions. The resulting product, or homogenate, is semisolid in nature and can essentially be treated in the same manner as plasma. Lengthening the homogenization

step or centrifuging the homogenate and decanting the supernatant will minimize large particles in the homogenate.

Homogenizers are small, compact and relatively inexpensive, and they require minimal training to operate; however, extended exposure to high-velocity blending can be irritating, so workers should wear ear-protection devices. The probe should be thoroughly and repeatedly rinsed between each sample to avoid cross contamination. The accompanying sidebar, "Homogenization Procedure for Analysis of Desipramine in Rat Brain Tissue," outlines a typical sample preparation procedure for the analysis of desipramine in brain tissue using homogenization.

Sonication is one alternative to homogenization. During sonication, the tissue sample is snap frozen and then immediately ground to a fine powder using a mortar and pestle in a liquid nitrogen bath. The weighed powder is stored and, when ready for analysis, mixed with a known volume of buffer and sonicated using a specially designed acoustical tool, horn or probe placed directly into the powder–buffer mixture. This method is more straightforward than homogenization, but powderizing tissue requires significant manual labour and can cause occupational health problems such as carpal tunnel syndrome. As with the homogenizer, the sonic probe should be thoroughly cleaned between samples.

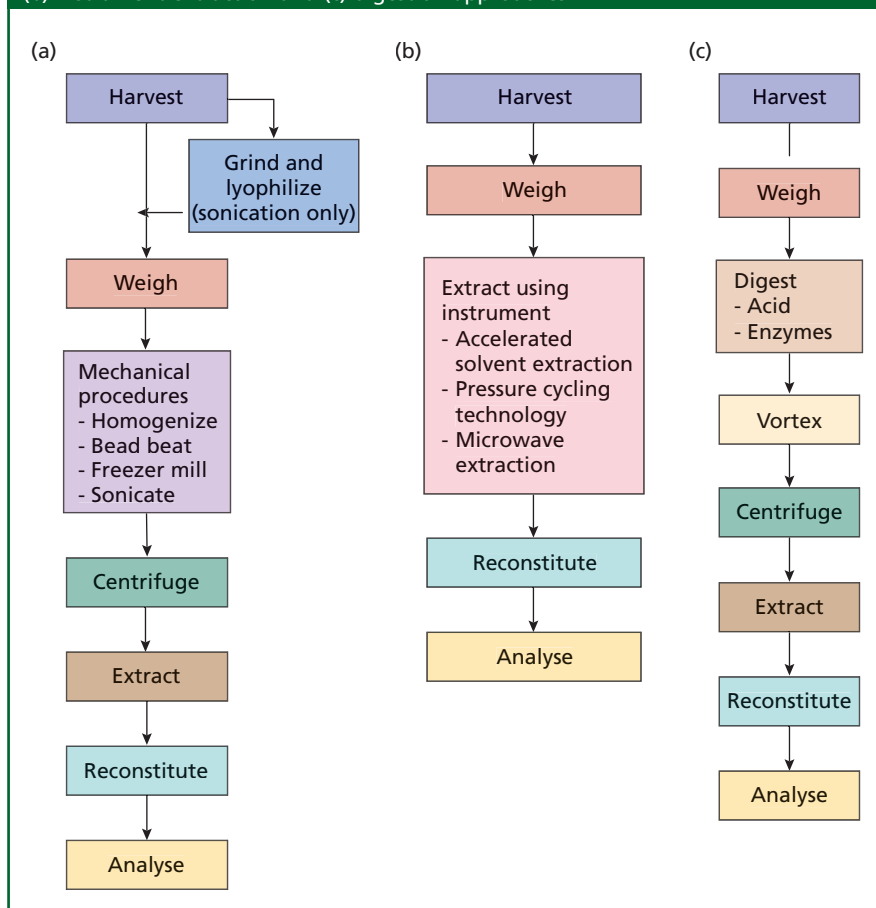
The bead beater represents a more hands-off approach to tissue sample preparation. Introduced 25 years ago by BioSpec Products Inc. (Bartlesville, Oklahoma, USA), the bead beater is a unique but simply designed apparatus that uses small beads and a high-speed rotor to rupture cells. A solid polytetrafluoroethylene impeller rotating at high speeds forces thousands of minute beads to collide in a specially shaped vessel. Cells are disrupted quickly, efficiently and safely. Chemists place individual samples in separate tubes with a defined amount of beads and buffer solution and then agitate them for 15–20 min. Homogenization inside the disposable microcentrifuge vials guarantees the minimization of cross-contamination. During longer agitation times, the unit is refrigerated to prevent sample heating from the beads' movement. Various bead sizes are available, and bead types include glass, stainless steel and ceramic.

This instrument's smaller cousin, the 96-well bead beater apparatus, is well suited for polymerase chain reaction (PCR) applications as it eliminates contamination

from extraneous DNA, even the slightest traces of which will ruin an entire experiment. In addition, the 96-well bead

beater allows sample processing for as many as two 96-well plates per batch. A variation of the bead beater is the

Figure 1: Workflow comparison for tissue preparation methods using (a) mechanical, (b) instrument extraction and (c) digestion approaches.



Homogenization Procedure for Analysis of Desipramine in Rat Brain Tissue

1. To prepare samples, place a weighed chunk of brain tissue in a vial. Add an appropriate amount of deionized water to achieve 0.1 g tissue/mL water.
2. To prepare standards and quality-control samples, place a weighed chunk of brain tissue in a vial. Spike an appropriate volume of 10 mg/mL stock solution of desipramine and deionized water to achieve 0.1 g tissue/mL water.
3. Homogenize at least 300 mg of blank tissue in 3 mL of deionized water to obtain enough blank matrix to prepare the standard curve, quality controls and blanks.
4. Homogenize the samples, initial working standard and quality controls for 3–5 min and clean the probe after each.
5. Dilute the working standard and quality control with blank homogenate to obtain standards and quality controls throughout the desired dynamic range. Vortex the mixtures well.
6. Mix a 100 μL aliquot of samples, standards, quality controls and blanks with 200 μL of 200 ng/mL haloperidol (internal standard) in acetonitrile (protein precipitation sample extraction).
7. Vortex the mixture for 5 min, centrifuge at 4000 rpm, and hold at 4 $^{\circ}\text{C}$ for 5–10 min.
8. Transfer the supernatants and inject them onto the LC–MS–MS system.

SPEX CertiPrep freezer mill (Metuchen, New Jersey, USA), which uses small magnetic bars, rather than beads, to pulverize the sample. Cooling is provided by immersing the sample chambers in a liquid nitrogen bath. The freezer mill is intended for samples larger than 500 mg, but it can be customized for smaller samples. Both the freezer mill and the bead beaters require that the sample be placed in secondary containers that might or might not be disposable. Cleaning non-disposable sample containers can be labour-intensive, so we recommend purchasing disposable devices.

Digestion Techniques

Mechanical techniques might do little to disrupt cellular structure and extract analytes from non-vascularized or low-water-content tissues such as bone, cartilage or hair. Extreme measures such as digestion with strong acid (e.g., 12 N hydrochloric acid) are used routinely for

DNA or nucleic acids, which can tolerate the harsh conditions. Alternatively, analysts can use certain enzymes to digest tissue samples. Commercial devices that contain digestion bombs fabricated from materials resistant to corrosive media are available. These digestion bombs can also be used as containers for the microwave extraction systems discussed below.

Enzymatic digestion, a technique commonly used for tissue dissociation and cell harvesting of proteins and DNA, offers the advantages of unattended sample preparation, potential automation and low cost. A range of enzymes is available with different digestive properties and efficiencies. The choice of enzyme can be driven by the desired tissue or component, such as cartilage, to be digested. Although the enzymatic digestion technique has been used for decades, only a few papers have been published that describe enzymatic digestion in tissue sample preparation of small molecules.⁶

We recently evaluated the feasibility of enzymatic digestion as an alternate tissue preparation technique for the bioanalysis of drugs.⁷ We chose two enzymes — collagenase and proteinase K — known to degrade connective tissues to allow tissue dissolution for evaluation. These enzymes were selected to represent a more conservative digestive enzyme (collagenase) and a more aggressive digestive enzyme (proteinase K). Our results indicated that enzymatic digestion has comparable extraction efficiency to homogenization and that enzymatic digestion using collagenase or proteinase K can be considered an alternative sample preparation method for the analysis of small molecules in tissue. The test compound levels of incurred rat brain tissue samples prepared by enzymatic digestion were in agreement with the values obtained by the conventional homogenization tissue preparation, which indicated that enzymatic digestion is an

Table 1: Types of tissue preparation techniques.

Technique	Typical Vendors*	Primary Tissue Types	Handles Whole Tissue Chunks	Analyte Stability Issues	Automation	Inexpensive	High Sample Throughput
Mechanical							
Homogenizer	Brinkmann Instruments	Small molecules, proteins, peptides, DNA and RNA	Yes	No	No	Yes	No
Ultrasonicator	Labcaire Systems	Small molecules, proteins, peptides, DNA and RNA	No	No	No	No	No
Bead beater and 96-well bead beater	BioSpec Products	DNA and nucleic acids	Yes	No	Yes	Yes	Yes
Freezer mill	SPEX CertiPrep	DNA and nucleic acids	Yes	No	Yes	No	Yes
Parallel homogenizer	TomTec	Small molecules, proteins, peptides, DNA and RNA	Yes	No	Yes	No	Yes
Digestion							
Acid or base	Parr Instrument	DNA and nucleic acids	Yes	Yes	No	Yes	Yes
Enzymatic	Worthington Life Sciences	Small molecules, DNA and nucleic acids	Yes	Maybe	Maybe	Yes	Yes
Extraction							
Accelerated solvent or pressurized-fluid extraction	Dionex and Applied Separations	Soil and foods	Yes	Yes	Yes	No	Yes
Microwave-accelerated extraction	CEM, Anton Parr and Milestone	Soil and foods	No	Yes	Yes	No	Yes
Pressure cycling technology	Boston Biomedica	DNA and nucleic acids	Yes	No	Yes	No	Yes

* Anton Parr (Graz, Austria); Applied Separations Inc. (Allentown, Pennsylvania, USA); BioSpec Products Inc. (Bartlesville, Oklahoma, USA); Boston Biomedica Inc. (West Bridgewater, Massachusetts, USA); Brinkmann Instruments Inc. (Westbury, New York, USA); CEM Corp. (Matthews, North Carolina, USA); Dionex Corp. (Sunnyvale, California, USA); Labcaire Systems Ltd (North Somerset, UK); Milestone Srl (Soriso, Italy); Parr Instrument Co. (Moline, Illinois, USA); SPEX CertiPrep (Metuchen, New Jersey, USA); TomTec (Hamden, Connecticut, USA); Worthington Life Sciences (Lakewood, New Jersey, USA).

appropriate tissue sample preparation method.⁷ Various other enzymes such as trypsin, papain or elastase could also be used, depending upon the degree of digestive strength needed.

Extraction Instruments

Some of the extraction instruments used for preparing other types of solid samples have been evaluated or at least considered for tissue samples.⁸ However, their intended uses can affect instrument design with respect to sample size. Chemists must consider the number and size of sample chambers or vials and the final extract volume when using these instruments. The ASE series of accelerated solvent extraction

Although some have reported using accelerated solvent extraction in the pharmaceutical area, accelerated solvent extraction is currently used most extensively in environmental analysis.

instruments (Dionex Corp., Sunnyvale, California, USA) use liquid solvents at elevated temperatures and pressures to extract analytes from solid or semisolid samples in very short periods of time and with small volumes of solvent. With accelerated solvent extraction, the sample is enclosed in a stainless steel vessel filled with an extraction solvent that is pressurized and heated. The sample is allowed to extract statically for 5–10 min, with the expanding solvent vented to a collection vial. Next, compressed nitrogen purges the remaining solvent into the same vial. Because the technique uses liquid solvents, it can be applied to any application in which liquid solvents are currently used. The entire procedure typically requires less than 15 min and approximately 15 mL of solvent for a 10 g sample.⁹ Although some have reported using accelerated solvent extraction in the pharmaceutical area,¹⁰ accelerated solvent extraction is currently used most extensively in environmental analysis.

Microwave-accelerated extraction has also been widely applied to solid samples as a means to speed the extraction process.^{5,6} Microwave heating can drive a variety of chemical processes, including acid digestion; however, the thermal instability of most types of tissue and analytes within the tissue has limited the applicability of this technique. Preliminary evaluations of microwave-accelerated extraction for extracting small molecules showed it was better suited to analytes that

can tolerate high-temperature conditions.⁸

Boston Biomedica Inc. (West Bridgewater, Massachusetts, USA) recently introduced a new type of extraction instrument that uses pressure cycling technology. Pressure cycling technology uses repeated cycles of ultrahigh and ambient pressure to extract proteins and nucleic acids from tissues.¹¹ Evaluating small-molecule extraction from tissues is an area of future work to expand the utility of this device.

Novel Technologies

An innovative technique well known to the food science community but less so to pharmaceutical researchers is matrix solid-phase dispersion, which was pioneered by

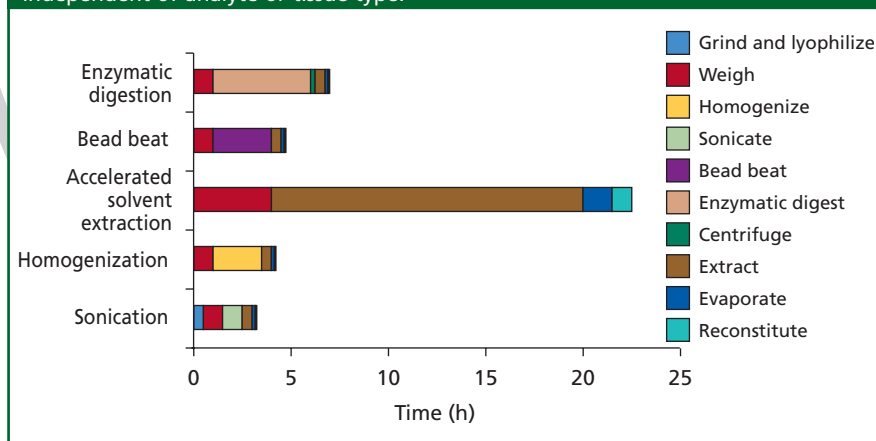
Steven Barker of Louisiana State University (USA).^{12,13} Similar to mechanical techniques such as bead beating, matrix solid-phase dispersion relies upon the shear forces generated by mixing tissue samples with silica particles larger than 50 µm and grinding them with a mortar and pestle. Analytes such as pesticides or drugs can be extracted from tissue using a range of stationary-phase chemistries. The analytes remain on the silica particles and can be eluted later with an appropriate solvent. The current multiple-step process requires repeated and careful manual intervention, but conceivably it could be automated or combined with some of the previously described devices.

Solid-phase microextraction (SPME), developed by Pawliszyn and co-workers¹⁴ from the University of Waterloo (Waterloo, Ontario, Canada), has also been used extensively for food and environmental applications and is now being used for biological samples. In SPME, a fused-silica fibre coated with an appropriate stationary phase is placed in contact with a sample. Agitation of the sample aids the kinetics of this equilibrium process. The fibre is removed after sufficient contact time, and the analytes can be thermally desorbed for gas chromatography (GC) or GC–mass spectrometry (MS) analysis or extracted for liquid chromatography (LC) or LC–MS. To date, most tissue samples have been homogenized before SPME, although headspace sampling can be used for volatile analytes.

A related technique, stir-bar sorptive extraction (SBSE), uses a polymer-coated, glass-encapsulated magnetic stirring bar instead of the coated fused-silica fibre.¹⁵ The stir bar is placed in contact with the sample with continuous agitation. Because the coated stir bar has a much greater mass of stationary phase compared with an SPME fibre, SBSE provides higher sample capacity and can achieve better detectable levels of trace substances. Although SBSE has been used for preparing biological fluids, sperm, foods and beverages, fruits and vegetables, and dairy products,¹⁵ to date it has not been used for analyte isolation from tissue samples.

The Autogizer parallel homogenizer (TomTec, Hamden, Connecticut, USA) was introduced as a means to speed the homogenization process using a multiprobe, parallel-processing approach.¹⁶ The parallel homogenizer features a four or

Figure 2: Comparison of total extraction time required for various tissue preparation methods. A sample set of 50 was used for calculations. Times shown are typical and independent of analyte or tissue type.



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six probe assembly of homogenizers or sonicators with a range of cutter sizes. The probes are cleaned automatically with three programmable wash stations. Fibrous material caught in the cutters may require manual intervention. The parallel homogenizer automates the time-consuming aspects of homogenizing and cleaning the probes and is by design four to six times as noisy as an individual homogenizer. Some solutions to the noise problem include locating the instrument in an isolated laboratory or placing sound-dampening material around it.

Considerations when Choosing a Tissue Preparation Technique

How to choose the best tissue preparation technique for everyone is a difficult topic to address. Sample throughput, analyte recovery, analyte thermal stability, amount of available sample, sample preparation techniques available, required precision and accuracy, manual labour involved and operator safety are only a few of the parameters that analysts must consider when selecting the optimum sample preparation technique. Figure 2 shows a comparison of the typical preparation times required for 50 tissue samples using various techniques. For homogenization, a parallel homogenizer can reduce the total preparation time by a factor of approximately 4–6.

In general, homogenization is the simplest approach and can be both the safest and the most cost-effective approach. However, if your laboratory needs to analyse dozens of tissue samples per month, investing in new technologies could be justifiable and cost-effective. As we have discussed, some instruments used for other types of solid samples can be modified to prepare tissues. However, scientists should consider judiciously whether the risks — cross-contamination, manual intervention and analyte instability — are outweighed by the benefits of using these instruments. Carryover between individual samples should be minimized, and the technique should be compatible with the analytical method you will be using (e.g., GC or LC–MS). The nature of the tissue itself should also be considered, because more fibrous connective tissues such as muscle will require more vigorous preparation than highly aqueous tissues such as cornea.

Tissue analysis presents its own set of unique challenges. A universal lack of reference material for any analyte in appropriate or comparable matrices exists, regardless of the application. Questions about spatial distribution heterogeneity of the analyte within the tissue matrix are rarely answered, unless chemists use an imaging technique such as autoradiography. Tissue quantification requires tedious sample weighing (slicing and dicing) for good accuracy and precision. Thus, techniques that mandate an exact amount of tissue, such as accelerated solvent extraction, will require significant time just to weigh the tissue. Although internal standards are used routinely, they are generally added as a solution to the homogenate, digest or extract after preparation. Unfortunately, researchers have discovered no physical means to disperse the internal standard into the tissue matrix or to determine the extraction efficiency of an internal standard in the matrix.

Extraction instruments for other solid samples often exhibit difficulty in scale-up or scale-down between various devices and require purchasing sample containers that are specific to the sample weight range. Correlation of extraction efficiency of the analyte between analytical standards and samples is always incomplete and often impossible. Despite these challenges, scientists have a choice between tried-and-true methods such as homogenization and more novel, yet cost-effective, approaches such as enzymatic digestion and the 96-well bead beater apparatus.

Summary

Although analytical chemists have performed traditional biological sample preparation methods for years, the demand for increased productivity, faster analysis and high-throughput assays has driven investigation of new technologies. Compared with the traditional approaches, these new techniques are expected to be faster and provide equivalent, if not superior, reproducibility and analyte extraction efficiency. Users have many choices for tissue sample preparation — from conventional homogenization or sonication to newer technologies such as enzymatic digestion, pressure cycling technology and parallel homogenization. Users should compare these techniques in terms of speed, cost and degree of automation.

References

1. P.J. Walter, S. Chalk and H.M. Kingston, in *Microwave-Enhanced Chemistry*, H.M. Kingston and E.J. Haswell, Eds. (American Chemical Society, Washington DC, 1997), 55–222.
2. G. LeBlanc, *LCGC* **17**(6S), S30–S37 (1999).
3. S.B. Hawthorne, Y. Yang and D.J. Miller, *Anal. Chem.*, **66**(18), 2912–2920 (1994).
4. R. Draisci et al., *J. Chromatogr. B*, **753**, 217–223 (2001).
5. B.E. Richter, *LCGC* **17**(6S), S22–S28 (1999).
6. A. Posyniak, J. Zmudzki and S. Semeniuk, *J. Chromatogr. A*, **914**, 89–94 (2001).
7. C. Yu et al., in *Proceedings of the 51st Annual Conference of the American Society of Mass Spectrometry and Allied Topics*, ThPM (Elsevier, New York, 2003), p. 257.
8. L. Cohen, unpublished data, 2000.
9. R. Draisci et al., *J. Chromatogr. B*, **753**, 217–223 (2001).
10. B. Benthin, H. Danz and M. Hamburger, *J. Chromatogr. A*, **837**, 211–219 (1999).
11. R.T. Schumacher et al., *Am. Lab.*, **34**(16), 38–44, (2002).
12. S.A. Barker, *J. Chromatogr. A*, **880**, 63–68 (2000).
13. S.A. Barker, *J. Chromatogr. A*, **885**, 115–127 (2000).
14. H. Kataoka, H.L. Lord and J. Pawliszyn, *J. Chromatogr. A*, **880**, 35–62, (2000).
15. F. David, B. Tienpont and P. Sandra, *LC•GC Europe* **16**(7), 410–417 (2003).
16. S. Wang et al., in *Proceedings of the 50th Annual Conference of the American Society of Mass Spectrometry and Allied Topics*, ThPJ (Elsevier, New York, 2002), 234.

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