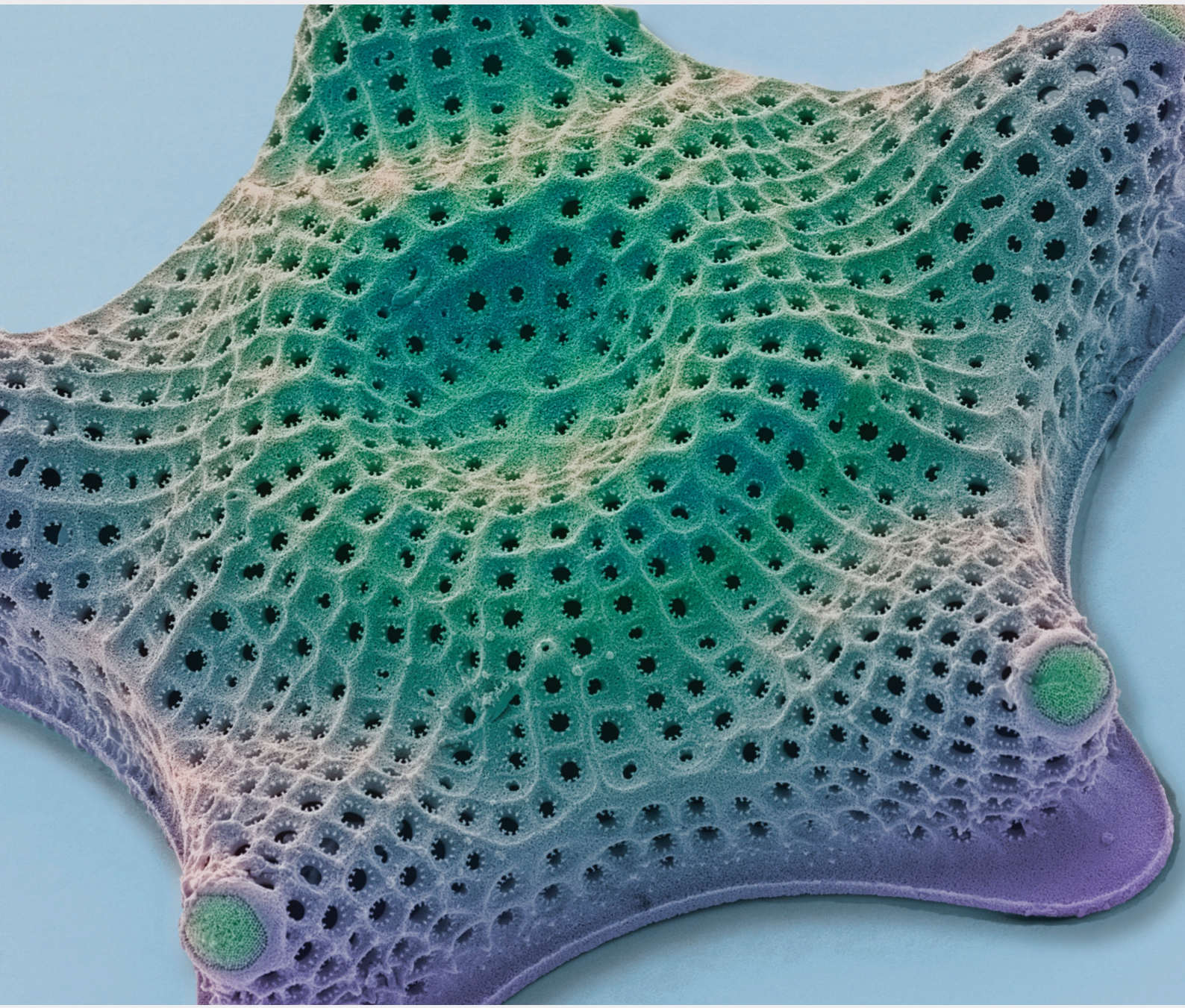
ISOLUTE® SLE+ User Guide

Supported Liquid Extraction



Biotage®

ISOLUTE® SLE+ User Guide

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Cover image:

Colored Scanning Electron Micrograph (SEM) of a *Triceratium* sp. Diatom

Diatomaceous earth materials are natural products made up of high silica content fossilized biominerals. Their properties have been exploited in many industrial processes. When packed into columns, they have been widely used as a support for chromatographic separations (GC) and can also be used for sample preparation applications.

ISOLUTE® SLE+
Supported Liquid Extraction Products

This guide describes how to develop supported liquid extraction methods using ISOLUTE® SLE+ products, along with hints and tips for optimizing performance and extending the range of analytes that can be extracted.

ISOLUTE® SLE+ plates and columns contain a modified form of diatomaceous earth, and are used for the extraction of a diverse range of analytes from aqueous samples such as biological fluids, using a simple **load-wait-elute** procedure. Various formats, both 96-well plates and columns are available for the extraction of aqueous sample volumes ranging from 10 µL* to 10 mL.

* See page 16 Hints, Tips and Troubleshooting.

Sample Preparation Using Supported Liquid Extraction

The Supported Liquid Extraction (SLE) process is analogous to traditional liquid-liquid extraction (LLE) and utilizes the same water immiscible solvent systems for analyte extraction. However, instead of shaking the two immiscible phases together, the aqueous phase is immobilized on an inert diatomaceous earth based support material and the water immiscible organic phase flows through the support, alleviating many of the liquid handling issues associated with traditional LLE such as emulsion formation. As a result recoveries are often higher and demonstrate better reproducibility from sample to sample (Figure 1).

In sample preparation, the principles of traditional LLE (partitioning of analytes between aqueous and water immiscible organic solvents) are well known and understood. Traditionally, analytes are extracted from aqueous samples through the addition of an appropriate water immiscible organic solvent. The two immiscible phases are shaken or mixed thoroughly in a separating funnel, and based on relative solubility of the analytes in the two phases, analytes will partition into the organic solvent. The efficiency of the extraction is enhanced by the shaking, which creates a high surface area for the extraction interface allowing partitioning to occur.

Liquid-liquid extraction can give particularly clean extracts of biological fluids, since matrix components such as proteins and phospholipids are not soluble in typical LLE solvents, and are therefore excluded from the final extract. The same benefits are true for supported liquid extraction (SLE) procedures.

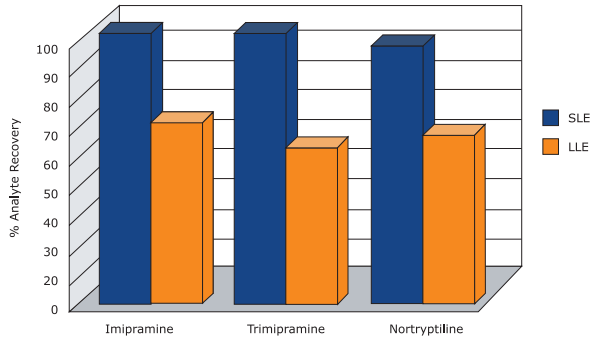


Figure 1. Recovery of tricyclic antidepressants from plasma, typical ISOLUTE® SLE+ procedure compared with the equivalent liquid-liquid extraction procedure.

Because the same water immiscible solvents are used in SLE, proteins and phospholipids are efficiently removed from the final extract, and no additional steps such as protein crash (precipitation) are required.

Using a fast, simple **load-wait-elute** procedure, supported liquid extraction using ISOLUTE® SLE+ products provides inherently cleaner extracts than other simple sample preparation techniques such as 'dilute and shoot' or protein precipitation.

The efficient extraction process combining high analyte recoveries, elimination of emulsion formation, and complete removal of matrix interferences such as proteins, phospholipids, and salts results in lower limits of quantitation compared to traditional LLE.

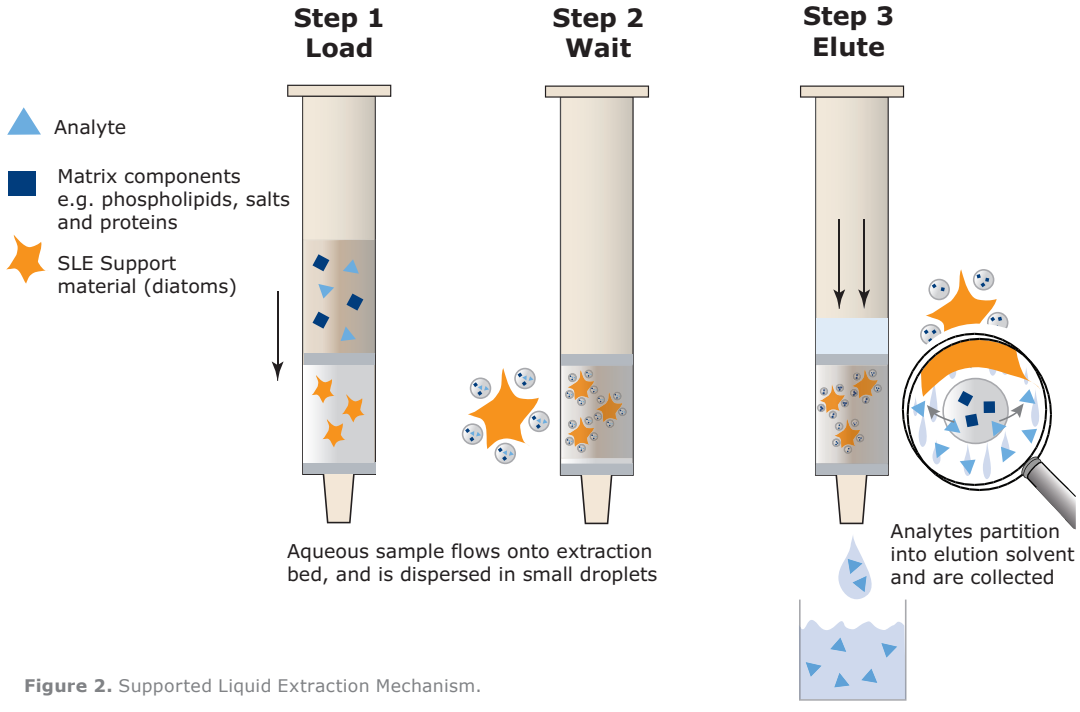


Figure 2. Supported Liquid Extraction Mechanism.

Supported Liquid Extraction Mechanism of Action

ISOLUTE® SLE+ products contain a modified form of diatomaceous earth, which provides a support for the liquid-liquid extraction process to occur, but does not interact chemically with the aqueous sample.

Application of the sample to the column results in the aqueous sample spreading over the surface of the material, forming an immobilized layer of small droplets held in place by a network of pores (Figure 2).

When the water immiscible extraction solvent is applied for the elution step, it flows over the aqueous droplets allowing efficient analyte partitioning. The large surface area of the extraction interface and flow through nature of the technique leads to a very efficient extraction procedure, because analytes come into contact with fresh solvent as the organic phase travels through the bed, mimicking a repeat LLE mechanism.

Extract Cleanliness

Excellent removal of phospholipids and proteins

Although supported liquid extraction is a very simple sample preparation technique, it provides much cleaner extracts than techniques such as 'dilute and shoot' and protein precipitation. Extract cleanliness can often be comparable with more selective SPE approaches. In supported liquid extraction, analytes are extracted by partitioning into a water immiscible organic solvent. Endogenous components such as salts, proteins peptides and phospholipids (present in blood related matrices) which cause matrix effects in LC-MS/MS are not soluble in these solvents. As a result they remain in the aqueous phase on the sorbent and are therefore eliminated from the final extract. High analyte recoveries along with low matrix components and reduced ion suppression result in better quantitation and method performance.

Phospholipid Removal Using ISOLUTE® SLE+

Phospholipids are matrix components found in blood based biological fluids such as plasma and serum. Their presence in sample extracts to be analyzed by LC-MS/MS is problematic as they often co-elute with analytes of interest causing significant ion suppression. Elimination of phospholipids from sample extracts is therefore essential for reliable analyte quantitation.

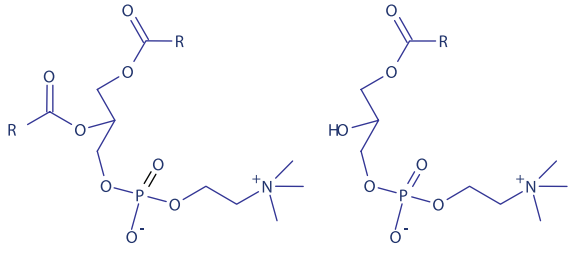


Figure 3. Examples of phospholipid structures.

Simple sample preparation techniques such as 'dilute and shoot' and protein precipitation do not significantly reduce phospholipid concentration in sample extracts, meaning analytical sensitivity and extract cleanliness can be compromised. Figure 4 compares the levels of phospholipids (PL) and lysophospholipids (lysoPL) in sample extracts prepared using protein precipitation (PPT) and ISOLUTE SLE+.

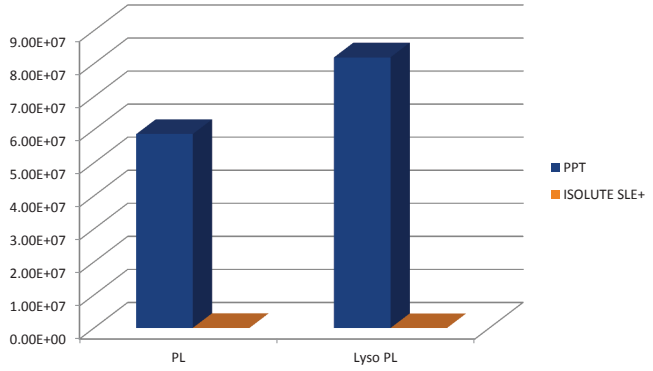


Figure 4. Comparison of phospholipids and lysophospholipids present in sample extracts prepared using protein precipitation (PPT, 100µL human plasma precipitated with 300µL acetonitrile) and ISOLUTE SLE+ (100 µL human plasma extracted using MTBE).

Phospholipid depletion products are designed to remove phospholipids from sample extracts. Figure 5 compares phospholipid (PL and lysoPL) levels in sample extracts prepared using two popular phospholipid depletion products with those in ISOLUTE SLE+ extracts. Using the fast, simple load-wait-elute procedure, ISOLUTE SLE+ reduces phospholipid concentration to significantly lower levels than phospholipid depletion products.

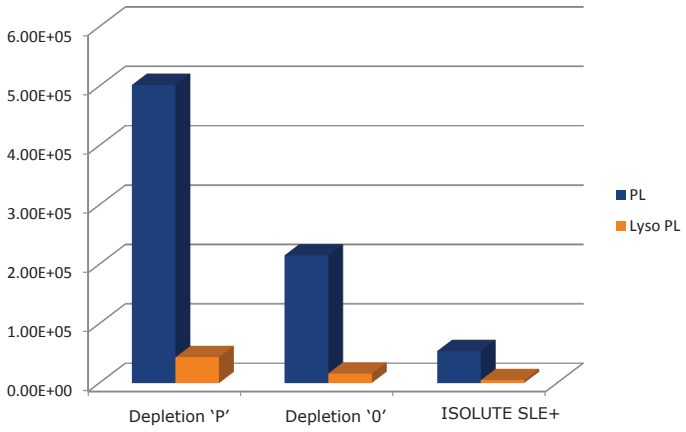


Figure 5. Comparison of phospholipids and lysophospholipids present in sample extracts prepared using phospholipid depletion (100 µL human plasma prepared using manufacturer's instructions) and ISOLUTE SLE+ (100 µL human plasma extracted using MTBE).

In supported liquid extraction using ISOLUTE® SLE+ plates, we have found that water immiscible solvents such as MTBE work effectively to extract a broad range of analytes. However, for analytes which do not elute well in these solvents, 5% (v/v) of a polar modifier can be added without sacrificing extract cleanliness.

The data below (Figure 6) illustrates the removal of phospholipids from plasma samples under various combinations of pre-treatment and extraction solvent conditions using ISOLUTE SLE+ plates. Similar results were seen for lysophospholipids. All extracts were compared to protein precipitated plasma samples as the 100% standard.

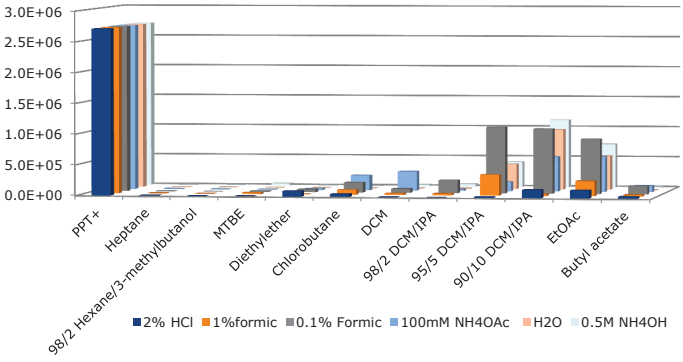
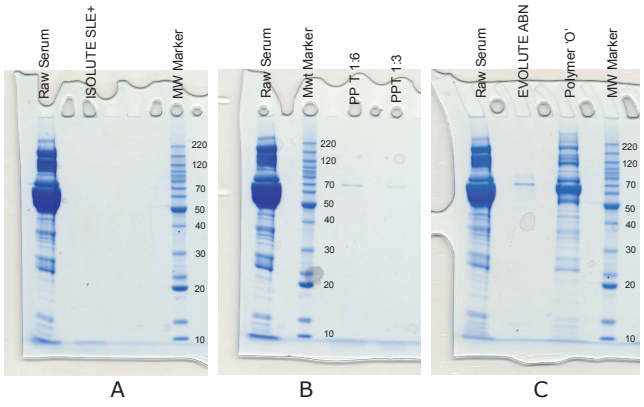


Figure 6. Levels of phospholipids in sample (human plasma, 100 µL) extracts prepared using ISOLUTE® SLE+ with a variety of sample pre-treatment and extraction solvents.

In figures 4, 5, and 6, Phospholipids were monitored by LC-MS/MS using electrospray ionization in the positive ion mode. The most abundant phospholipids (selected from full scan, SIR and precursor ion scan data) were quantified using the 184 product ion in the multiple reaction monitoring mode (MRM).

Protein Removal Using ISOLUTE® SLE+

To demonstrate the effectiveness of ISOLUTE SLE+ in removing proteins from biological fluid samples, serum was prepared using ISOLUTE SLE+, protein precipitation and solid phase extraction. Residual protein in the extracts was analyzed using gel electrophoresis. Complete protein removal was seen with ISOLUTE SLE+.



A ISOLUTE® SLE+: Rat serum pre-treated 1:1 (v/v) with water, loaded onto ISOLUTE SLE+ plate and extracted with MTBE. All extracts were evaporated to dryness for gel electrophoresis work up.

B Protein precipitation: rat serum precipitated with acetonitrile (1:3 and 1:6 v/v)

C Solid phase extraction: 100 µL rat serum extracted using manufacturers recommended generic methods

Gel electrophoresis: NuPAGE Novex 12% Bis-Tris mini gel with MOPS SDS running buffer at 200V, 120mA and 12.5V. Gels were run for approximately 65 minutes to ensure complete protein migration.

Figure 7. Protein removal, ISOLUTE® SLE+ vs. protein precipitation and solid phase extraction.

Productivity in Automation

In addition to being easy to use on both vacuum and positive pressure processing manifolds, ISOLUTE SLE+ plates and columns are well suited for use in automated, higher throughput applications.

Procedures are confined to straightforward liquid (sample or elution solvent) dispensing and vacuum or pressure application. No off-line shaking or mixing, centrifuging, flash freeze/pour or other manual steps necessary for traditional liquid-liquid extraction are required.

The simple load-wait-elute procedure allows ISOLUTE SLE+ plates and columns to be processed in half the time of traditional liquid-liquid extraction (see table 1) or solid phase extraction procedures.

ISOLUTE SLE+ support material gives excellent loading and elution characteristics. Due to the unique extraction mechanism of supported liquid extraction, with the whole sample being absorbed onto the support, there is no need to collect waste. The extract collection plate can be in place throughout the procedure. The organic elution solvent is the only solvent that flows through the well/column, ensuring even flows and minimizing the possibility of well or column blockage.

In addition to 96-well plate formats for low volume biological fluid samples, ISOLUTE SLE+ columns are available in both 400 µL and 1 mL sample capacity, with the tabless format option compatible with most automation systems.

Table 1: Comparison of Automated Extraction

Technique	Time for processing 96 plasma samples
Automated* ISOLUTE SLE+	12.5 min
Automated* liquid-liquid extraction	22.5 min

* Using the Quadra-96™ liquid handling system, typical ISOLUTE® SLE+ procedure compared with the equivalent liquid-liquid extraction procedure.

Processing ISOLUTE® SLE+ Products

Processing Conditions

Processing ISOLUTE® SLE+ columns and 96-well plates is largely performed under gravity, with a pulse of vacuum or positive pressure used to initiate loading of the sample, and to maximize solvent recovery (leading to more reproducible analyte recovery) after elution. Both manual and automated, vacuum or positive pressure systems can be used.

Volume Guidelines

Unlike SPE, where the sample is loaded onto the column and flows through the sorbent to waste, in SLE the ENTIRE sample volume is absorbed onto the extraction bed. It is therefore vital to use a format with sufficient capacity to absorb the whole sample volume.

Note: the 'sample volume' refers to the aliquot of raw sample matrix plus any dilution buffer used. For example, to extract 200 µL of plasma, diluted 1:1 with buffer, a 400 µL capacity product should be used.

Table 2 below provides guidelines for sample load and elution volume for each ISOLUTE SLE+ format.

The recommended workflow for processing ISOLUTE® SLE+ columns and plates is shown below

1. Pre-treat sample as required (including internal standard addition)
2. Ensure appropriate collection vessel is in place
3. Load sample onto ISOLUTE SLE+ column or plate
4. Apply vacuum (-0.2 bar) or pressure (3 psi) for 2–5 seconds to initiate loading
5. Wait 5 minutes for sample to completely absorb and form extraction layer
6. Apply water immiscible extraction solvent and allow to flow for 5 minutes under gravity
7. Apply vacuum (-0.2 bar) or pressure (10 psi) for 10–30 seconds to complete elution
8. Evaporate eluate to dryness and reconstitute as required.

Table 2: Maximum Sample and Elution Volumes for ISOLUTE® SLE+ Products

Product Capacity	Maximum Load Volume	Elution Protocol/ Volume
200 µL 96-well plate	200 µL	1 x 1 mL
400 µL 96-well plate	400 µL	2 x 900 µL
200 µL Array column/plate	200 µL	2 x 600 µL
400 µL Array column/plate	400 µL	3 x 750 µL
400 µL column	400 µL	2 x 900 µL
1 mL column	1 mL	2 x 2.5 mL
2 mL column	2 mL	2 x 5 mL
5 mL column	5 mL	3 x 8 mL
10 mL column	10 mL	2 x 20 mL

Table 2. The elution volumes described represent a starting point for method development, and may be reduced during method optimization, particularly for freely soluble analytes.

Internal Standards

When using internal standards, they should be added to the raw sample, mixed, and allowed to equilibrate prior to any other sample pre-treatment. When standards are added in water miscible organic solvents, ensure that the volume added is as low as possible (for example, 10 µL standard solution in 200 µL sample). For optimum performance, even when higher sample volumes are used, the volume of internal standard solution should be kept as low as possible.

This will minimize any solvent bridge effect, avoiding matrix effects due to increased transfer of water soluble matrix components into the elution solvent.

Scalability

A method developed on one ISOLUTE SLE+ format can easily be transferred to other formats if larger or smaller sample volumes are to be extracted (see below).

β-blockers: A suite of β-blockers (Figure 8) was used in recovery experiments from plasma using the relevant sample volumes diluted 1:1 (v/v) 0.5M NH_4OH and eluted with recommended elution volumes. As can be seen, consistent recoveries in excess of 80% were evident across all formats.

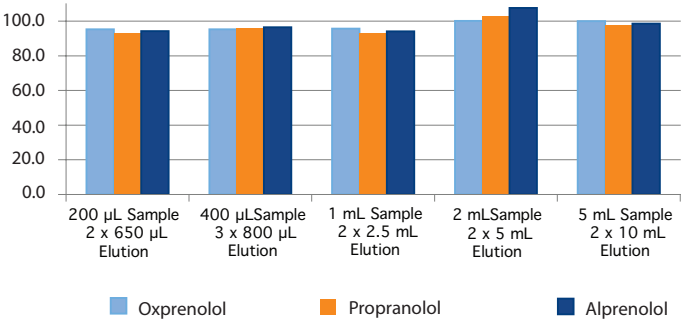


Figure 8. Scalability of columns using β-Blocker analyte suites.

Method Development Strategies

Supported Liquid Extraction is based on the partitioning of the desired analyte(s) from an aqueous sample phase into a water immiscible organic extraction solvent. Key factors affecting analyte partitioning are:

- Analyte functionality (pK_a and LogD)
- Solubility of the desired analyte(s) in the water immiscible extraction solvents

Typical extraction solvents used in supported liquid extraction are MTBE, ethyl acetate, dichloromethane, hexane or mixed solvents. Many other water immiscible extraction solvents are also applicable. Solvent selection depends largely on the range of analytes to be extracted, in terms of polarity (LogP or LogD), and their solubility characteristics. The range of analytes that can be extracted is extended using some of the strategies described in this section.

If a liquid-liquid extraction method exists, similar extraction conditions (solvent and pH conditions) are a good starting point for method development using ISOLUTE® SLE+ products.

Sample Pre-Treatment

In order to promote partitioning of analytes into the organic phase in supported liquid extraction, the charge on any acidic or basic groups should be suppressed wherever possible. This is particularly important for more polar analytes.

Extraction efficiency can be optimized based on drug functionality by modification of the aqueous sample pH (see '2 pH unit rule', opposite, for theoretical considerations).

For acidic analytes, it is often preferential to acidify the sample with an appropriate buffer prior to loading to ensure that the analyte charge is suppressed (non-ionized) and therefore more soluble the organic phase.

Conversely, for basic analytes, higher recoveries may be obtained by adding a small amount of basic modifier to raise the sample pH prior to loading.

pH control is most important for polar analytes that are only sparingly soluble in water immiscible solvents when charged.

The 2 pH Unit Rule

The pK_a of an ionizable functional group is defined as the pH at which 50% of this group in solution is charged, and 50% is uncharged (neutral). Each pH unit change affects the percentage of charged or uncharged groups by a factor of 10, so it is optimal to perform extractions at a pH at least 2 pH units from the pK_a value, to ensure that 99.5% of the functional groups are in the desired state of ionization.

e.g. Effect of pH on the dissociation of weak acid with a pK_a value of 4.0

pH	% free acid (uncharged)	% dissociated (charged)
2.0	99.5	0.5
3.0	95	5.0
ACID pK_a = 4.0	50	50
5.0	5.0	95
6.0	0.5	99.5

Therefore, for the highest solubility of a weakly acidic analyte with pK_a of 4.0 in an organic solvent, adjust sample to pH 2.0 or less (2 pH units BELOW the pK_a).

e.g. Effect of pH on the dissociation of the conjugate acid of a weak base with a pK_a value of 9.0

pH	% base (uncharged)	% dissociated (charged)
11.0	99.5	0.5
10.0	95	5.0
ACID pK_a = 9.0	50	50
8.0	5.0	95
7.0	0.5	99.5

Therefore, for the highest solubility of a weakly basic analyte with pK_a of 9.0 in an organic solvent, adjust sample to pH 11.0 or higher (2 pH units ABOVE the pK_a).

Note that it is not always necessary to reach the theoretical optimum pH to achieve high recoveries using ISOLUTE SLE+ products, due to the highly efficient extraction process.

Buffer Recommendations for LC-MS/MS Analysis

The following pages outline general strategies for method development for extraction of neutral, acidic and basic analytes using ISOLUTE® SLE+. A screening approach to method development is also described (page 12).

When developing methods for LC-MS/MS analysis, we recommend the use of volatile (MS friendly) buffers wherever possible.

Recommended	Not recommended*
Hydroxide	Phosphate
Acetate	Carbonate
Formate	Other non-volatile buffers

*Where the use of non-volatile buffers is necessary, consider reducing the sample load volume to ~3/4 of the recommended maximum, to minimize any adverse effects. This is particularly important when elution solvents in which water is partially soluble (e.g. ethyl acetate).

Method Development for Neutral (Non-Ionizable) Compounds

For true neutral analytes that do not have a pK_a value, pH control is not a major factor as these analytes should extract across the pH range. The choice of the correct extraction solvent is the most important factor. A water immiscible organic solvent in which the analyte is freely soluble is a good choice.

For very non-polar analytes, non-polar solvents such as heptane, hexane, or dichloromethane may be most appropriate.

For more polar compounds, more polar extraction solvents such as MTBE, dichloromethane or ethyl acetate are useful.

For polar molecules that do not elute well in these solvents, 5% (v/v) of a polar modifier such as isopropanol can be added to the extraction solvent to enhance extraction efficiency and raise analyte recovery. Note however that the use of higher amounts of polar (water miscible) modifiers in the extraction solvent can lead to co-extraction of some matrix components such as phospholipids.

Typical extraction conditions for neutral (non-ionizable) analytes, using a 200 µL ISOLUTE® SLE+ plates (part number 820-0200-P01)

Sample pre-treatment	Dilute plasma sample 1:1 (v/v) with water
Load	Dispense sample (200 µL). Apply vacuum (-0.2 bar) for 2–5 seconds to initiate flow.
Wait	Wait 5 mins for sample to completely absorb
Elute	Apply extraction solvent (1 mL). Allow solvent to flow for 5 mins under gravity. Apply vacuum (-0.2 bar) for 10–30 seconds to complete elution
Post-extraction	Evaporate to dryness and reconstitute in mobile phase prior to analysis

Example:

Extraction of endogenous steroids from human plasma

In this example, pH adjustment of the sample is not required, as the analytes do not have any acidic or basic functional groups.

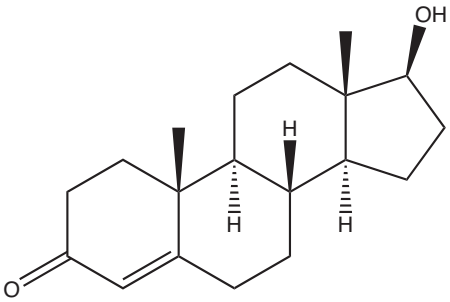


Figure 9. Structure of Testosterone.

Note:

For neutral analytes that are protein bound, pH adjustment may improve recovery. See Hints, Tips and Troubleshooting, page 16.

LogP of endogenous steroids

Analyte	Log P
Aldosterone	0.71
21-deoxycortisol	2.07
Androstenedione	2.72
11-deoxycortisol	2.74
17-hydroxyprogesterone	3.04
Testosterone	3.18
DHEA	3.3
Progesterone	3.83
Androsterone	3.93

Results

Recoveries of endogenous steroids from human plasma using a 200 µL ISOLUTE SLE+ plate are shown below. For neutral analytes, choice of elution solvent is the most important factor affecting analyte recovery.

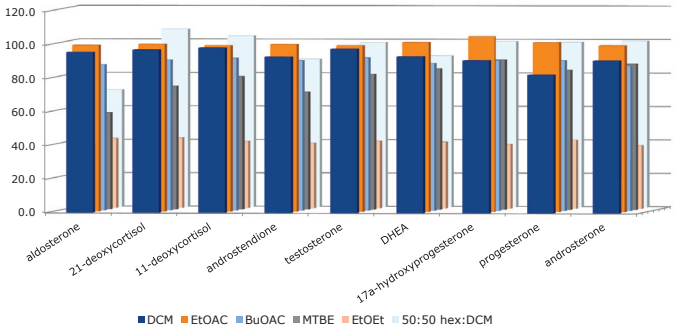


Figure 10. Recoveries of endogenous steroids from human plasma using various elution solvents.

Method Development for Basic Compounds

A water immiscible organic solvent in which the analytes are highly soluble should be chosen as extraction solvent.

For basic analytes, recovery may be enhanced by increasing sample pH to deprotonate the analyte increasing the solubility in the water immiscible organic solvent. This can be achieved by dilution of the sample (typically 1:1, v/v) with a basic buffer such as 0.5M ammonium hydroxide. This will raise pH >10 for most biological fluids. For strongly basic compounds (e.g. quaternary amines), consider the use of ion pair reagents (see page 14).

pH control is most important when dealing with polar analytes.

For very non-polar analytes, non-polar solvents such as heptane, hexane, or dichloromethane may be most appropriate.

For more polar compounds, more polar extraction solvents such as MTBE, dichloromethane or ethyl acetate are useful.

For polar molecules that do not elute well in these solvents, 5% (v/v) of a polar modifier such as isopropanol can be added to the extraction solvent to enhance extraction efficiency and raise analyte recovery. Note however that the use of higher amounts of polar (water miscible) modifiers in the extraction solvent can lead to co-extraction of some matrix components such as phospholipids.

Typical extraction conditions for basic analytes, using a 200 µL ISOLUTE® SLE+ plate (part number 820-0200-P01)

Sample pre-treatment	Dilute plasma sample 1:1 (v/v) with 0.5M NH_4OH (approximate pH 10.4)
Load	Dispense sample (200 µL). Apply vacuum (-0.2 bar) for 2–5 seconds to initiate flow.
Wait	Wait 5 mins for sample to completely absorb.
Elute	Apply extraction solvent (1 mL). Allow solvent to flow for 5 mins under gravity. Apply vacuum (-0.2 bar) for 10–30 seconds to complete elution.
Post-extraction	Evaporate to dryness and reconstitute in mobile phase prior to analysis.

Example:

Extraction of β-blockers from human plasma

In this example, the plasma sample is adjusted to basic pH using 0.5 M ammonium hydroxide.

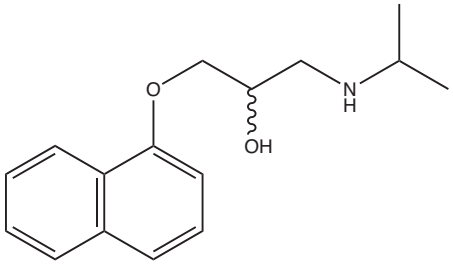


Figure 11. Structure of propranolol.

pK_a and LogP of selected β-blockers

Analyte	pK_a	Log P
Atenolol	9.5	0.57
Sotalol*	8.3/9.2	0.85
Nadolol	9.67	1.23
Pindolol	8.8	2.17
Propranolol	9.45	3.17

Results

Recoveries of β-blockers from human plasma using a 200 µL ISOLUTE® SLE+ plate are shown below.

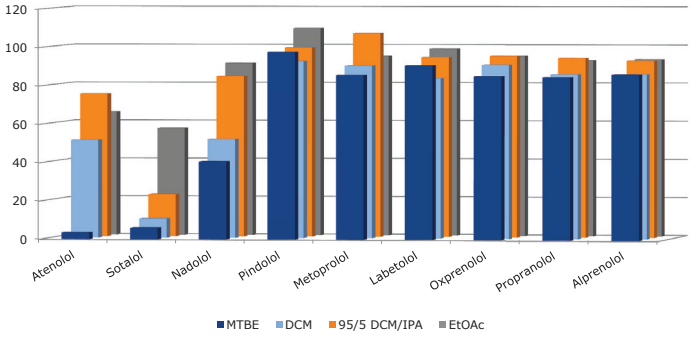


Figure 12. Recoveries of endogenous steroids from human plasma using various elution solvents.

*Note that Sotalol is an amphoteric compound, so further method optimization is required to achieve high analyte recoveries. See 'Hints, Tips and Troubleshooting' (Page 16).

Method Development for Acidic Compounds

A water immiscible organic solvent in which the analytes are highly soluble should be chosen.

For acidic analytes, recovery may be enhanced by lowering sample pH to suppress ionization of the analyte, allowing it to exist in the neutral form. This can be achieved through the addition of an acidic buffer such as 1% (v/v) formic acid.

pH control is more critical when dealing with more polar analytes.

For very non-polar analytes, non-polar solvents such as heptane, hexane, or dichloromethane may be most appropriate.

For more polar compounds, more polar extraction solvents such as MTBE, dichloromethane or ethyl acetate are useful.

For polar molecules that do not elute well in these solvents, 5% (v/v) of a polar modifier such as isopropanol can be added to the extraction solvent to enhance extraction efficiency and raise analyte recovery. Note however that the use of higher amounts of polar (water miscible) modifiers in the extraction solvent can lead to co-extraction of some matrix components such as phospholipids.

Typical extraction conditions for acidic analytes, using a 200 µL ISOLUTE® SLE+ plate (part number 820-0200-P01)

Sample pre-treatment	Dilute plasma sample 1:1 (v/v) with 1% formic acid (approx. pH 3.4).
Load	Dispense sample (200 µL). Apply vacuum (-0.2 bar) for 2–5 seconds to initiate flow.
Wait	Wait 5 mins for sample to completely absorb.
Elute	Apply extraction solvent (1 mL). Allow solvent to flow for 5 mins under gravity. Apply vacuum (-0.2 bar) for 10–30 seconds to complete elution.
Post-extraction	Evaporate to dryness and reconstitute in mobile phase prior to analysis.

Example:

Extraction of NSAIDs from human plasma

In this example, the plasma sample is adjusted to acidic pH using 1% formic acid.

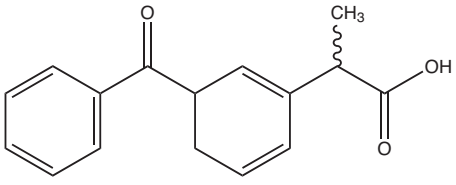


Figure 13. Structure of ketoprofen.

pK_a and LogP of selected NSAIDs

Analyte	pK_a	Log P
Acetaminophen	9.7	0.46
Salicylic acid	2.97	2.21
Naproxen	4.2	2.8
Mefenamic acid	4.2	5.1

Results

Recoveries of NSAIDs from human plasma using a 200 µL ISOLUTE® SLE+ plate are shown below. Recoveries of more polar analytes are enhanced by the use of more polar elution solvents.

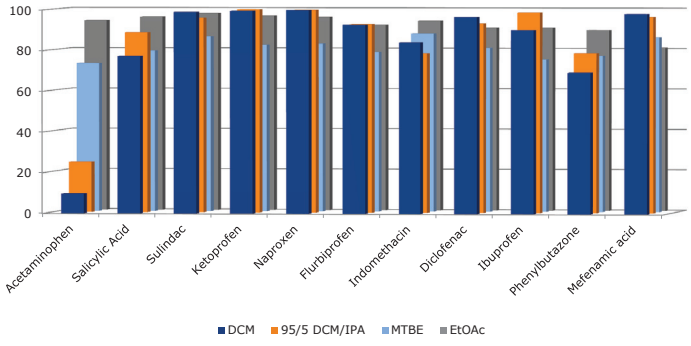
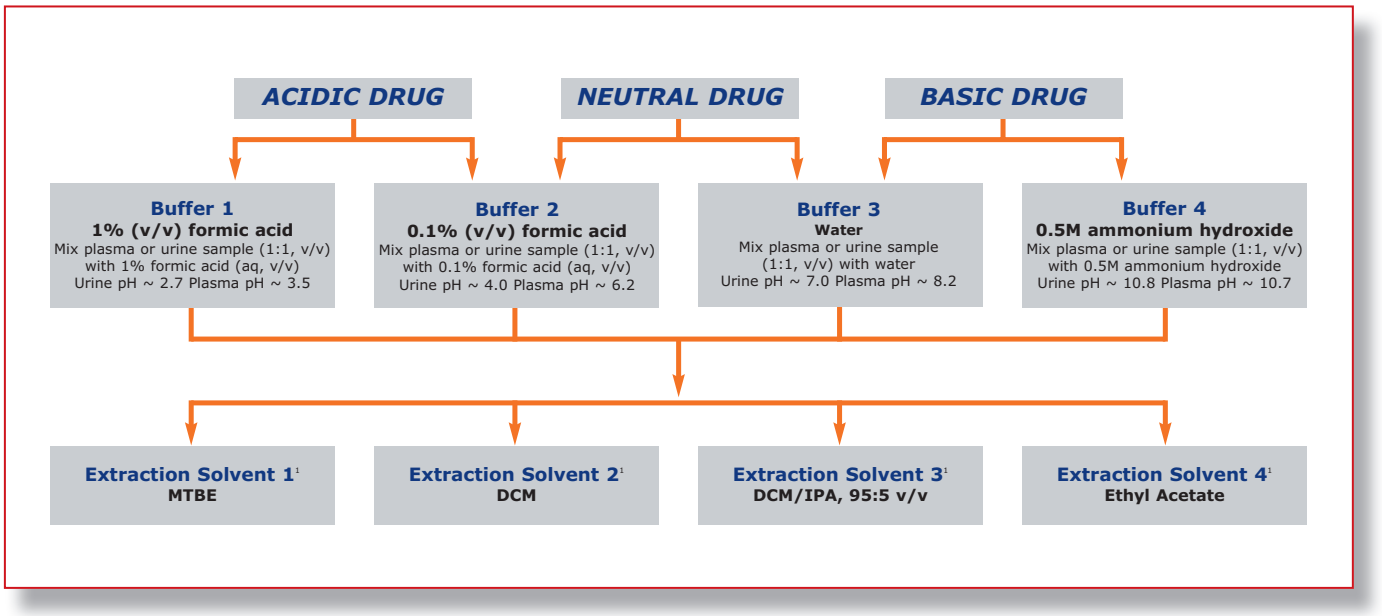


Figure 14. Recoveries of NSAIDs from human plasma using various elution solvents.

Streamlined Method Development Recommendations

Biotage have developed a simple screening approach based on the principles described in this section, according to analyte(s) functionality. By simply screening 2 pHs, combined with 4 extraction solvents, you can develop a method in minutes.

ISOLUTE® SLE+ Supported Liquid Extraction Method Selection



Using the Chart

- Recommendations can be applied to any format of ISOLUTE® SLE+ product. Appropriate load and elution volumes are detailed on page 7.
- Analyte functionality: according to whether your analyte is acidic, neutral or basic, various pH conditions are recommended. For best results, analytes should be in their non-ionized form if possible.
- For acidic drugs, low and intermediate sample pH conditions should be evaluated.
- For basic drugs, intermediate and high sample pH conditions should be evaluated.
- Due to the rapid partition and equilibration of analytes into fresh solvent as the elution solvent passes through the ISOLUTE SLE+ column, extraction efficiency compared to LLE is increased.
- Precise pH control/adjustment may not always be required for low polarity analytes. Optimized pH control is more important for more polar analytes.
- Neutral analytes with no pK_a value can be extracted across the pH range. If protein binding is apparent then the use of acid or base in the sample pre-treatment can help to disrupt protein binding, or minimize extraction of unwanted sample components.
- The 4 extraction solvents selected give a wide range of solvent characteristics and polarity.

Extending the Range of Extractable Analytes

For some analytes, standard extraction conditions may not be suitable. This is often due to the low solubility of certain analytes in non-water miscible solvents. For example, small polar acids, and analytes with strongly acidic or basic groups, which cannot be neutralized by pH control, may need further method optimization.

This section describes strategies developed in our R&D laboratories to enhance recovery of these analytes, and extend the range of analytes that can be extracted using supported liquid extraction.

Example 1:

Extraction of small, polar acids using advanced pH control

Small, polar acids may not give high recoveries using standard pre-treatment conditions in supported liquid extraction, because of their poor solubility in water immiscible solvents when in their ionized state, combined with the extreme pH conditions needed to neutralize the analytes and improve their solubility.

Methylmalonic acid (see below) is a small, extremely polar, water soluble acid. Using standard sample pre-treatment conditions for acids, recoveries of <40% were achieved (see Figure 16).

Analyte	pK_a	Log P
Methylmalonic acid	3.07	0.17

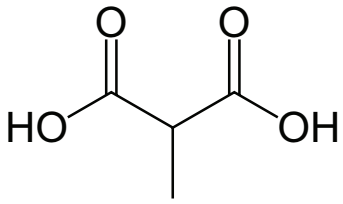


Figure 15. Properties and structure of methylmalonic acid.

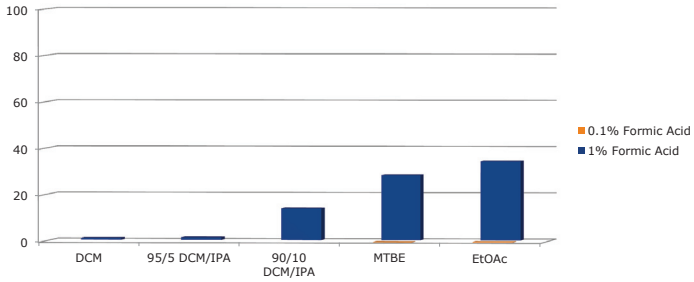


Figure 16. Methylmalonic acid recovery from human plasma using 'standard' sample pre-treatment conditions.

We therefore investigated advanced pH control in order to improve analyte recovery. Using the extraction solvents which gave some analyte recovery in the first experiment (MTBE and ethyl acetate), a range of acidic sample pre-treatment conditions were evaluated:

Sample pre-treatment	Sample pH
Dilution 1:1 (v/v) with HCl (2%)	1.71
Dilution 1:1 (v/v) with HCl (5%)	0.89
Dilution 1:1 (v/v) with H_3PO_4 (4%)	1.71
Dilution 1:1 (v/v) with H_3PO_4 (5%)	1.55

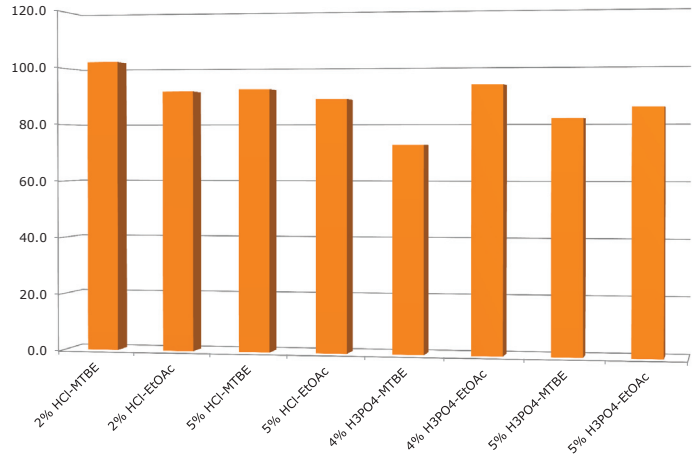


Figure 17. Methylmalonic acid recovery from human plasma using advanced sample pre-treatment conditions.

Using advanced pH control for plasma-pre-treatment, acceptable recoveries of methymalonic acid were achieved. However, use of strong, non-volatile acids for sample pre-treatment did lead to increased ion suppression when using solvents with higher water solubility (e.g. ethyl acetate). To minimize this effect, the use of solvents with low water solubility (e.g. MTBE) are recommended.

Alternatively, consider the use of a decreased load volume, or a larger capacity product (Hints, Tips and Troubleshooting, page 16).

Example 2

Use of ion pair reagents

The use of ion pair reagents can be a very useful approach, especially when dealing with matrices and/or analytes that are susceptible to hydrolysis or other degradation when exposed to extreme pH conditions. This approach can also be used to help with the simultaneous extraction of acid, neutral and basic drugs in a single extraction protocol.

In this example, the optimized, simultaneous extraction of 11-nor-9-carboxyΔ^9THC and 11-nor-9-carboxyΔ^9THC-glucuronide from urine is described. The parent compound extracts well under acidic conditions, but the highly water soluble glucuronide metabolite is not recovered (see Figure 18).

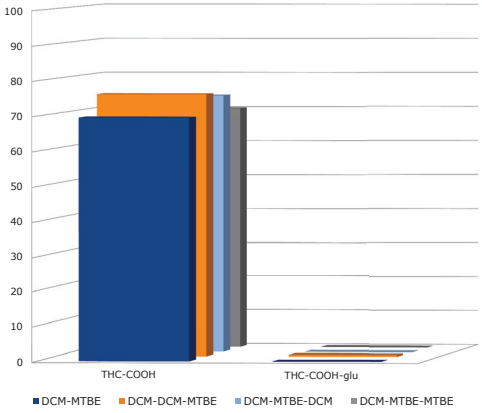


Figure 18. Recovery of THC and THC-Glucuronide from urine using standard 1% formic acid pre-treatment, and a variety of extraction solvents.

The use of a 'neutral' ion pair reagent allows ion pairing of the carboxylic acid groups, increasing their solubility in water immiscible solvents.

Procedure for simultaneous extraction of 11-nor-9-carboxy-Δ⁹-THC and 11-nor-9-carboxy-Δ⁹-THC glucuronide from urine, using a 200 µL supported liquid extraction plate, (part number 820-0200-P01).

Sample pre-treatment[1]	Dilute urine 1:1 (v/v) using 25mM dibutylammonium acetate.
Load	Dispense sample (200 µL). Apply vacuum (-0.2 bar) for 2–5 seconds to initiate flow.
Wait	Wait 5 mins for sample to completely absorb.
Elute	Apply ethyl acetate (1 mL). Allow solvent to flow for 5 mins under gravity. Apply vacuum (-0.2 bar) for 10–30 seconds to complete elution.
Post-extraction[2]	Evaporate to dryness at 40 °C and reconstitute in mobile phase prior to analysis.

[1] These pre-treatment conditions allow ion pairing of the carboxylic acid groups while avoiding any pH related hydrolysis of the glucuronide or the matrix.

[2] Blowdown at 40 °C ensures that the ion pair reagent does not affect subsequent chromatography.

Using the ion pair reagent as described gave high recoveries of both analytes, and no losses of the glucuronide metabolite were observed. 25mM dibutylammonium acetate demonstrated optimum recovery (see Figure 19).

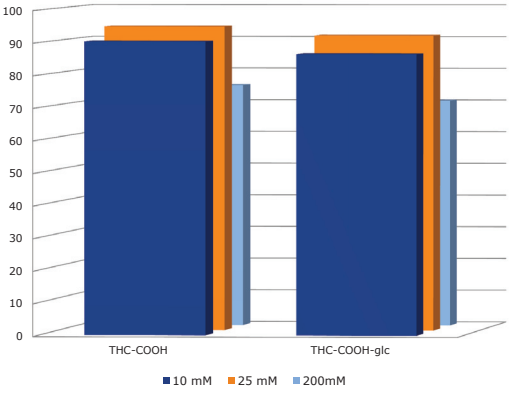


Figure 19. Recovery of THC and THC-Glucuronide from urine using dibutylammonium acetate as an ion pair reagent (10mM, 25mM, 200mM).

Ion pair pre-treatment recommendations

Functional group to be paired	Recommended ion pair reagent	Final sample pH
Basic (+ve charge)	Heptafluorobutyric acid, (10-100mM, 1:1, v/v)	Acidic
Basic (+ve charge)	Trifluoroacetic acid (TFA) (up to 1% aq v/v, 1:1, v/v)	Acidic
Acidic (-ve charge)	Dibutyl ammonium acetate, (25mM, 1:1, v/v)	Neutral
Acidic (-ve charge)	Tetrabutylammonium acetate (25mM, 1:1, v/v)	Basic

For best results, volatile ion pair reagents should be used. These are removed at the extract evaporation stage, and will not therefore impact on the subsequent chromatographic separation.

Example 3

Analytes with extreme protein binding

Because proteins are insoluble in typical SLE elution solvents, strongly protein bound analytes cannot partition freely into the organic elution solvent, leading to reduced analyte recovery. Where analytes are strongly protein bound, it may therefore be necessary to disrupt this binding prior to sample load, in order to achieve high analyte recoveries.

Protein binding disruption strategies include:

- pH adjustment, for example addition of trichloroacetic acid
- addition of $ZnSO_4$ solutions (aqueous or aqueous/solvent combination)
- addition of various concentrations of water miscible organic solvent.

In this example, the extraction of 25-OH Vitamin D2 and D3 from human serum is described. Due to the strongly protein bound nature of the analytes, standard aqueous pre-treatment conditions only resulted in analyte recoveries of approximately 65%.

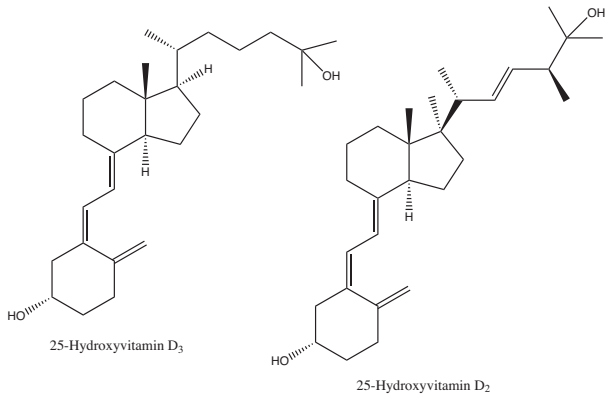


Figure 20. Structure of 25-OH Vitamins D2 and D3.

LogP of 25-OH Vitamin D2/D3

Analyte	Log P
25-OH Vitamin D2	~ 7.0
25-OH Vitamin D3	~ 7.0

In the final optimized procedure, serum was pre-treated by dilution (1:1, v/v) with a mixture of water/isopropanol (1:1, v/v) resulting in a total solvent concentration of 25% (v/v) in the load. This relatively high organic content was sufficient to induce disruption of the 25-OH Vitamin D from its binding without causing precipitation of the proteins in the sample.

Due to the high organic content in the treated sample, a 'solvent bridge' effect is possible, potentially leading to increased solubility of the aqueous portion of the sample in the extraction solvent, and ultimately breakthrough of some matrix components into the final extract. In order to counteract this effect, it was necessary to under load the extraction plate. We loaded a maximum of 300 µL of pre-treated sample per well onto a 400 µL plate. This afforded extra capacity to the plate and avoided aqueous breakthrough, as well as maintaining excellent extract cleanliness. In addition, the samples demonstrated excellent flow characteristics through the plate.

Optimized procedure for extraction of highly protein bound 25-OH Vitamin D from serum, using a 400 µL supported liquid extraction plate, (part number 820-0400-P01)

Sample pre-treatment	Dilute serum 1:1 (v/v) using water/ isopropanol (1:1, v/v).
Load	Dispense sample (300 µL). Apply vacuum (-0.2 bar) for 2–5 seconds to initiate flow.
Wait	Wait 5 mins for sample to completely absorb.
Elute	Apply heptane (750 µL). Allow solvent to flow for 5 mins under gravity. Apply a further aliquot of heptane (750 µL). Apply vacuum (-0.2 bar) for 10–30 seconds to complete elution.
Post-extraction	Evaporate to dryness at room temperature and reconstitute in a suitable solvent prior to analysis.

Results

Using the optimized procedure described, high recoveries were achieved and excellent extract cleanliness was maintained.

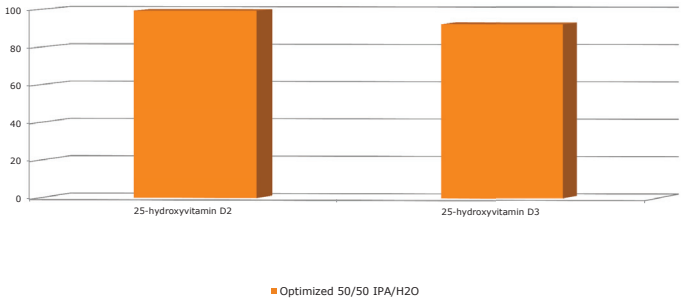


Figure 21. Recovery of 25-OH Vitamin D2 and D3 using sample pre-treatment conditions optimized to disrupt protein binding of strongly hydrophobic analytes.

Hints, Tips and Troubleshooting

Situation	Solution
To maximize the volume of raw sample that can be loaded onto an ISOLUTE® SLE+ product.	The standard sample pre-treatment recommendation is a 1:1 (v/v) sample dilution prior to sample loading. However, to load a larger volume of raw sample without exceeding capacity, we recommend the addition of a smaller volume (e.g. 1:3 or 1:9 (v/v)) of more concentrated buffer or pH control reagent.
To maximize the volume of raw sample that can be loaded onto an ISOLUTE SLE+ product for NEUTRAL analytes such as steroids and amides.	Sample pre-treatment may not be required as neutral analytes often extract well at physiological pH, so raw sample can be loaded directly (with internal standard, as required).
To load low sample volumes (<150 µL).	Use a high dilution factor. Ensure load volume is sufficient to completely cover the top frit of the column or plate. This will facilitate reliable flows during sample processing. For plate formats, the minimum volume required to cover the frit is ~150 µL. For example, for a 10 µL sample, dilute to a minimum of 150 µL with a suitable pre-treatment buffer.
To disrupt protein binding of hydrophobic analytes in serum or plasma.	Dilution of the sample with 1:1 (v/v) using water/ isopropanol (1:1, v/v) can help to disrupt the protein without causing full precipitation of proteins.
To improve extract cleanliness for very low concentration analytes in biological fluids.	Decrease load volume. For example, load 300 or 350 µL pre-treated sample onto a 400 µL capacity plate or column.
To improve extract cleanliness where a higher polarity extraction solvent is required.	Decrease load volume. For example, load 300 or 350 µL pre-treated sample onto a 400 µL capacity plate or column.
To remove lipids and improve extract cleanliness for low level analysis.	Once sample is loaded and absorbed, use a 2 step elution process. Selectively elute non-polar lipids using hexane (discard), then elute analytes of interest with a suitable solvent.
To improve extract cleanliness when extracting whole blood, and avoid breakthrough of red blood cells.	Decrease load volume (use approximately ¾ of maximum recommended volume). For example, load 300 µL pre-treated sample onto a 400 µL capacity plate or column.
Sample pre-treatment tips for whole blood samples.	» Avoid precipitation of proteins with precipitating agents. » Avoid the use of high concentrations of $ZnSO_4$. » Vortex samples thoroughly after addition of pre-treatment reagents.

Situation	Solution
To increase recovery of analytes present inside red blood cells.	Centrifuge sample following sample pre-treatment to lyse red blood cells.
To improve extract cleanliness and avoid phospholipid contamination of the final extract, when using polar extraction solvent such as ethyl acetate.	Decrease load volume. For example, load a maximum of 300 µL pre-treated sample onto a 400 µL capacity plate or column, and optimize extraction solvent volume to obtain desired extract cleanliness and/or analyte recovery.
Extraction of amphoteric analytes.	Evaluate loading at pH corresponding to the isoelectric point of the analyte. Note that this approach is highly dependent on analyte polarity and pK_a values.
To increase recovery of strongly acidic analytes (e.g. $-SO_3^-$, $-PO_4^-$) or strongly basic analytes.	Evaluate the use of an ion pair reagent during sample pre-treatment. See page 14 for further details on ion pair reagents.
To increase recovery of very water soluble analytes.	Evaluate the use of a saturated salt solution in sample pre-treatment. Note that this may reduce extract cleanliness.
To extract both acidic and basic analytes in the same procedure. (I)	Load aqueous sample (~pH5) (50% of total well/column capacity). Elute with first aliquot of elution solvent. Add 10 µL conc. ammonium hydroxide (NH_4OH), and wait 5 mins, then elute with second aliquot of elution solvent.
To extract both acidic and basic analytes in the same procedure. (II)	Alternatively, increase sample pH using ammonium hydroxide (1-2%), load sample, then elute using solvent containing up to 1% (v/v) trifluoroacetic acid (TFA). Evaporate to dryness (>40 °C) to remove any traces of TFA and avoid any ion pair effect in subsequent chromatography. Alternatively, evaluate the use of ion pair reagents (see page 14).
To extract analytes with a wide range of polarity or solubility characteristics in a single procedure.	Evaluate the use of a series of aliquots of different extraction (elution) solvents, or blended solvents.
To avoid breakthrough of aqueous phase into final extract.	Ensure maximum sample volume is not exceeded. See page 7 (load volumes) for further details, and reduce load volume if necessary. If a polar modifier is used – evaluate the effect of reducing the % of polar modifier, or reduce load volume if recoveries are adversely affected.

Applications

Application Number	Title
AN601	Extraction of Tricyclic Anti-depressants from Plasma Using ISOLUTE® SLE+ Supported Liquid Extraction Plates
AN602	Extraction of Corticosteroids from Plasma using ISOLUTE SLE+ Supported Liquid Extraction Plates
AN603	Extraction of Non-steroidal Anti-inflammatory Drugs (NSAIDs) from Plasma using ISOLUTE SLE+
AN721	Extraction of Tamoxifen and Metabolites from Urine Using ISOLUTE SLE+
AN734	Method for the Extraction of Warfarin From Human Plasma Using ISOLUTE SLE+
AN738	Extraction of Methylmalonic Acid (MMA) and Succinic Acid (SA) from Human Serum Using ISOLUTE SLE+ in 96-Well Plates and Columns
AN740	Extraction of Testosterone and Other Steroid Hormones From Human Plasma Using ISOLUTE SLE+ 96-Well Plates
AN741	Extraction of Opiates From Human Urine Using ISOLUTE SLE+ 96-Well Plates and Columns
AN742	Extraction of Amphetamines From Urine Using ISOLUTE SLE+ 96-Well Plates
AN746	Extraction of Amphetamines From Urine Using ISOLUTE SLE+ Columns
AN747	How to Process ISOLUTE SLE+ Plates and Columns with Pressure+ 96 & 48 Positive Pressure Manifolds
AN751	Extraction of Benzodiazepines From Human Urine Using ISOLUTE SLE+ 96-Well Plates and Columns Prior to LC-MS/MS Analysis
AN752	Extraction of Barbiturates From Human Urine Using ISOLUTE SLE+ Columns with GC-MS Analysis

Application Number	Title
AN753	Extraction of Retinol, β-Carotene (Vitamin A) and α-Tocopherol (Vitamin E) from Serum using ISOLUTE SLE+ 96-Well Plates with APCI-LC-MS-MS Analysis
AN754	Extraction of Retinol, β-Carotene (Vitamin A) and α-Tocopherol (Vitamin E) from Whole Blood using ISOLUTE SLE+ 96-Well Plates with APCI-LC-MS-MS Analysis
AN756	Extraction of Benzodiazepines From Human Urine Using ISOLUTE SLE+ in Column Format Prior to GC-MS analysis
AN757	Extraction of Vitamin D Metabolites From Human Serum Using ISOLUTE SLE+ in 96-Fixed Well Plate Format Prior to LC-MS-MS analysis
AN758	Extraction of a Range of Immunosuppressants From Whole Blood Using ISOLUTE SLE+ for LC-MS/MS Analysis
AN761	Extraction of 1, 25 Dihydroxyvitamin D From Human Serum Using ISOLUTE SLE+ Prior to LC-MS/MS Analysis
AN762	Extraction of Low Level Testosterone and Androstenedione From Human Serum Samples Using ISOLUTE SLE+
AN764	Extraction of 22 Pain Management Drugs from Urine using ISOLUTE SLE+ in 96-Fixed Well Plate Format Prior to LC-MS-MS
AN770	Extraction of a range of Opiates and Metabolites from human urine using ISOLUTE SLE+ columns prior to GC-MS analysis
AN771	Extraction of Cocaine and Metabolites From Hydrolyzed Urine Using ISOLUTE SLE+ prior to LC-MS/MS Analysis
AN772	Extraction of Cocaine and Metabolites From Urine Using ISOLUTE SLE+ prior to LC-MS/MS Analysis
AN776	Extraction of Bath Salts (substituted cathinones) From Human Urine Using ISOLUTE SLE+ Columns prior to GC-MS Analysis

Biotage is constantly developing new applications using ISOLUTE® SLE+ products. Visit the literature database on www.biotage.com

Sample Processing Accessories

ISOLUTE® SLE+ plates and columns can be processed manually using vacuum or positive pressure manifolds. For automated processing, ISOLUTE SLE+ plates and columns are compatible with and easy to use on leading automation systems.

Biotage® VacMaster™ Sample Processing Stations

The VacMaster range of manual processing stations offers versatility, a small footprint and cost effective sample throughput from 96 well plates through to large column applications. Designed to meet the most demanding criteria for safety, extract purity, flexibility and ease of use, the VacMaster range of vacuum manifolds can be readily incorporated into the laboratory workflow.

Biotage® VacMaster™-96

The VacMaster-96 manifold is ideal for processing 96-well plates. The compact design and lightweight construction make it suitable for manual processing or for integrating with automated liquid handling system.

Two control units are available for use with either a vacuum source or for use with lab air to generate the vacuum.

Biotage®VacMaster™-10 and -20

VacMaster-10 and -20 manifolds are ideal for processing up to 10 (VacMaster-10) or 20 (VacMaster-20) samples in parallel using ISOLUTE SLE+ columns, and are compatible with all 400 µL to 10 mL capacity columns.

A range of stopcock options and spare parts for VacMaster manifolds are available. Contact your local representative or visit www.biotage.com for further details.

Biotage® Pressure+ Positive Pressure Manifolds

Biotage® PRESSURE+ manifolds offer positive pressure, parallel processing for all ISOLUTE SLE+ 96-well and Array plates, and 400 µL-1mL sample capacity ISOLUTE SLE+ columns. The systems utilize a consistent, uniform flow of positive pressure to move both samples and solvents on and off ISOLUTE SLE+ products. Each port of the PRESSURE+ manifold independently maintains constant pressure, increasing the overall reproducibility of analyte recoveries. This unique design allows for partially populated racks to be used without sacrificing extraction efficiency. The intuitive Biotage PRESSURE+ is easily incorporated into laboratory work flow.

Biotage® PRESSURE+ 96

The self-adjusting upper manifold of the PRESSURE+96 manifold is compatible with all 96 well plate formats in addition to the popular 1 mL and 2 mL Array modular well formats without the need to purchase supplementary gaskets. Biotage collection plates are recommended for the most consistent and reliable results.

Biotage® PRESSURE+ 48

The same self-adjusting technology utilized in the PRESSURE+ 96 manifold allows the PRESSURE+ 48 to utilize all columns up to 1 mL volume ISOLUTE SLE+ columns without the need to purchase supplementary gaskets. In addition, the unique design allows for between 1 and 48 columns to be processed in parallel without empty ports affecting flow rates. Tabless or flangeless columns should be used for full population and optimum sealing. The modular rack system accommodates most popular collection vessels.

Evaporation Products

Biotage® SPE Dry 96 and 96 Dual

Microplate Sample Evaporation System

Designed for high throughput laboratories, the SPE Dry 96 and SPE Dry 96 Dual Sample Concentrator Systems provide efficient solvent evaporation in microplate format and are compatible with 96 well collection plates. Heated gas flow from above and below the collection plate ensures efficient solvent evaporation. An easy-to-use front panel display allows accurate temperature programming and gas flow control. The systems are supplied as standard with stainless steel needles and are also available with PTFE-coated needles for applications using aggressive solvents, acids and bases. Biotage delivers the most efficient solvent evaporation laboratory equipment for method development, sample extractions and much more.

TurboVap®

Automated Solvent Evaporation Systems

TurboVap® 96

The TurboVap 96 Concentration Evaporator Workstation is a microprocessor-controlled evaporation system for simultaneous, automated concentration of multiple samples with unattended operation, convenience and speed. It can accommodate one or two 96-well collection plates.

TurboVap® LV

The TurboVap LV Concentration Evaporator Workstation provides simultaneous evaporation of up to 50 samples. This evaporation system offers many interchangeable tube racks giving you the flexibility for automated low volume sample preparation ranging in volumes size from 1.5 mL to 30 mL. The microprocessor-control provides monitoring of the timed operation and water bath temperature. It also provides automatic gas shutoff and operational diagnostics.

Ordering Information

ISOLUTE® SLE+ Well Plates and Columns

Description	Pack Qty.	Part Number
ISOLUTE SLE+ 200 µL Supported Liquid Extraction Plate	1	820-0200-P01
ISOLUTE SLE+ 400 µL Supported Liquid Extraction Plate	1	820-0400-P01
ISOLUTE SLE+ 200 µL Array Wells	100	820-0200-T
ISOLUTE SLE+ 400 µL Array Wells	100	820-0400-T
ISOLUTE SLE+ 400 µL Sample Volume Columns	50	820-0055-B
ISOLUTE SLE+ 400 µL Sample Volume Columns (tabless)	50	820-0055-BG
ISOLUTE SLE+ 1 mL Sample Volume Columns	30	820-0140-C
ISOLUTE SLE+ 1 mL Sample Volume Columns (tabless)	30	820-0140-CG
ISOLUTE SLE+ 2 mL Sample Volume Columns	20	820-0290-D
ISOLUTE SLE+ 5 mL Sample Volume Columns	20	820-0690-E
ISOLUTE SLE+ 10 mL Sample Volume Columns	16	820-1420-F

Array Accessories

Description	Pack Qty.	Part Number
ISOLUTE Array base plate	1	120-1000-P01
ISOLUTE base plate sealing strips (strips of 8)	50	120-1200
Luer Adaptors (to fit any vacuum manifold)	25	120-1201
Well removing tool	1	120-1202

Deep Well Collection Plates

Description	Pack Qty.	Part Number
Collection plate, 1 mL	50	121-5202
Collection plate, 2 mL	50	121-5203

Sample Processing Manifolds and Evaporation Products

Description	Part Number
VacMaster-96	
VacMaster-96 Sample Processing Manifold	121-9600
Vacuum Control Unit VCU-1	121-9601
Vacuum Control and Generation Unit VCU-2	121-9602
VacMaster-10 and -20	
VacMaster-10 Sample Processing Manifold	121-1016
VacMaster-20 Sample Processing Manifold	121-2016
Pressure+ 96 and 48	
PRESSURE + 96 Positive Pressure Manifold	PPM-96
PRESSURE + 48 Positive Pressure Manifold	PPM-48
SPE Dry 96 Sample Concentrator System	
SPE Dry 96 Sample Concentrator System 110V	SD-9600-DHS-NA
SPE Dry 96 Sample Concentrator System 220V	SD-9600-DHS-EU
SPE Dry 96 Sample Concentrator System, with with TEFLON coated needles (Top Head Only), 110V	SD-9600-DHS-T-NA
SPE Dry 96 Sample Concentrator System, with with TEFLON coated needles (Top Head Only), 220V	SD-9600-DHS-T-EU
SPE Dry 96 Dry Dual Sample Concentrator System	
SPE Dry 96 Dual Concentrator System 110V	SD2-9600-DHS-NA
SPE Dry 96 Dual Concentrator System 220V	SD2-9600-DHS-EU
SPE Dry 96 Dual Concentrator Sample Concentrator System, with TEFLON coated needles (Top Head Only), 110V	SD2-9600-DHS-T-NA
SPE Dry 96 Dual Concentrator Sample Concentrator System, with TEFLON coated needles (Top Head Only), 220V	SD2-9600-DHS-T-EU
TurboVap®	
TurboVap 96 (120 VAC)	C103263
TurboVap 96 (220 VAC)	C103264
TurboVap LV (120V)	C103198
TurboVap LV (230V)	C103199

Notes

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ordermailbox@biotage.com

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Fax: +81 3 5627 3121
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