Solid Phase Extraction using the 96-well Format ISOLUTE®-96 Fixed Well Plates and ISOLUTE Array 96-well Plates

Chemistry Data Sheet

New approaches to the quantitation of drugs and metabolites in biological fluids have increased the need for high throughput methodologies to avoid the traditional laboratory bottleneck of sample preparation. Solid phase extraction in a 96-well plate format such as ISOLUTE®-96 Fixed Well Plates or ISOLUTE Array 96-well Plates is a solution to this problem. See Figure 1 for schematic of SPE 96-well plate.

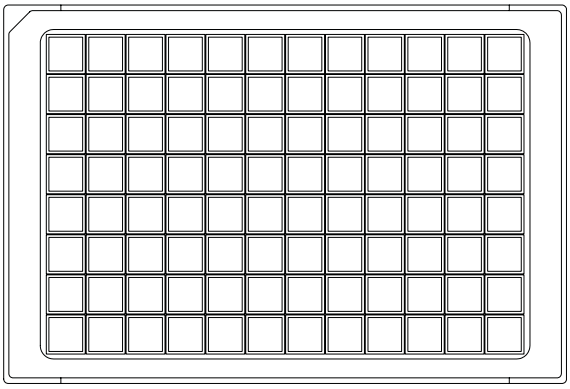

Figure 1. *Schematic of a 96-well SPE plate. Each cell represents a SPE 'column' with reservoir, sorbent bed, and outlet.*

SPE using the 96-well Format

The principles of SPE are the same, whether you are using standard format SPE columns, fixed well format ISOLUTE-96 plates, individual ISOLUTE Array columns/wells or complete (or partially complete) ISOLUTE Array plates.

The main difference between SPE in columns (or individual wells) compared to plates is that it is not possible to individually control the flow of the various solvents through the cells of the plate. When using individual columns/wells, solvent flow through the column can be controlled using stopcocks.

96-well plates are processed on a vacuum manifold that applies vacuum to the whole plate, not the individual cells. The flow of solvents is therefore not controlled for each individual well, but over the whole plate. For this reason, certain physical handling parameters should be optimized to ensure trouble-free SPE in this format.

Under optimized conditions, the variation in liquid flow across a 96-well SPE plate (either ISOLUTE-96 or ISOLUTE Array plates) has been found to be very similar to that found for the equivalent standard column format processed on a standard vacuum manifold. Typical RSDs are approximately 15% for all formats.

As well as small changes to typical operating procedures during the SPE stages, care should also be taken during post SPE manipulations (e.g. sample evaporation/concentration, reconstitution, transfer to autosampler vials etc.) to minimize any potential problems in the plate format.

It is useful to refer to individual wells by their row/column identifier (e.g. A3, H4).

Biotage

Sample Processing/liquid Handling

The speed of manually dispensing the conditioning solvent, equilibration solvent, sample, interference elution solvents and analyte elution solvent using the 96-well format can be greatly enhanced using a standard multi-port (8 or 12 position) dispenser. Solvent is dispensed simultaneously into every well of a column (i.e. A1 to H1) or row on the plate.

There are also a number of automated liquid handling systems available which are suitable for SPE use.

The Different 96-well SPE Plate Formats Available

ISOLUTE-96 Fixed Well Plates

ISOLUTE-96 fixed-well plates consist of 96 (8 x 12 matrix) fixed position wells packed with SPE sorbent – the industry standard format. The nominal volume of each well is approximately 1.2 mL, and sorbent bed masses typically range from 25–100 mg per well (10 mg for resin based sorbents).

ISOLUTE Array 96-well Plates

ISOLUTE Array 96-well Plates and SPE columns have been designed for scientists who require a versatile SPE format that is compatible with both high and low sample throughput.

The ISOLUTE Array system consists of individual SPE columns (1 or 2 mL volume, typically 10–100 mg sorbent bed mass) that can be processed using a traditional vacuum manifold (e.g. VacMaster™-10 or -20 Sample Processing Manifolds) in exactly the same way as standard 1 mL ISOLUTE SPE Columns.

Alternatively the individual ISOLUTE Array columns can be fitted into a 96 position (8 x 12) base plate for processing as a standard 96-well SPE plate using a plate format vacuum manifold (e.g. VacMaster-96 Sample Processing Manifold). The plate assembly can be complete (8 x 12 wells in place) or partially complete (any number of columns up to 96 in place). If using a partially complete ISOLUTE Array plate, the empty positions **must** be blocked off using base plate plugs.

Populating the Array Base Plate

Populate the ISOLUTE Array base plate with Array columns of the chosen configuration as follows:

- Place the base plate on the VacMaster-96 manifold lid.
- Fill positions A1 up to A12 as required, ensuring the columns are aligned correctly using the well alignment/removal tool, and press firmly into place.
- Fill positions B1 to H1 ensuring the columns are aligned correctly, and press firmly into place.
- Using the filled positions as a guide, place the remaining columns in position until the desired number of wells are filled. Using the leveling tool, press the columns firmly into place.
- Seal any remaining empty positions with strips of 8 base plate plugs.

The ISOLUTE Array plate is now ready for use.

Processing the 96-well SPE Plates

The VacMaster-96 manifold is designed for processing both the ISOLUTE-96 and ISOLUTE Array plate formats.

Six-step SPE

The most commonly used mode of solid phase extraction in bio-analytical applications is 'six-step SPE'. It can produce extracts of biological fluids (plasma, serum, urine) having high purity, and concentrated analytes (drugs and/or metabolites). This mode involves 6 steps as follows:

- Sample pre-treatment
- Solvation (conditioning)
- Equilibration
- Sample loading
- Interference elution
- Analyte elution

(A brief description of each of these steps is given below. Please request Chemistry Data Sheet **TN110** for a full description of each of these steps, and other modes of operation of SPE). Chemistry Data Sheets describing the use of specific ISOLUTE sorbents are also available.

Sample Pre-treatment

See relevant Chemistry Data Sheets for specific sorbents. As stated previously the chemistry of SPE remains the same for 96-well format.

Dilution Factors

As biological fluids (particularly plasma or serum samples) can vary significantly in their viscosity from sample to sample, it is important to use dilution to lower and standardize the viscosity of the samples, to ensure even, reproducible flow from well to well, and minimize well blockage. In general, the greater the dilution factor, the more even the results.

In order to maintain the control of both chemical and physical parameters, dilution with a suitable BUFFER is recommended. See Figure 2 for the effect of dilution on flow rate. As plasma varies in viscosity, this is a guideline to dilution only.

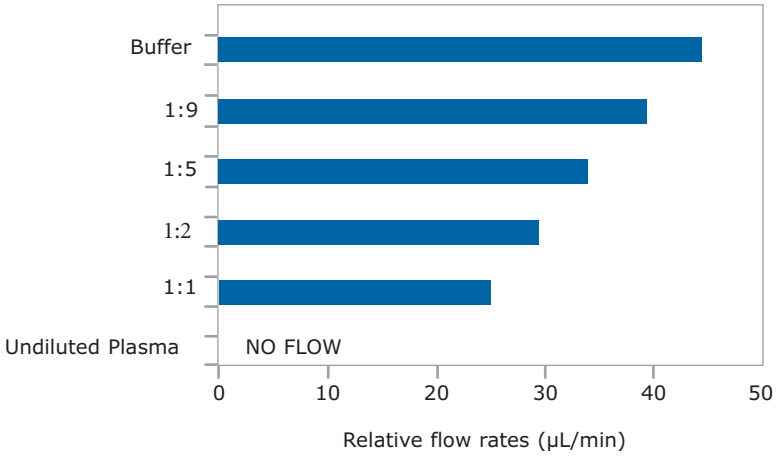

Figure 2. Flow rates of 250 µL of undiluted plasma, and plasma diluted with buffer, using 25 mg ISOLUTE Array plate

Solvation

Solvation is usually necessary to prepare the sorbent for the extraction process. For certain sorbents the sorbent bed should not be allowed to dry out after solvation.

It is recommended that wells are initially conditioned with an excess of methanol (or other suitable solvent) loaded under gravity or low vacuum (-2 "Hg). This has the effect of making the flow of subsequent solvent/sample loading steps more even across the plate.

Typical Volumes

Bed Mass	Solvation Volume
10 mg	220 µL
25 mg	500 µL
100 mg	2 mL

It has been noted that if the top frit is allowed to become dry (i.e. the level of solvation solvent drops below the surface of the frit material), it can be difficult to induce the flow of the next aqueous step using a low vacuum. **This is due to the hydrophobic nature of the frit material, and is not related to the sorbent type (applies equally to silica based and resin based sorbents).** The following procedures apply to each subsequent aqueous step of the method.

There are various procedures that can help minimize this. Processing regimes differ slightly between low bed mass (10 and 25 mg) and higher bed mass (50 and 100 mg) plates.

Processing Procedures

10 mg	In general, all steps can be loaded under gravity. Under these conditions, frits are unlikely to dry out, and flows of subsequent steps will be unaffected.
25 mg	In general, all steps can be loaded under gravity, although some variation in flow may occur under these conditions. If necessary, a low vacuum (–2 "Hg) is ample to give a good flow rate and is not high enough to dry the frits.
50 and 100 mg	For best results, approximately 5% methanol should be added to all subsequent aqueous steps (provided this does not affect analyte recovery). All wells should be loaded with no vacuum, and a pulse of high vacuum (–10 "Hg, 2 seconds) applied to initiate flow. The vacuum should then be reduced to –2 "Hg.

In general we recommend that as long as sufficient capacity is obtained from a 10 or 25 mg bed mass, that this configuration be the bed mass of choice for 96-well plate based SPE methods.

- If the subsequent step has a high % organic component, the hydrophobicity of the frit will be overcome by the solvent, and flow should occur without problems.
- For ISOLUTE Array plates, it is possible to optimize vacuum levels on individual wells on a standard vacuum manifold (eg VacMaster-10 or -20 manifold), and transfer directly to the plate for increased throughput.

Equilibration

To maximize retention of the analyte by the sorbent, the column is treated with a solvent that is as 'matrix-like' as possible. A typical volume of equilibration solvent is 0.2–1 mL/50 mg of sorbent.

Sample Loading

Load the diluted sample as follows:-

10 mg	Load under gravity
25 mg	Load under gravity, **OR** apply a low vacuum of –2" Hg, either during or after sample application
50 and 100 mg plate	Apply a low vacuum of –2" Hg, either during or after sample application, **OR** load sample into all wells with no vacuum, followed by a pulse of –5 to –10 "Hg, reduced to –2" Hg after 2 seconds

Interference Elution

The purpose of interference elution is to selectively remove undesired compounds from the sorbent without eluting the analytes. A typical volume of interference elution solvent is 0.2–1 mL /50 mg of sorbent. Interference elution should be carried out under minimum vacuum.

Sorbent drying may be necessary to remove excess solvent prior to elution. In the plate format, it is not easy to visually judge whether an individual well is dry. Any drying regime therefore should be clearly specified in terms of drying time and vacuum required.

A typical drying time for subsequent elution with water miscible solvents is about 30 seconds per 10 mg sorbent at -10 "Hg. If a water immiscible solvent is used for elution, drying times would be significantly longer. We recommend that the optimum conditions be determined experimentally for individual applications.

Elution

Prior to elution, ensure that the collection plate has been inserted into the VacMaster-96 manifold, in the correct alignment (i.e. position A1 on the extraction plate is aligned directly above position A1 on collection plate). This will ensure that individual samples are not confused.

Replace the polypropylene lid of the VacMaster-96 manifold and ISOLUTE-96 or ISOLUTE Array plates. Ensure that the extraction plate outlets are correctly positioned, and inserted into the collection plate wells. Spacers are available to raise collection plates, and ensure penetration. This is important to prevent cross contamination between wells.

Apply the elution solvent and allow to flow under suitable vacuum conditions as appropriate to bed mass. For all bed masses, a pulse of vacuum at the end of elution will ensure that all elution solvent is collected.

Minimum elution volumes as low as 100 µL for 25 mg sorbent mass may be achieved with robust/optimized SPE methods. Flow control is important to ensure reproducibility.

Post SPE Sample Manipulation

When using either ISOLUTE-96 or ISOLUTE Array plates, the analytes are eluted into a collection plate (consisting of an 8 x 12 matrix of collection wells). These plates are available in 1 and 2 mL volumes as standard. The collection plate can be sealed for storage purposes if analysis is not to be performed immediately.

Direct Injection

To minimize sample manipulation following SPE, analytes should be eluted using a solvent system that is directly compatible with the analytical technique. If the elution solvent is too strong for compatibility with the analytical technique, and further concentration is not required, the eluent should be diluted with mobile phase buffer.

The collection plate can then be placed directly onto a 96-position autosampler.

Eluent Evaporation/Concentration

Depending on the analytical technique/methodology to be used to analyze the extracted samples, it may be necessary to further concentrate, or evaporate and reconstitute the eluent, prior to the analytical stage.

Using a 96-well sample concentrator (e.g. SPE Dry™ 96), this step can be performed in the collection plate. In the plate format, it is not easy to visually judge whether an individual well is dry, or what volume of solvent remains. Any drying regime therefore should be clearly specified in terms of instrument, temperature, drying time and gas type and flow rate.

See **page 7** for solvent evaporation times using the SPE Dry 96 system.

SPE Dry 96 solvent evaporation times

Solvent	* 500 µL/40 ˚C	** 1 mL/40 ˚C	500 µL/60 ˚C	** 1 mL/40 ˚C
Water	90	165	46	86
Methanol	14	28	11	20
Methanol/Water 50/50 (v/v)	46	95	28	56
Propan-2-ol	16.5	26	11.5	16.5
Acetonitrile/Water 50/50 (v/v)	51	60	31	36
Methanol/Acetonitrile 50/50 (v/v)	19	24	12	15
Acetonitrile	17	20	12	15.5
Methanol/1% (v/v) NH_4OH	18	28	13.5	16
DMF	73	105	33	41
Methanol/Methylene Chloride 50/50 (v/v)	12	15.5	7.5	9.5
Methylene Chloride/1% (v/v) NH_4OH	8.5	11.5	6	8

** 500 µL volumes were in a 1 mL collection plate*
*** 1 mL volumes were in a 2 mL collection plate*
1. Experiments were conducted with flow rate at 50 L/min at the upper manifold and 30 L/min at the lower manifold.
2. Drying gas used was compressed air. Only standard moisture trap was used within the system. Moisture content of ambient air will affect results.
3. All temperatures listed in above table refer to the top head. Bottom unit was 20 °C higher.

Typical evaporation times (minutes) for commonly used solvents. Forced gas convection heating from above and below each microplate well evaporates solvents up to five times more rapidly than other microplate sample evaporation systems.

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