# EVOLUTE® User Guide




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# EVOLUTE® SPE Products from Biotage®

| | |
|---|---|
| **EVOLUTE ABN** | for simultaneous extraction of acidic, basic and/or neutral analytes |
| **EVOLUTE CX** | for extraction of basic analytes |
| **EVOLUTE WCX** | for extraction of strongly basic analytes |
| **EVOLUTE AX** | for extraction of acidic analytes |
| **EVOLUTE WAX** | for extraction of strongly acidic analytes |

Whether working in the field of bio-analysis in drug development, clinical or forensic analysis, or with the diversity of samples in food safety and environmental applications, the tools and methods used for sample preparation have a clear impact on productivity during method development, validation and routine analysis. EVOLUTE SPE sorbents and extraction methodologies have been developed to extract a wide range of analytes while reducing or eliminating matrix components that can cause ion suppression in LC-MS analysis.


## Faster method development – the EVOLUTE approach

- Faster method development using well tested generic methods
- Method validation – easier validation through matrix effects reduction / elimination
- Routine analysis – less down time through consistent elimination of matrix components

In developing the EVOLUTE SPE family, Biotage's goal has been to improve productivity in method development with a combination of sorbents and methods that can be applied to as wide a range of analytes as possible, and at the same time reduce or eliminate unwanted matrix components from the final extracts.

EVOLUTE Express products combine the powerful EVOLUTE chemistry with optimized features which can further enhance productivity by eliminating the need for some traditional steps in the SPE procedure.

**Part 1** of this guide describes detailed methods for use with each EVOLUTE sorbent. These generic methods have been extensively tested to provide a great starting point for SPE method development for a wide range of analytes of varying functionality. The optimized methods contain steps that ensure high analyte recoveries, while minimizing the presence of unwanted endogenous sample components. Practical hints and tips from Biotage's R&D laboratories are also included.

**Part 2** outlines the use of the sorbent selection plate to identify the best solution to complex sample preparation problems such as extraction of unknowns, peptides, etc. Once the most promising sorbent has been identified, the method can be optimized if required using the methods described in part 1.

Ordering Information — see pages 36–38

## An Introduction to the EVOLUTE SPE Family Chemistry

EVOLUTE sorbents have been developed to provide robust, reliable SPE. They are based on a modified non-polar polystyrene-divinylbenzene polymer which incorporates polar hydroxyl groups. These non-ionizable hydroxyl groups ensure that the polymer is both water wettable, and also able to extract a diverse range of analytes through non-polar (van der Waals) interactions. No secondary interactions exist, so EVOLUTE retention and elution characteristics are completely predictable and extractions on these robust sorbents are not affected by drying of the sorbent bed during sample processing.

EVOLUTE SPE sorbents are manufactured with a very narrow pore size distribution (**Figure 1**), tailored to eliminate the co-extraction of plasma proteins and provide cleaner extracts for analysis. This pore size optimization dramatically reduces protein concentration in biological fluids extracts (**Figure 2**).

Native serum contains many protein bands when stained with Coomassie blue. When a sample of native serum is passed through an EVOLUTE ABN column and the extract is examined by gel electrophoresis, these protein bands are virtually eliminated. This demonstrates the ability of EVOLUTE products to remove protein contaminants from your sample.

Removal of proteins from sample extracts avoids transfer into the analytical system, reducing matrix effects in LC-MS analysis. Additionally increased backpressure in UPLC systems due to protein contamination is eliminated, avoiding the need for column back flushing and frequent replacement of guard columns.

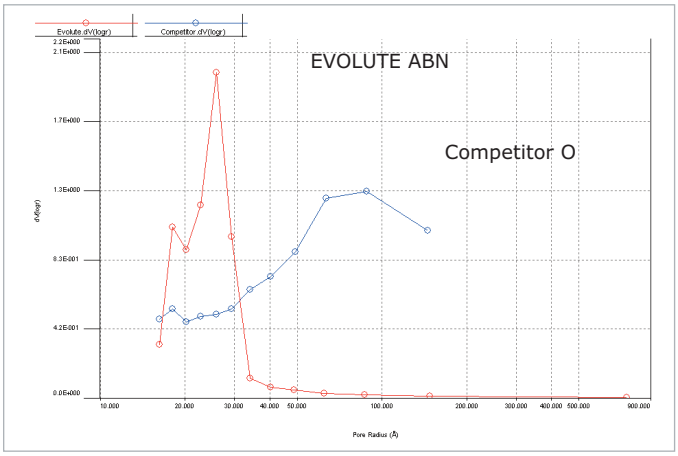



**Figure 1.** Pore size distribution of EVOLUTE sorbents compared with a competitor polymer based SPE column.

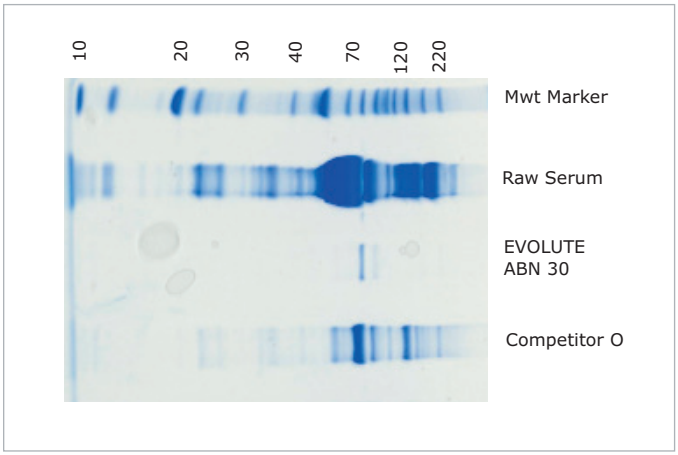



**Figure 2**. Gel electrophoresis showing protein removal from serum using the EVOLUTE ABN generic method.

## EVOLUTE ABN for clean extracts of acidic, basic and neutral analytes

EVOLUTE ABN consists of a modified polystyrene-divinylbenzene polymer for reversed phase (hydrophobic) retention, incorporating non-ionizable hydroxyl groups (**Figure 3**) which impart excellent wettability without secondary interactions. EVOLUTE ABN can be used to extract a wide range of acidic, basic and neutral analytes from aqueous matrices including biological fluids.

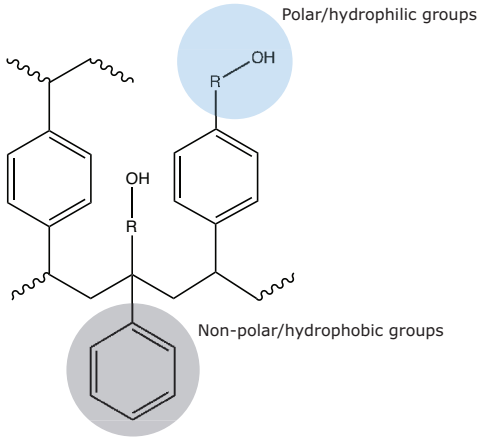



**Figure 3.** Structure of EVOLUTE ABN sorbent

## Mixed–Mode SPE for Additional Selectivity and Extract Cleanliness

To extend analyte selectivity and extract cleanliness beyond that achievable with EVOLUTE ABN, EVOLUTE mixed-mode sorbents are available. EVOLUTE mixed-mode sorbents consist of the EVOLUTE polymer 'backbone' modified with ionic functional groups. Mixed-mode SPE utilizes a dual retention mechanism to provide additional selectivity and clean-up for complex samples. The mixed-mode sorbents are primarily intended for the extraction of ionizable analytes. These mixed-mode sorbents can also be used for the extraction of non-ionizable analytes through retention of ionizable matrix components on the sorbent bed. Mixed-mode EVOLUTE SPE sorbents are:

### EVOLUTE CX

Combines non-polar and strong[1] cation exchange functionality ($-SO_3^-$ , always fully ionized) for extraction of basic analytes from aqueous samples. Exchange capacity ~0.5 mmol/g.

### EVOLUTE WCX

Combines non-polar and weak[2] cation exchange functionality ($-COOH/-COO^-$, $pK_a$~5) for extraction of strongly basic analytes from aqueous samples. Exchange capacity ~0.4 mmol/g.

### EVOLUTE AX

Combines non-polar and strong[1] anion exchange functionality ($-N(CH_3)_3^+$, always fully ionized) for extraction of acidic analytes from aqueous samples. Exchange capacity ~0.7 mmol/g.

### EVOLUTE WAX

Combines non-polar and weak[2] anion exchange functionality ($-NH_2/NH_3+$) $pK_a$~10), for extraction of strongly acidic analytes from aqueous samples. Exchange capacity ~0.3 mmol/g.

[1]Strong ion exchange sorbents (anion or cation) carry a permanent charge, under all pH conditions.
[2]Weak ion exchange sorbents carry a charge which can be eliminated by changing pH.

The dual retention mechanism exhibited by mixed-mode sorbents allows the use of 100% organic solvent in the interference wash step thus removing problem interferences, without reducing analyte recovery (**Figure 4**).

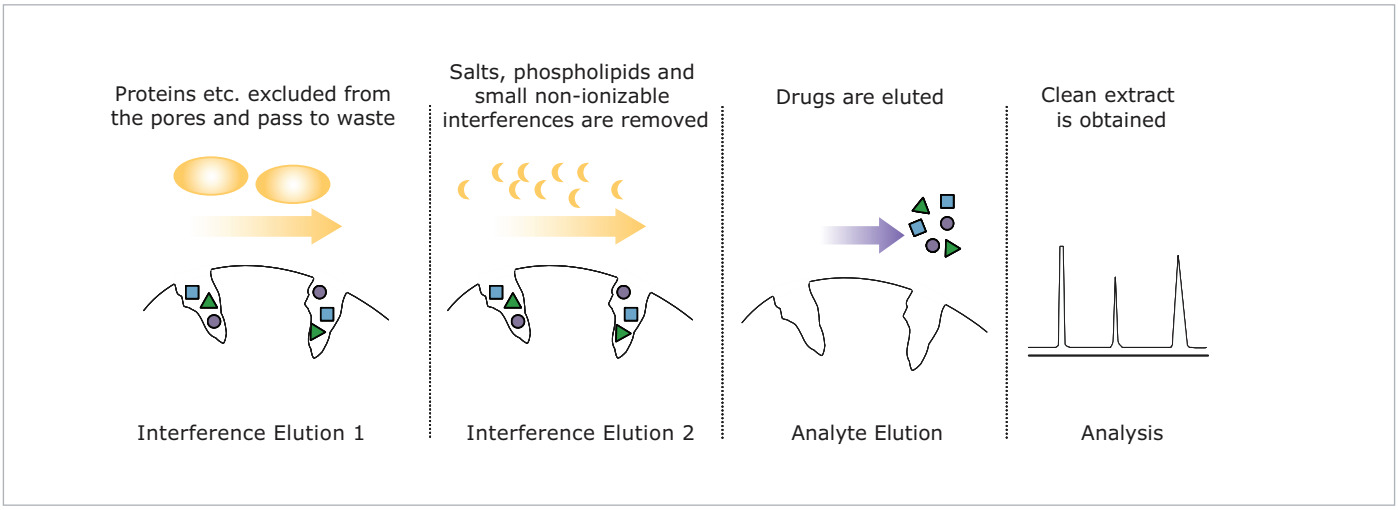


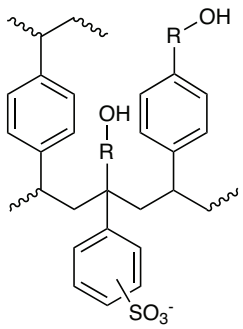

**Figure 4.** SPE using EVOLUTE mixed-mode sorbents

For extraction of **basic** analytes, two options are available:

**EVOLUTE CX** is modified with sulfonic acid groups. This negatively charged sorbent retains basic analytes through non-polar and strong **C**ation e**X**change retention mechanisms. Analyte elution is achieved at high pH by eliminating the positive charge on the ANALYTE.

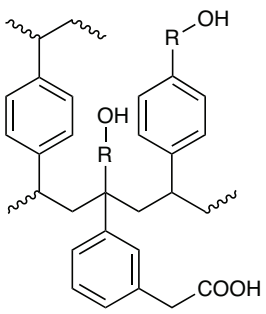
**EVOLUTE WCX** is modified with carboxylic acid groups. This sorbent retains basic analytes through non-polar and **W**eak **C**ation e**X**change retention mechanisms. Analyte elution is achieved at low pH by eliminating the negative charge on the SORBENT.

**EVOLUTE CX**

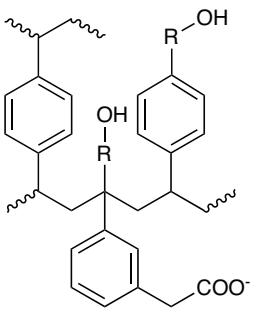



All pH conditions

**EVOLUTE WCX**




Low pH




High pH

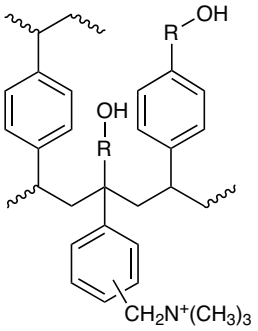
**Figure 5.** EVOLUTE mixed-mode cation exchange sorbents for extraction of basic analytes

For extraction of **acidic** analytes, two options are available:

**EVOLUTE AX** is modified with quaternary amine groups. This positively charged sorbent retains acidic analytes through non-polar and strong **A**nion e**X**change retention mechanisms. Analyte elution is achieved at low pH by eliminating the negative charge on the ANALYTE.

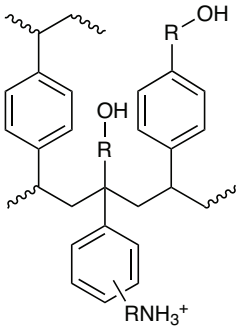
**EVOLUTE WAX** is modified with amino groups. This sorbent retains acidic analytes through non-polar and **W**eak **A**nion e**X**change retention mechanisms. Analyte elution is achieved at high pH by eliminating the positive charge on the SORBENT.

**EVOLUTE AX**

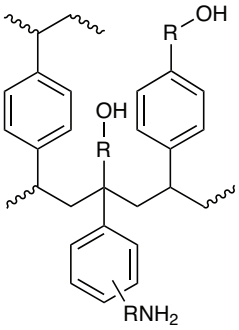



All pH conditions

**EVOLUTE WAX**




Low pH




High pH

**Figure 6.** EVOLUTE mixed-mode anion exchange sorbents for extraction of acidic analytes

## Strong vs. Weak Ion Exchange Functionality

Strong ion exchange sorbents retain a permanent charge across the pH range, whereas weak ion exchange sorbents can be ionized or neutral, depending on the pH conditions. Many ionizable analytes can be successfully extracted using either weak or strong ion exchange sorbents. Analyte $pK_a$ can predict the sorbent options available using the 2 pH unit rule (see appendix 1).

**EVOLUTE CX** has strong cation exchange functionality due to its $-SO_3^-$ group. This has a permanent negative charge under all pH conditions, and can be used to extract a wide range of charged basic/cationic species. However, strong bases (quats) are irreversible bound to the sorbent, and cannot be eluted using pH control.

**EVOLUTE WCX** has weak cation exchange functionality, due to its carboxylic acid group. This has $pK_a$ of ~5, so at pH of 7 and above, 100% of the WCX sorbent is negatively charged, whereas at pH of 3 and below, 100% of the sorbent is neutralized. It too can be used to extract a wide range of charged basic/cationic species, including strong bases or quaternary amines, as they can be eluted under low pH conditions. This is illustrated in **Figure 5**.

**EVOLUTE AX** has strong anion exchange functionality due to its $-N(CH_3)_3^+$ group. This has a permanent positive charge under all pH conditions and can be used to extract a wide range of acidic/anionic species. However, strong acids such as sulfonic acids are irreversibly bound to the sorbent, and cannot be eluted using pH control.

**EVOLUTE WAX** has weak anion exchange functionality, due to its amine group. This has a $pK_a$ of approximately 10, so at pH of 8 and below, 100% of the sorbent is positively charged, whereas at pH of 10 and above it is neutralized. It too can be used to extract a wide range of acidic/anionic species, including strong acids, as they can be eluted under high pH conditions. This is illustrated in **Figure 6**.

The control of pH during the sample loading and interference elution steps is particularly important for the weak ion exchangers. For example, when the pH equals the $pK_a$, only 50% of the sorbent carries a charge (half the ion exchange capacity).

Weak cation exchange sorbents have greatest capacity at pH 2 units above $pK_a$ (pH 7 and above for EVOLUTE WCX). Weak anion exchangers have greatest capacity at 2 pH units below $pK_a$ (pH 8 or below for EVOLUTE WAX).

Other factors to consider when choosing between weak and strong ion exchange sorbents include analyte stability at the pH of the generic method elution solvents as well as compatibility of elution solvents with the analytical method. For example, if a basic analyte is not stable under the high pH conditions of the EVOLUTE CX sorbent, the use of EVOLUTE WCX and the associated acidic elution solvent can be considered for many basic compounds.

## Fractionation of Complex Mixtures Using EVOLUTE Mixed-Mode Sorbents

Mixed-mode sorbents can also be used to fractionate complex mixtures. For example, for a sample containing a mixture of acidic, neutral and basic analytes, extracted on EVOLUTE CX, wash 2 can be used to elute acidic and neutral compounds, with analytes eluted in the analyte elution step.

# EVOLUTE SPE Products from Biotage


## EVOLUTE SPE Formats

EVOLUTE SPE sorbents are available in a variety of formats to match a wide range of application requirements, from 96-well plates to 6 mL columns.

EVOLUTE EXPRESS 96-well plates, available packed with 10 mg or 30 mg of all EVOLUTE sorbents, are ideal for high throughput extraction of biological fluid samples. The plates process plasma, serum and urine samples at fast flow rates, with excellent flow consistency from well to well. Well clogging is eliminated, leading to high analyte recoveries and reduced variability.

EVOLUTE EXPRESS plates can be processed under vacuum or positive pressure conditions, and are compatible with leading automated sample preparation platforms.

## Load-Wash-Elute Procedure

For many applications, EVOLUTE EXPRESS plates can save time and increase productivity by eliminating the need for the conditioning and equilibration steps required in traditional SPE. All the generic methods in this user guide indicate where steps may be omitted during method development when using EVOLUTE EXPRESS plates.

EVOLUTE EXPRESS plates containing 10 mg sorbent can be eluted with as little as 100–200 µL of solvent for many applications, further increasing productivity by avoiding the need for evaporation prior to analysis.

Identification of the best sorbent for a particular application is simple and fast using the [screening plate], and methods can then be optimized using the information in this guide.

For higher volume samples, EVOLUTE sorbents are available in a range of bed masses and columns sizes. Sorbent particle size is tightly controlled to ensure trouble free processing on vacuum or positive pressure manifolds, and automated sample preparation instruments.

## Method Development

EVOLUTE SPE products and methods can be used to extract any of the following compound classes, either singly or as multiple analytes, from aqueous samples using either non-polar (hydrophobic) or mixed-mode SPE.

- Neutral
- Weakly acidic ($pK_a$ 2–8)
- Strongly acidic ($pK_a$<2)
- Weakly basic ($pK_a$ 2–10)
- Strongly basic ($pK_a$>10)
- Amphoteric (with both acidic and basic groups)
- Mixtures of acids, bases and neutral
- Fractionation of acidic, neutral and basic compounds

The EVOLUTE family offers a full range of SPE options, with sorbents and methodologies optimized for different sample preparation solutions. A summary of the functionality of the EVOLUTE family is shown in **Table 1**.

| EVOLUTE Sorbent | Functionality | Non-polar | Strong cation exchange | Weak cation exchange | Strong anion exchange | Weak anion exchange |
|---|---|---|---|---|---|---|
| EVOLUTE ABN | Non-polar | ● | | | | |
| **Mixed-mode sorbents** | | | | | | |
| EVOLUTE CX | Non-polar and $-SO_3^-$ | ○ | ● | | | |
| EVOLUTE WCX | Non-polar and $-COO^-$/-COOH | ○ | | ● | | |
| EVOLUTE AX | Non-polar and $-N(CH_3)_3^+$ | ○ | | | ● | |
| EVOLUTE WAX | Non-polar and $-NH_3$+/$-NH_2$ | ○ | | | | ● |

**Table 1.** Summary of EVOLUTE family functionality ● primary interaction ○ secondary interaction

Successful sample preparation depends on the correct choice of sorbent and methodology for the application. There is often more than one option for a particular analyte or group of analytes. Biotage provide simple guidelines based on analyte and sample clean up needs to allow selection of the correct sorbent and methodology to meet your sample preparation requirements.

# EVOLUTE Method Development

When characterizing a sample preparation problem, it is worth considering both analyte and matrix factors as well as other practical components such as the number of samples to be run.

For analytes with some **hydrophobic (non-polar)** functionality (whether acidic, basic or neutral), a sorbent with a single non-polar retention mechanism such as **EVOLUTE ABN** is ideal for extraction from aqueous samples. Depending on the degree of hydrophobic character, and complexity of the matrix, the method can be optimized to reduce the amount of co-extracted matrix components.

The generic EVOLUTE ABN method has been optimized to simultaneously extract a broad range of acidic, basic and neutral analytes.

For **acidic** or **basic** analytes containing ionizable functional groups, **mixed-mode** sorbents combining non-polar and ion exchange retention mechanisms can provide additional selectivity and clean up. This approach is particularly useful for extractions from biological fluids, as the dual retention mechanism allows strong interference elution solvents to be used, which eliminate phospholipids and other unwanted co-extracted species from the final extract.

## Basic Analytes

**For basic analytes**, two mixed-mode (non-polar plus cation exchange) options are available. For extraction of basic analytes with $pK_a$ 2–10, **EVOLUTE CX** combines non-polar with strong cation exchange retention mechanisms. EVOLUTE CX is also recommended for extraction of amphoteric species, and fractionation of complex mixtures. **For strongly basic analytes** (quaternary amines or $pK_a$>10) **EVOLUTE WCX** should be used.

## Acidic analytes

**For acidic analytes** two mixed-mode (non-polar plus anion exchange) options are available. For acidic analytes with $pK_a$ 2–8, **EVOLUTE AX** combines non-polar with strong anion exchange retention mechanisms. For strongly acidic analytes ($pK_a$<2) **EVOLUTE WAX** should be used.

**Table 2.** summarizes the EVOLUTE sorbent choices based on analyte functionality. Robust generic methodology is available for each sorbent to facilitate rapid method development (see pages 11–23).

| | EVOLUTE sorbent selection | | | | |
|---|---|---|---|---|---|
| Analytes | **ABN** page 11 | **CX** page 15 | **WCX** page 17 | **AX** page 20 | **WAX** page 22 |
| Neutral | ● | | | | |
| Weakly acidic $pK_a$ 2–8 | ○ | | | ● | |
| Strongly acidic $pK_a$<2 | ○ | | | | ● |
| Weakly basic $pK_a$ 2–10 | ○ | ● | | | |
| Strongly basic $pK_a$>10 | ○ | | ● | | |
| Amphoteric | ○ | ● | | ● | |
| Acidic, neutral, basic mix | ● | | | | |
| Fractionation acidic/neutral/basic | | ● | | | |

**Table 2.** EVOLUTE sorbent selection ● First choice ○ Alternative

Once the most appropriate sorbent is selected, methods can be further optimized using the practical hints and tips described for each sorbent type.

# EVOLUTE ABN

## Optimized Method Development Using EVOLUTE ABN

EVOLUTE ABN is designed to extract a diverse range of acidic, basic or neutral analytes from aqueous samples using a robust hydrophobic (non-polar or reversed phase) retention mechanism. No secondary interactions are present, ensuring that retention and elution can be achieved using simple solvent systems to provide high analyte recovery. Additionally, the narrow pore size distribution eliminates proteins from the extract when extracting biological samples (see **Figure 7**).

## When to use EVOLUTE ABN

- When a simple, fast, robust SPE method is required
- For complex samples (e.g. biological fluids)
  - Neutral analytes
  - Mixtures of acids, bases and neutrals
- Clean samples (e.g. drinking water)
  - Multi analyte suites
  - Acidic, Basic or neutral analytes,
- When analyte stability issues mean it is necessary to avoid high or low pH conditions
- For simple solvent exchange procedures (from aqueous to organic solvent)

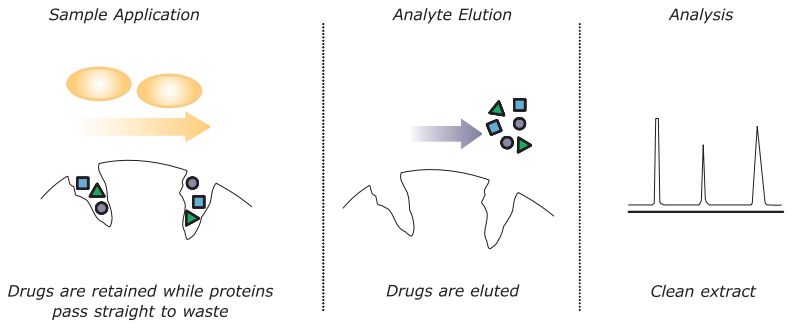



**Figure 7.** Retention and elution using EVOLUTE ABN.

**EVOLUTE ABN Generic Method for Combined Extraction of Acidic, Basic and Neutral Analytes from Biological Fluids.**

| Step | Conditions | Effect |
|---|---|---|
| Pre-treatment | Dilute sample 1:3 (v/v) with 2% formic acid | Reduces or eliminates protein binding |
| *Conditioning | Methanol (1 mL) | |
| *Equilibration | 0.1% formic acid (1 mL) | For acid labile compounds, this method can be run using neutral or basic pH conditions |
| Sample load | 400 µL–2 mL diluted plasma | |
| Interference wash | Water/methanol (95/5, v/v, 1 mL) | Removes polar interferences (salts, proteins and larger phospholipids). |
| Elution | Methanol (500 µL) | Elutes analytes |
| Evaporate and re-constitute as necessary for analysis | | |

Volumes suitable for 25/30 mg formats, see table 4 on page 24 for guidelines for other formats.
*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

## Optimizing Phospholipid Removal Using EVOLUTE ABN

Whilst polymer-based SPE products that utilize a single non-polar (hydrophobic) retention mechanism are extensively used for the extraction of drugs from biological fluids, the non-selective nature of these polymers can lead to co-extraction of high levels of unwanted endogenous sample components such as phospholipids.

The retentive nature of phospholipids makes them a common challenge in analytical sample preparation for LC–MS. Unless removed at the sample preparation stage, phospholipid species can co-elute with analytes of interest, causing ion suppression or enhancement in the analytical system. The EVOLUTE ABN generic method removes greater than 90% of larger molecular weight phospholipids (PLs) but only approximately 35% of lysophospholipids (Lyso PLs) compared with protein precipitation.

Modification of the generic method, depending on analyte properties, can significantly improve removal of phospholipids and result in cleaner extracts and more reliable quantification.

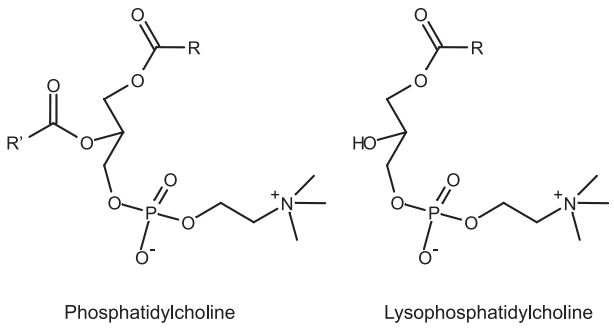



**Figure 8**. Typical Phospholipid Structures

### Case A: For non-polar (hydrophobic) analytes

When analytes of interest are strongly retained on the SPE sorbent, increasing the strength of the interference elution solvent can significantly reduce the phospholipid content of the extract, without affecting analyte recovery. Using an interference wash of 60/40 (v/v) water/acetonitrile removes approx. 95% of PLs and lysoPLs from the final extract. This is illustrated in **Figure 9**.

| Case A: EVOLUTE ABN Generic Method Modified for Reduction of Phospholipids in the Final Extract when Analytes are Strongly Retained (i.e. non-polar analytes) | | |
|---|---|---|
| **Step** | **Conditions** | **Effect** |
| Pre-treatment | Dilute sample 1:3 (v/v) with 2% formic acid | Reduces or eliminates protein binding |
| *Conditioning | Methanol (1 mL) | |
| *Equilibration | 0.1% formic acid (1 mL) | For acid labile compounds, this method can be run using neutral or basic pH conditions |
| Sample load | 400 µL–2 mL diluted plasma | |
| Interference elution | Water/acetonitrile (60/40, v/v, 1 mL) | Removes polar interferences (salts, proteins plus 94% of PLs and 96% of lysoPLs) |
| Analyte elution | Methanol (500 µL) | Elutes analytes |
| Evaporate and re-constitute as necessary for analysis | | |

Volumes suitable for 25/30 mg formats, see table 4 on page 24 for guidelines for other formats.
*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

### Case B: For polar analytes

When analytes are weakly retained on the sorbent, modifying the elution solvent to incorporate more than 20% water yields the greatest reduction in phospholipid contamination without affecting analyte recovery. This is illustrated in **Figure 9**.

| Case B: EVOLUTE ABN Generic Method modified for Reduction of Phospholipids in the Final Extract when Analytes are Weakly Retained (i.e. polar analytes) | | |
|---|---|---|
| **Step** | **Conditions** | **Effect** |
| Pre-treatment | Dilute sample 1:3 (v/v) with 2% formic acid | Reduces or eliminates protein binding |
| *Conditioning | Methanol (1 mL) | |
| *Equilibration | 0.1% formic acid (1 mL) | For acid labile compounds, this method can be run under neutral or basic pH conditions |
| Sample load | 400 µL–2 mL diluted plasma | |
| Interference elution | Water/methanol (95/5, v/v, 1 mL) | Removes polar interferences (salts, proteins and larger phospholipids) |
| Analyte elution | Methanol/water (80/20, v/v, 500 µL) | Elutes analytes, reduces LysoPLs in the final extract by leaving them on the column |
| Evaporate and re-constitute as necessary for analysis | | |

Volumes suitable for 25/30 mg formats, see table 4 on page 24 for guidelines for other formats.
*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

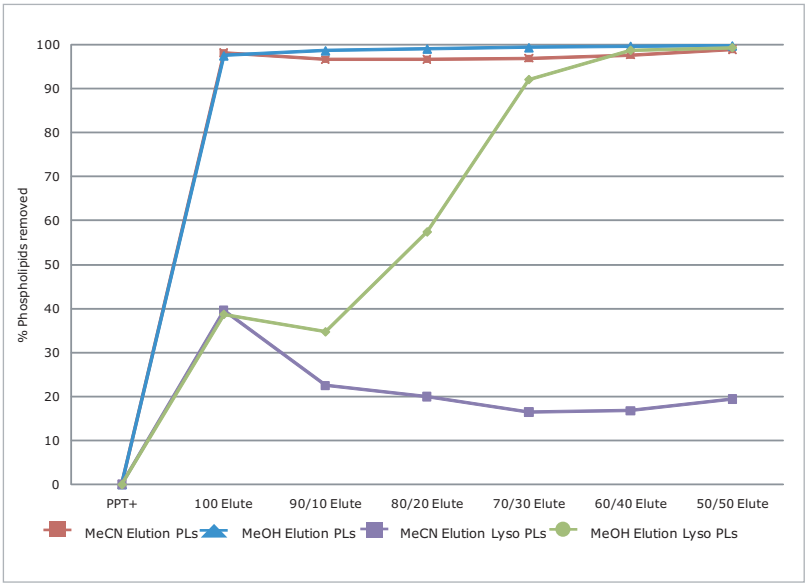



**Figure 9.** Effect of different solvent combinations on phospholipid content of extracts. A wash of 60:40 (v/v) acetonitrile:water will remove 94% of PLs and 96% of lysoPLs from the final extract (case A). Elution using 80:20 (v/v) methanol:water reduced lyso PL concentration in the final extract by 50% (case B).

## More Method Optimization Tips for EVOLUTE ABN

| | |
|---|---|
| **To improve retention of polar or weakly retained acidic analytes during sample loading and interference elution steps:** | Ensure sample pH is 2 pH units[2] below the $pK_a$ of the analyte. Evaluate the use of buffer at this pH for equilibration and interference elution steps. |
| **To improve elution of acidic analytes:** | Analyte elution depends on analyte solubility in the elution solvent. Addition of a volatile acid can maximize analyte recovery. Acidic analytes will have greater solubility at 2 pH units[2] below the $pK_a$ of the analyte. Evaluate the use of up to 0.1% formic acid in methanol. |
| **To improve retention of polar or weakly retained basic compounds during the sample loading and interference elution steps:** | Ensure sample pH is 2 pH units[2] above the $pK_a$ of the analyte. Evaluate the use of buffer at this pH for equilibration and interference elution steps. |
| **To improve elution of basic compounds:** | Analyte elution depends on analyte solubility in the elution solvent. Addition of a volatile base to the elution solvent can maximize analyte recovery. Basic analytes will have greater solubility at 2 pH units[2] above the $pK_a$ of the analyte. Evaluate the use of up to 5% ammonia in methanol. |
| **Viscous samples**: | Additional dilution may be required to improve flow characteristics of particularly viscous samples. |
| **Acid labile compounds:** | For biological fluid samples containing high levels of endogenous proteins, such as plasma, the use of formic acid in the pre-treatment can improve recovery of strongly protein bound analytes. **However, care should be taken when extracting acid labile compounds (e.g. lactones) if using this approach.** If desired, a neutral sample pH can also be used. We recommend 0.1 M ammonium acetate for sample dilution. If buffering of urine samples is necessary, use a lower ionic strength buffer (20–50 mM) for sample pre-treatment and column equilibration. |

[2]See appendix 1 for more detailed explanation of the 2 pH unit rule

# EVOLUTE CX

## Optimized Method Development for Basic Analytes Using EVOLUTE CX

EVOLUTE CX is designed to extract basic analytes from biological fluids and other aqueous samples using mixed-mode non-polar/strong cation exchange retention mechanisms. The sorbent consists of the EVOLUTE backbone surface modified with a sulfonic acid functional group (0.5 mmol/g capacity) (see **Figure 10**). By using both non-polar and strong cation exchange retention mechanisms, basic analytes are selectively retained. The simple wash steps in the EVOLUTE CX generic method remove matrix components such as salts, non-ionizable interferences, proteins and phospholipids. In fact EVOLUTE CX removes greater than 98% of both proteins and phospholipids from plasma samples.

### When to use EVOLUTE CX

- Basic analytes ($pK_a$ 2–10)
- Basic compounds that are too polar for a single non-polar retention mechanism using EVOLUTE ABN
- Amphoteric analytes
- Fractionation of complex mixtures (separates acidic and neutrals from bases)
- For analytes stable in high pH elution solvent
- To selectively retain basic interferences

**EVOLUTE CX** combines non-polar and strong cation exchange in a mixed-mode retention mechanism for improved recovery of basic compounds and cleaner extracts. Optimal retention is obtained at least 2 pH units below the $pK_a$ of the analyte.

Analyte elution is achieved by eliminating analyte charge i.e. by using a high pH.

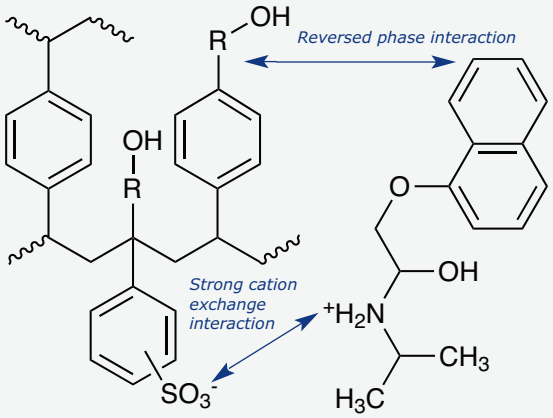



**Figure 10.** Structure of EVOLUTE CX with interactions

| EVOLUTE CX Generic Method for Extraction of Basic Analytes from Biological Fluids. | | |
|---|---|---|
| **Step** | **Conditions** | **Effect** |
| Pre-treatment | Dilute sample 1:3 (v/v) with 0.05M ammonium acetate pH 6.0. **For protein bound drugs in plasma or serum, dilute with 2% formic acid** | Reduces or eliminates protein binding |
| *Conditioning | Methanol (1 mL) | |
| *Equilibration | 0.05M ammonium acetate pH 6.0 (1 mL) | |
| Sample load | 400 µL–2 mL diluted plasma | Analyte retained by hydrophobic interaction |
| Interference elution 1 | 0.05M ammonium acetate pH 6.0 (1 mL) | Removes polar interferences (salts, proteins and larger phospholipids) |
| Interference elution 2 | Methanol (1 mL) | Analyte retained by cation exchange interactions. Removes remaining PLs plus neutral and acidic interferences. Alternatively, collect to analyze acidic and neutral compounds of interest. |
| Analyte elution | Methanol /ammonium hydroxide (95/5, v/v) (500 µL–1 mL) | Elutes basic analytes by eliminating ANALYTE charge |
| Evaporate and re-constitute as necessary for analysis | | |

Volumes suitable for 25/30 mg formats, see table 4 on page 24 for guidelines for other formats.
*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

### Reagents

- 0.05M ammonium acetate pH 6.0. Dissolve 3.854 g of ammonium acetate in 950 mL of deionized water. Adjust to pH 6.0 with acetic acid (ACS reagent grade). Make up to 1 L with deionized water and mix thoroughly.
- Methanol /ammonium hydroxide (95/5, v/v) solution. Take 5 mL of ammonium hydroxide (28%) and add 95 mL methanol. Mix thoroughly.

## Method Optimization Tips for EVOLUTE CX

| | |
|---|---|
| **Amphoteric compounds.** For optimum cleanliness without compromising analyte recovery of amphoteric compounds with dual carboxylic acid/amine functionality (e.g. benzoylecgonine): | Use an additional wash using 2% formic acid after interference wash 1. This step ionizes the amine while neutralizing the acid functionality ensuring a true cation exchange effect occurs. |
| **Polar basic drugs:** For optimum cleanliness without compromising analyte recovery of polar basic drugs with $pK_a$~8 and above: | Use an additional wash using 2% formic acid after interference wash 1. This step removes lysophospholipids and ensures that the amine functionality is "locked on" such that analyte/s is not eluted in the subsequent methanol wash. |
| **For whole blood samples:** | Pre-treat the sample by dilution with 0.05M ammonium acetate, pH6, followed by sonication and centrifugation, to denature (lyse) the blood. Do not use 2% formic acid, as this can cause discoloration of the sample. |
| **For very polar basic analytes:** | **To enhance recoveries of very polar basic analytes, where 2% formic acid pre-treatment cannot be used due to the nature of the matrix** (e.g. milk or whole blood), a buffer of intermediate pH e.g. pH 5 can be used for sample pre-treatment, equilibration and interference elution 1. |

# EVOLUTE WCX

## Optimized Method Development for Strongly Basic Analytes Using EVOLUTE WCX

EVOLUTE WCX is designed to extract strongly basic analytes (including quaternary amines) from biological fluids and other aqueous samples using mixed-mode non-polar/weak cation exchange retention mechanisms and mildly acidic elution conditions. EVOLUTE WCX is the sorbent of choice when basic analytes are retained too strongly using the EVOLUTE CX sorbent. The sorbent consists of the EVOLUTE backbone surface modified with a carboxylic acid functional group (~0.4mmol/g capacity) (see **Figure 11**). Utilization of non-polar and weak cation retention mechanisms allows for strongly basic analytes to be selectively retained. The simple wash steps in the EVOLUTE WCX generic method remove matrix components such as proteins, salts, non-ionizable interferences and phospholipids delivering cleaner extracts with reproducible recoveries for reliable, accurate quantification. The ability to elute using acidic conditions makes EVOLUTE WCX invaluable for the extraction of bases which are unstable under basic elution conditions.

## When to use EVOLUTE WCX

- Quaternary amines
- Strong bases ($pK_a$ >10) (use method 1)
- Mixtures of bases including strong bases (use method 2)
- Bases unstable at high pH (use method 2)
- Bases, when acidic elution conditions are preferred due to LC-MS/MS compatibility
- When basic compounds are difficult to elute from EVOLUTE CX

**EVOLUTE WCX** combines non-polar and weak cation exchange in a mixed-mode retention mechanism for improved recovery of basic compounds and cleaner extracts.

Analyte elution is achieved by eliminating sorbent charge i.e. by using a low pH

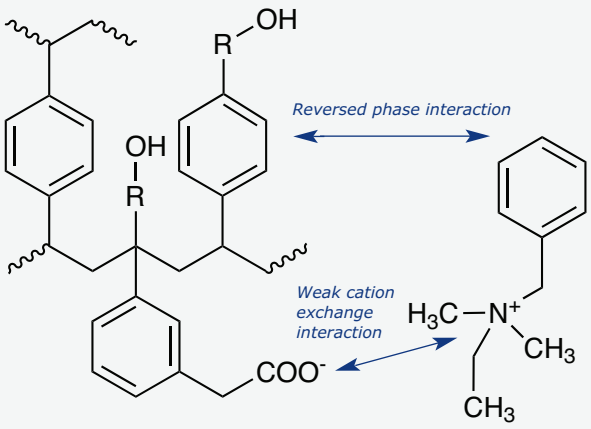



**Figure 11.** Structure of EVOLUTE WCX with interactions

## EVOLUTE WCX

| EVOLUTE WCX Generic Method 1: For Extraction of Strong Bases $pK_a$ >10 (including quaternary amines) from Biological Fluids. | | |
|---|---|---|
| **Step** | **Conditions** | **Effect** |
| Pre-treatment | Dilute sample 1:3 (v/v) with water/ammonium hydroxide (95:5, v/v). **For protein bound drugs in plasma or serum, dilute with 2% formic acid** | Reduces or eliminates protein binding |
| *Conditioning | Methanol (1 mL) | |
| *Equilibration | Water/ammonium hydroxide (95:5, v/v) (1 mL) | |
| Sample load | 400 µL–2 mL diluted plasma | Analyte retained by hydrophobic interaction |
| Interference elution 1 | Water/ammonium hydroxide (95:5, v/v) (1 mL) | Removes polar interferences (salts, proteins and larger phospholipids) |
| Interference elution 2 | Methanol (1 mL) | Analyte retained by cation exchange interactions. Removes remaining PLs plus neutral, acidic and weakly basic interferences |
| Analyte elution | Methanol /formic acid (98/2, v/v) (500 µL–1 mL) | Elutes strongly basic analytes by eliminating SORBENT charge |
| Evaporate and re-constitute as necessary for analysis | | |

Volumes suitable for 25/30 mg formats, see table 4 on page 24 for guidelines for other formats.
*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

### Reagents

- Water/ammonium hydroxide (95:5, v/v). Take 5 mL of 28% ammonium hydroxide solution, and make up to 100 mL with water. Mix thoroughly.
- Methanol /formic acid (98/2, v/v). Take 2 mL of 98% formic acid solution, and add 98 mL methanol. Mix thoroughly.

| EVOLUTE WCX Generic Method 2: For Extraction of Mixtures of Weak and Strong Bases or Basic Analytes Unstable at High pH from Biological Fluids. | | |
|---|---|---|
| **Step** | **Conditions** | **Effect** |
| Pre-treatment | Dilute sample 1:3 (v/v) with 0.05M ammonium acetate pH 7.0. **For protein bound drugs in plasma or serum, dilute with 2% formic acid** | Reduces or eliminates protein binding |
| *Conditioning | Methanol (1 mL) | |
| *Equilibration | 0.05M ammonium acetate pH 7.0 (1 mL) | |
| Sample load | 400 µL–2 mL diluted plasma | Analyte retained by hydrophobic interaction |
| Interference elution 1 | 0.05M ammonium acetate pH 7.0 (1 mL) | Removes polar interferences (salts, proteins and larger phospholipids) |
| Interference elution 2 | Methanol (1 mL) | Analyte retained by cation exchange interactions. Removes remaining PLs plus neutral and acidic interferences |
| Analyte elution | Methanol /formic acid (98/2, v/v) (500 µL–1 mL) | Elutes strongly basic analytes by eliminating SORBENT charge |
| Evaporate and re-constitute as necessary for analysis | | |

Volumes suitable for 25/30 mg formats, see table 4 on page 24 for guidelines for other formats.
*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

**Reagents**

- 0.05M ammonium acetate pH 7.0. Dissolve 3.854 g of ammonium acetate in 950 mL of deionized water. Make up to 1 L with deionized water and mix thoroughly. Adjust to pH 7.0 with ammonium hydroxide.
- Methanol /formic acid (98/2, v/v) solution. Take 2 mL of 98% formic acid solution, and add 98 mL methanol. Mix thoroughly.

## Method Optimization Tips for EVOLUTE WCX

**For sample containing weakly basic interferences:**

Use EVOLUTE WCX generic method 1. This allows elution of weak bases in interference wash 2, so they do not contaminate the final extract.

For analytes with $pK_a$ ~9 and below, elution can be achieved using high pH solvent (e.g. methanol/ammonium hydroxide (95/5, v/v). This may improve extract cleanliness where interfering species elute using acidic elution conditions

# EVOLUTE AX

## Optimized Method Development for Extraction of Acidic Analytes Using EVOLUTE AX

EVOLUTE AX is designed to extract acidic analytes from biological fluids and other aqueous samples using mixed-mode non-polar/strong anion exchange retention mechanisms. The sorbent consists of the EVOLUTE backbone surface modified with a quaternary amine functional group with a chloride counter ion (0.7 mmol/g capacity) (see **Figure 12**). By using both non-polar and strong anion exchange retention mechanisms, acidic analytes are selectively retained. The simple wash steps in the EVOLUTE AX generic method remove matrix components such as proteins, salts, non-ionizable interferences and phospholipids delivering cleaner extracts with reproducible recoveries for reliable, accurate quantification.

### When to use EVOLUTE AX

- For aqueous samples including biological fluids
  - Acidic analytes ($pK_a$ 2–8)

**EVOLUTE AX** combines non-polar and strong anion exchange in a mixed-mode retention mechanism for improved recovery of acidic compounds, and cleaner extracts.

Analyte elution is achieved by eliminating analyte charge ('low' pH)

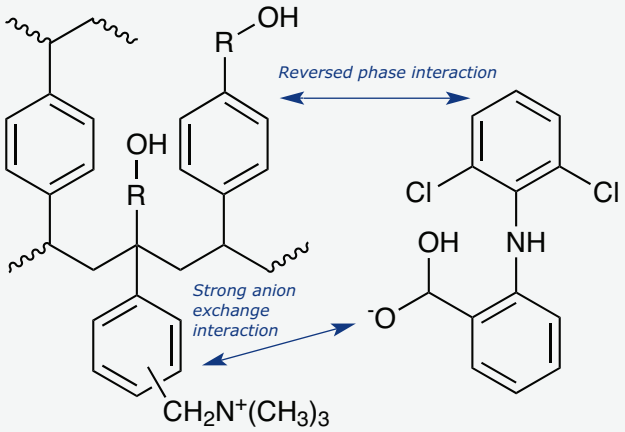



**Figure 12.** Structure of EVOLUTE AX with interactions

| EVOLUTE AX Generic Method for Extraction of Acidic Analytes from Biological Fluids. | | |
|---|---|---|
| **Step** | **Conditions** | **Effect** |
| Pre-treatment | Dilute sample 1:3 (v/v) with 2% formic acid | Reduces or eliminates protein binding |
| *Conditioning | Methanol (1 mL) | |
| *Equilibration | Water (1 mL) | |
| Sample load | 400 µL–2 mL diluted plasma | Analyte retained by hydrophobic interactions |
| Interference elution 1 | Ammonium acetate (0.05M pH 7.0 )/ methanol (95/5, v/v) (1 mL) | Removes polar interferences (salts, proteins and larger phospholipids) |
| Interference elution 2 | Methanol (1 mL) | Analyte retained by cation exchange interactions. Removes remaining PLs plus neutral and basic interferences |
| Analyte elution | Methanol /formic acid (98/2, v/v) (500 µL–1 mL) | Elutes acidic analytes by eliminating ANALYTE charge |
| Evaporate and re-constitute as necessary for analysis | | |

Volumes suitable for 25/30 mg formats, see table 4 on page 24 for guidelines for other formats.
*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

**Reagents**

- 0.05M ammonium acetate pH 7.0. Dissolve 3.854 g of ammonium acetate in 950 mL of deionized water. Make up to 1 L with deionized water and mix thoroughly. Adjust to pH 7.0 with ammonium hydroxide.
- Methanol /formic acid (98/2, v/v) solution. Take 2 mL of 98% formic acid solution, and add 98 mL methanol. Mix thoroughly.

## Method Optimization Tips for EVOLUTE AX

**For urine samples:**

Dilute sample with 50 mM ammonium acetate pH 7.0 (avoid acidification of sample). This prevents co-elution of polar urinary acids in the elution step, and can lead to a cleaner extract.

In extreme cases the use of a polar aprotic solvent such as ACN for sample pre-treatment can help retention of very polar analytes e.g. EtG, providing advanced cleanliness. See application note AN718 for further details.

For very polar acids, reducing the salt and acid concentration in the loading and wash steps can provide better retention.

# EVOLUTE WAX

## Optimized Method Development for Extraction of Strongly Acidic Analytes Using EVOLUTE WAX

EVOLUTE WAX is designed to extract strongly acidic analytes ($pK_a$<2) from biological fluids and other aqueous samples using mixed-mode non-polar/weak anion exchange retention mechanisms. The phase consists of the EVOLUTE backbone surface modified with a primary-secondary amine functional group in the free base form (0.3 mmol/g capacity[a]) (see **Figure** 13). By using non-polar and weak anion exchange retention mechanisms, strongly acidic analytes are selectively retained. The simple interference elution steps in the EVOLUTE WAX generic method remove matrix components such as proteins, salts, non-ionizable interferences and phospholipids delivering cleaner extracts with reproducible recoveries for reliable, accurate quantification.

[a]0.7 mmol/g for 50 µm material

## When to use EVOLUTE WAX

- For aqueous samples including biological fluids
- Strongly acidic analytes ($pK_a$<2) such as alkyl phosphates, alkyl sulfonates
- Acidic analytes unstable at the low pH used for EVOLUTE AX elution step but stable under basic pH conditions
- Acidic analytes difficult to elute from EVOLUTE AX

**EVOLUTE WAX** combines non-polar and weak anion exchange in a mixed-mode retention mechanism for improved recovery of acidic compounds, and cleaner extracts.

Analyte elution is achieved by eliminating sorbent charge ('high' pH).

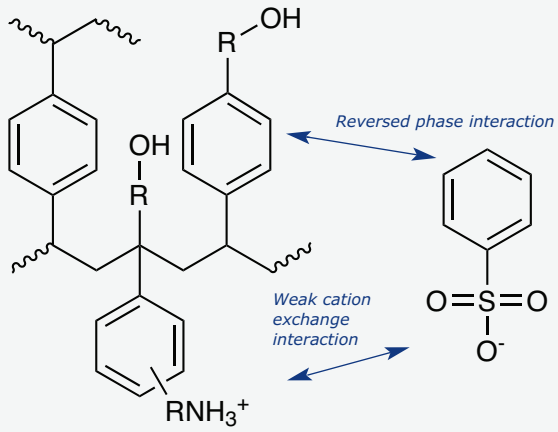



**Figure 13.** Structure of EVOLUTE WAX with interactions

| EVOLUTE WAX Generic Method for Extraction of Acidic Analytes from Biological Fluids. | | |
|---|---|---|
| **Step** | **Conditions** | **Effect** |
| Pre-treatment | Dilute sample 1:3 (v/v) with 2% formic acid (aq) | Reduces or eliminates protein binding. Strong acids will retain their negative charge under these pH conditions |
| *Conditioning | Methanol (1 mL) | |
| *Equilibration | 2% formic acid (aq) (1 mL) | |
| Sample load | 400 µL–2 mL diluted plasma | Analyte retained by hydrophobic interactions |
| Interference elution 1 | 2% formic acid (aq) (1 mL) | Removes polar interferences (salts, proteins and larger phospholipids) |
| Interference elution 2 | Methanol (1 mL) | Analyte retained by cation exchange interactions. Removes remaining PLs plus neutral and basic interferences |
| Analyte elution | Methanol /ammonium hydroxide (95/5, v/v), (500 µL–1 mL) | Elutes acidic analytes by eliminating SORBENT charge |
| Evaporate and re-constitute as necessary for analysis | | |

Volumes suitable for 25/30 mg formats, see table 4 on page 24 for guidelines for other formats.
*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

**Reagents**

- 2% formic acid. Take 2 mL of 98% formic acid, and make up to 100 mL with water. Mix thoroughly
- Methanol /ammonium hydroxide (95/5, v/v) solution. Take 5 mL of ammonium hydroxide (28%) and add 95 mL methanol. Mix thoroughly.

## Method optimization tips for EVOLUTE WAX

**To maximize recoveries of low molecular weight, polar acids**

Evaluate the use of deionized water for sample pre treatment, equilibration and/or wash steps to prevent analyte breakthrough

# Processing Conditions for EVOLUTE Cartridges and Plates

These guidelines for flow rates (table 3) and volumes (table 4) should be used for method development using the optimized EVOLUTE methods described on pages 11–23.

## Flow Guidelines for Plates and Cartridges

Biotage plates and cartridges can be used with industry standard vacuum and positive pressure sample processing manifolds or centrifuge equipment.

| Column Size | 96-well plate | 1 mL and 10 mL "G" columns | 3 mL and 10 mL "H" columns | 6 mL "C" columns |
|---|---|---|---|---|
| **Flow rate** | 1 mL/min | 1 mL/min | 3 mL/min | 7 mL /min |

## Flow optimization tips

Before processing, set the vacuum or pressure to provide the appropriate flow rate. If possible, turn off the vacuum or pressure while samples are loaded. This will provide consistent flow rates and the best analytical precision. Once optimum chemistry has been achieved, flow rate can be adjusted for maximum throughput. Final flow rate should be set at 10–20% lower than the breakthrough limit.

For the 100 mg/3 mL format, a vacuum of –1" Hg produces a flow of 2 mL/minute.

For the 50 µm particle size most steps give flow rates greater than or equal to 1 mL/min under gravity, however, for viscous samples higher vacuum levels may be required upon sample loading and the first aqueous wash.

## Volume Guidelines for Plates and Cartridges

| Step | Sorbent bed mass | | | | | |
|---|---|---|---|---|---|---|
| | **10 mg** | **25 and 30 mg** | **50 mg** | **100 mg** | **200 mg** | **500 mg** |
| Column conditioning** | 500 µL | 1 mL | 2 mL | 3 mL | 6 mL | 6 mL |
| Column equilibration** | 500 µL | 1 mL | 2 mL | 3 mL | 6 mL | 6 mL |
| Sample loading | 200 µL | 200–400 µL | Application specific, based on analyte concentration in sample | | | |
| Washes 1 and 2 | 500 µL | 1 mL | 2 mL | 3 mL | 6 mL | 6 mL |
| Elution | 200 µL | 500 µL–1 mL | Dependent on analyte and choice of elution solvent. Minimum elution volume 2–5 bed volumes* | | | |

**Table 4.** Volume guidelines

*1 bed volume is approximately 200 µL per 100 mg of sorbent.
** These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

## Volume optimization tips

Elution volumes can be minimized by successive aliquot elution instead of a single volume, i.e. 2 x 1 mL instead of 1 x 2 mL

Once optimum conditions for high analyte recovery and extract cleanliness are determined, further optimization of solvent volumes may result in reduced solvent usage.

# Streamlined Method Development Using EVOLUTE SPE Products

When faced with a complex mixture of analytes, unknowns or zwitterions, it may not be straight forward to identify which of the optimized methods to evaluate first. Biotage have developed a simple approach to method development using the EVOLUTE family of SPE sorbents. This section of the guide outlines the options available for fast development of a robust sample preparation method.

### Option 1: Non-polar SPE using EVOLUTE ABN

In many instances, use of a non-polar retention mechanism (EVOLUTE ABN) will provide good results, and should be screened using the methodology described on page 12.

### Option 2: Mixed-mode SPE using the EVOLUTE EXPRESS sorbent selection plate

Alternatively, use the EVOLUTE EXPRESS sorbent selection plate for sorbent screening. Using a simplified approach (just 2 methods), all four of the EVOLUTE mixed-mode sorbents can be screened to identify the best solution to a complex sample preparation problem.

In this streamlined process, method selection is based on analyte functionality only. Where analyte functionality is known, select methods as described below:

### Method selection

| | |
|---|---|
| **Use method 1 for extraction of** | • Bases using EVOLUTE CX<br>• Strong acids using EVOLUTE WAX |

| | |
|---|---|
| **Use method 2 for extraction of** | • Acids using EVOLUTE AX<br>• Strong bases using EVOLUTE WCX |

Neutrals will be eluted in the 'elute 1 solvent' for both methods, so this should be collected and analyzed when neutrals or unknown analytes are to be extracted. We recommend that EVOLUTE ABN is used for further optimization of methods for neutral compounds (see option 1).

**Method 1: Use for extraction of bases using EVOLUTE CX or strong acids using EVOLUTE WAX**

| **EVOLUTE CX**<br>Bases ($pK_a$ 2–10) | **Method 1** | **EVOLUTE WAX**<br>Strong acids ($pK_a$<2) |
|---|---|---|
| | Prepare sample: dilute plasma sample 1:1 with 4% $H_3PO_4$ in water | |
| | *Condition: Methanol | |
| | *Equilibrate: Water | |
| | Load sample | |
| | Wash: 2% formic acid (aq) | |
| Neutrals and acids eluted | Elute 1: Methanol | Neutrals and bases eluted |
| Bases eluted by eliminating charge on analyte | Elute 2: 5% $NH_4OH$ in methanol | Strong acids eluted by eliminating charge on sorbent |

- Collect Elute 1 solvent and analyze for neutrals or unknowns
- Collect Elute 2 solvent and analyze for bases or strong acids

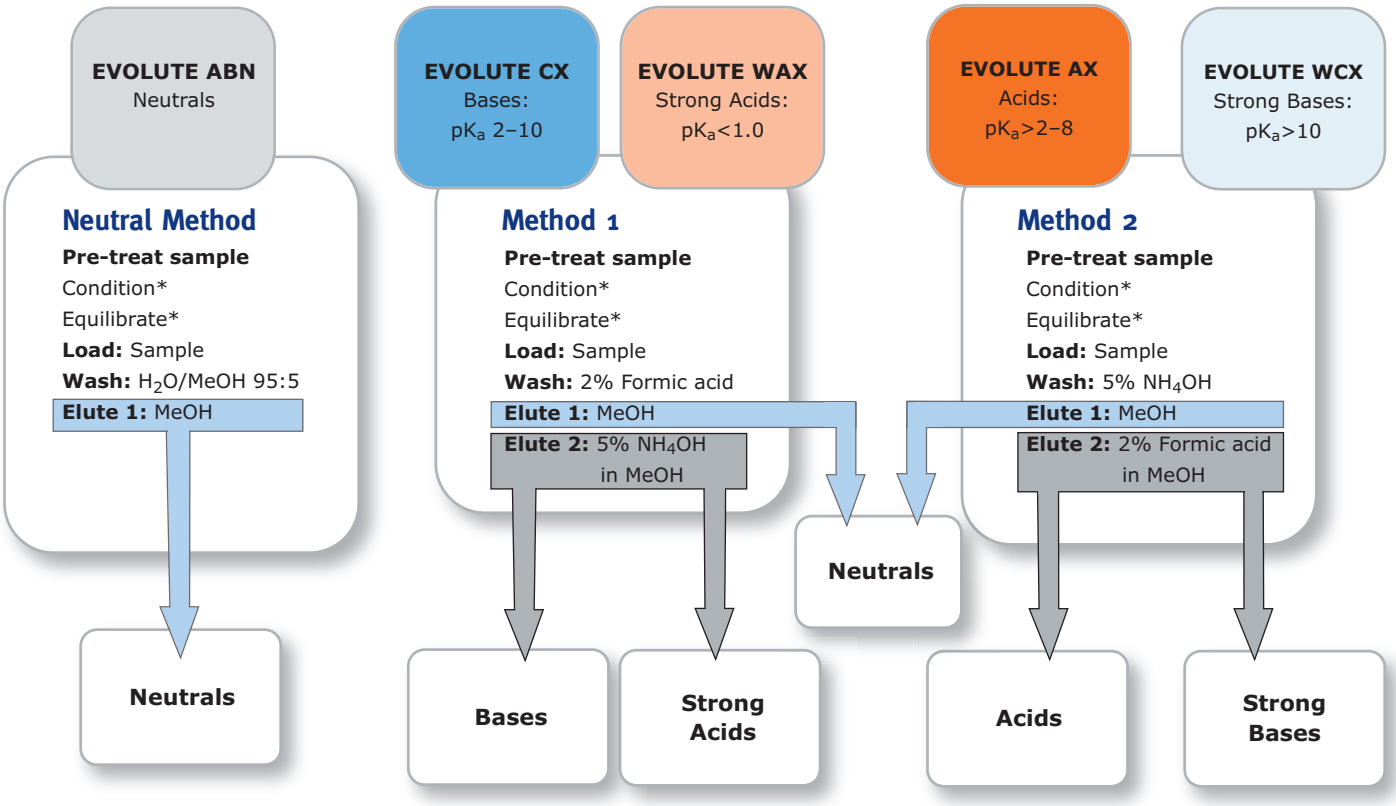
*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

| Method 2: Use for extraction of acids using EVOLUTE AX or strong bases using EVOLUTE WCX | | |
|---|---|---|
| **EVOLUTE AX**<br>Acids ($pK_a$ 2–8) | **Method 2** | **EVOLUTE WCX**<br>Strong bases ($pK_a$>10) |
| | Prepare sample: dilute plasma sample 1:1 with 4% $H_3PO_4$ in water | |
| | *Condition: Methanol | |
| | *Equilibrate: Water | |
| | Load sample | |
| | Wash: 5% $NH_4OH$ (aq) | |
| Neutrals and acids eluted | Elute 1: Methanol | Neutrals and bases eluted |
| Acids eluted by eliminating charge on analyte | Elute 2: 2% formic acid in methanol | Strong bases eluted by eliminating charge on sorbent |

- Collect Elute 1 solvent and analyze for neutrals or unknowns
- Collect Elute 2 solvent and analyze for acids or strong bases

*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

This approach is summarized below:




*These steps can be removed when evaluating the Load-Wash-Elute procedure using EVOLUTE EXPRESS products.

**Figure 14.** Summary of streamlined sorbent and method selection protocols.

## EVOLUTE EXPRESS Sorbent Selection Plate

The EVOLUTE Express sorbent selection plate contains the 4 EVOLUTE mixed-mode sorbents. Each well contains 30 mg of sorbent. It has been designed to provide a single plate for streamlined method development using methods 1 and 2 on page 25–26.

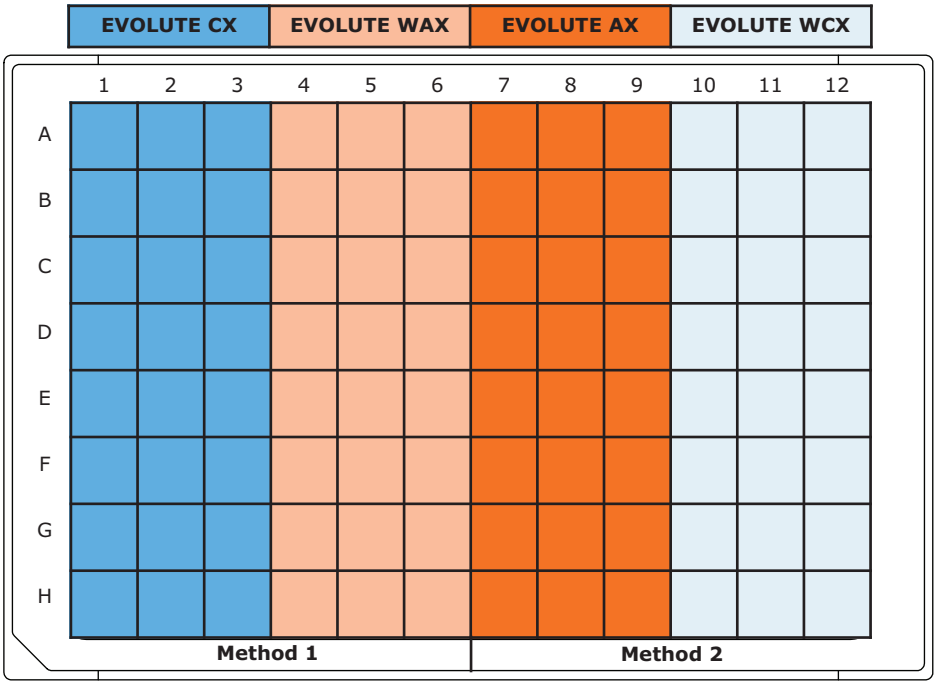



**Figure 15.** EVOLUTE EXPRESS sorbent selection plate layout showing correct method for each sorbent.

When using the EVOLUTE EXPRESS sorbent selection plate, the following protocol should be used:

**Columns 1–3 contain EVOLUTE CX: use method 1**

- Collect Elute 1 solvent and analyze for acids, neutrals and unknowns
- Collect Elute 2 solvent and analyze for **bases**

**Columns 4–6 contain EVOLUTE WAX: use method 1**

- Collect Elute 1 solvent and analyze for bases, neutrals and unknowns
- Collect Elute 2 solvent and analyze for **strong acids**

**Columns 7–9 contain EVOLUTE AX: use method 2**

- Collect Elute 1 solvent and analyze for bases, neutrals and unknowns
- Collect Elute 2 solvent and analyze for **acids**

**Columns 10–12 contain EVOLUTE WCX: use method 2**

- Collect Elute 1 solvent and analyze for acids, neutrals and unknowns
- Collect Elute 2 solvent and analyze for **strong bases**

Once the best method / sorbent for your analyte(s) has been identified using this streamlined process, further optimization may be required to increase analyte recovery or improve extract cleanliness.

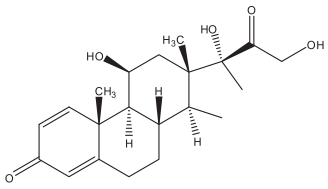
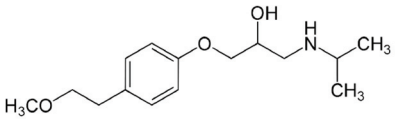
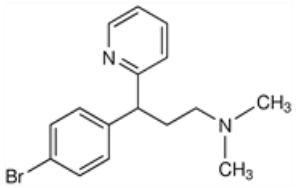
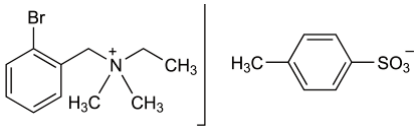
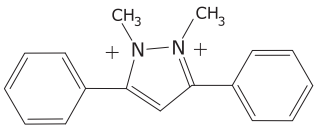
In this situation, we recommend that you evaluate the optimized methods for each EVOLUTE sorbent described on pages 11–23, with the optimization tips provided.

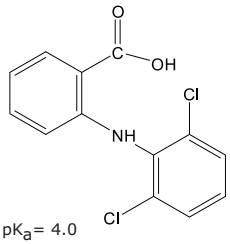
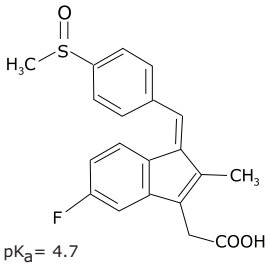
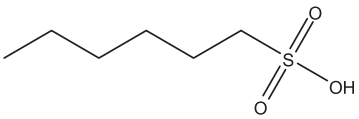
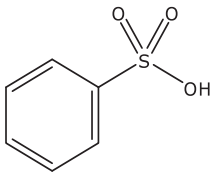
## Streamlined Method Development Using EVOLUTE SPE Products

**Case study: Use of the EVOLUTE EXPRESS sorbent selection plate**

To illustrate the use of this approach, the streamlined method development process was evaluated using the EVOLUTE EXPRESS sorbent selection plate, using a suite of test analytes with typical class functionality. Results achieved (without any further method optimization) are shown in **Table 5**.

### Structures of test analytes

| Analyte Functionality | Structure |
|---|---|
| Neutral |  Prednisolone |
| Base |  Metoprolol, $pK_a$ =9.5 |
| Base |  Brompheniramine $pK_a$ = 3.5 |
| Strong Base |  Bretylium tosylate $pK_a$ >10 |
| Strong Base |  Diphenzoquat $pK_a$>10 |

| Analyte Functionality | Structure |
|---|---|
| Acid |  Diclofenac $pK_a$= 4.0 |
| Acid |  Sulindac $pK_a$= 4.7 |
| Strong Acid |  Hexanesulfonic acid $pK_a$<1 |
| Strong Acid |  Benzenesulfonic acid $pK_a$<1 |

## Results

| Analyte | Class/functionality | Sorbent | Method | Average recovery (n=7) | RSD |
|---|---|---|---|---|---|
| Prednisolone | Neutral (present in ELUTE 1) | Any | 1 or 2 | 97.6 (n=21) | 3.9 |
| Metoprolol | Base | EVOLUTE CX | 1 | 95.8 | 5.4 |
| Brompheniramine | Base | EVOLUTE CX | 1 | 108.7 | 4.5 |
| Bretylium tosylate | Strong base | EVOLUTE WCX | 2 | 102.0 | 4.8 |
| Difenzoquat | Strong base | EVOLUTE WCX | 2 | 99.6 | 3.9 |
| Sulindac | Acid | EVOLUTE AX | 2 | 93.2 | 4.3 |
| Diclofenac | Acid | EVOLUTE AX | 2 | 92.6 | 2.4 |
| Hexanesulfonic acid | Strong acid | EVOLUTE WAX | 1 | 88.1 | 6.9 |
| Benzenesulfonic acid | Strong acid | EVOLUTE WAX | 1 | 93.9 | 3.3 |

**Table 5:** Typical analyte recoveries using the streamlined method development process

## Conclusion

The streamlined method development process using the EVOLUTE EXPRESS sorbent selection plate provides a fast, simple approach to development of SPE methods for a diverse range of analyte types, giving high analyte recoveries and clean extracts, with no complex method development required.

# EVOLUTE Applications

The EVOLUTE SPE family has been successfully used for extraction of a wide range of analytes from many matrices, including plasma, urine, whole blood, water, milk and tissue extracts.

We are continually developing new applications using EVOLUTE SPE products. Visit the Sample Preparation Applications Database (**www.biotage.com/applications**) to find the latest information.

**Analytes extracted using EVOLUTE sorbents include:**

Acetazolamide
Amiloride
Amitriptyline
Anhydroecgonine methyl ester
Atenolol
Bendoflumethiazide
Benzenesulfonic acid
Benzoylecgonine
Bretylium tosylate
Brompheniramine
Bumetanide
Carbamazepine
Chlormequat
Citalopram
Cocaethylene
Cocaine
Diclofenac
Diethyldithiophosphate (DEDTP)
Diethylphosphate
Diethylthiophosphate (DETP)
Difenzoquat
Dimethyldithiophosphate (DMDTP)
Dimethylphosphate (DMP)
Dimethylthiophosphate (DMTP)
Dopamine
Ecgonine methyl ester
Epinephrine
Erythromycin
Ethacrynic acid
Ethyl glucuronide
Fluoxetine
Fluvoxamine
Furosemide
Hexanesulfonic acid
Hydrochlorothiazide
Hydroflumethiazide
Ibuprofen
Labetalol
Mefenamic acid
Melamine
Mephedrone
Methazolamide
Metoprolol
Mianserin
Naltrexone
Neostigmine
Norcocaine
Norepinephrine
Oxprenolol
Paroxetine
Procainamide
Propranolol
Quinidine
Ranitidine
Salbutamol
Sotalol
Spironolactone
Sulfamethoxazole
THC-COOH
Trimethoprim

# Appendix 1

## The 2 pH unit rule

The $pK_a$ of a molecular functional group is defined as the pH at which 50% of this group in solution are charged, and 50% are uncharged. Each pH unit change affects the percentage of charged or uncharged groups by a factor of 10, so it is sensible 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 a 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 |

For strongest retention of a weakly acidic analyte with $pK_a$ of 4.0, using a:

- Non-polar (hydrophobic) retention mechanism – adjust sample to pH 2.0 (2 pH units BELOW the $pK_a$ )
- Anion exchange retention mechanism - adjust sample to pH 6.0 (2 pH units above 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 | % free base (uncharged) | % dissociated (charged) |
|---|---|---|
| 11.0 | 99.5 | 0.5 |
| 10.0 | 95 | 5.0 |
| BASE $pK_a$ = 9.0 | 50 | 50 |
| 8.0 | 5.0 | 95 |
| 7.0 | 0.5 | 99.5 |

For strongest retention of a weakly basic analyte with $pK_a$ of 9.0, using a:

- Non-polar (hydrophobic) retention mechanism – adjust sample to pH 11.0 (2 pH units **ABOVE** the $pK_a$)
- Cation exchange retention mechanism - adjust sample to pH 7.0 (2 pH units **BELOW** the $pK_a$)

# Sample Preparation Products

## EVOLUTE Format Options

### 96-well Formats

Process small volume biological fluid samples with 96-well plates for high throughput sample preparation. Biotage offers two 96-well plates; the industry standard fixed well plate design and the modular Array style.


### EVOLUTE EXPRESS 96-Well Plates

Ideal for assays where sample numbers are high. The one piece molded plate holds up to 2 mL per well and is compatible with all commonly used liquid handling systems. Process EVOLUTE EXPRESS plates manually or on instrument decks using the VacMaster-96 Sample Processing Manifold.

Alternatively, EVOLUTE EXPRESS 96-well plates can be processed using the PRESSURE+ 96 positive pressure manifold.


### Modular Array Plates

Used for method development, sorbent screening and for assays where sample numbers are variable. Minimize cost and avoid the problem of partially used plates by populating the base plate with as many wells as required. Available in 1 or 2 mL options, the format is compatible with many liquid handling systems or can be processed manually using the VacMaster-96 manifold.

Array wells can also be processed on a VacMaster-10 or -20 Sample Processing manifold, using PTFE adapters.

### Column Formats

Traditional 'syringe barrel' style reservoirs used for SPE range in volume from 1 to 150 mL.

The columns can be packed with sorbent masses ranging from 10 mg (for small volume biological fluid samples) up to 10 g for high capacity applications. Process these columns using VacMaster-10 or -20 Sample Processing Manifolds.

Sample size is not limited to the reservoir volume. Load larger samples by stacking an empty reservoir above the extraction column using ISOLUTE Column Adaptors, or use the Large Volume Extraction Kit.

## Biotage Sample Processing Accessories

### VacMaster™ 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.


### VacMaster 96

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

The VacMaster-10 and -20 manifolds are ideal for processing up to 10 (VacMaster-10) or 20 (VacMaster-20) samples in parallel using standard EVOLUTE SPE columns. 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.

Two control units are available for use with either a vacuum source or for use with lab air to generate the vacuum. For ordering information please visit www.biotage.com or request a catalog from your Biotage representative.

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

## PRESSURE+ Positive Pressure Manifolds


Biotage PRESSURE+ manifolds offer positive pressure, parallel processing for 96 well plates, 1 mL 3 mL and 6 mL EVOLUTE column formats. The systems utilize a consistent, uniform flow of positive pressure to move both low and high viscosity liquids through EVOLUTE plates and columns. 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 regardless of SPE format.

### 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.

### PRESSURE+ 48

The same self-adjusting technology utilized in the PRESSURE+ 96 manifold allows the PRESSURE+ 48 to utilize all columns up to 6 mL 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.

## SPE Automation


### RapidTrace®

RapidTrace is the perfect complement to high-speed analytical techniques, a powerful high-throughput workstation dedicated specifically to SPE extraction. In its full modular configuration, ten RapidTrace modules can process up to 100 SPE columns per hour. Available in 1 mL, 3 mL and 6 mL formats, RapidTrace eliminates tedious and time-consuming manual extraction methods. Capable of handling up to ten samples per module, a 10 module RapidTrace Workstation delivers the throughput to match 2–5 minute LC/MS/MS cycle times. All sample and solvent flow rates are individually controlled under positive pressure. Simple to use software enables users to develop methods in minutes such that SPE conditions can be optimized in a fraction of the time required using conventional techniques.

## Evaporation and Reconstitution

Designed for high throughput laboratories, the SPE-Dry 96 and TurboVap® 96 sample concentrator systems provide efficient evaporation in microplate format.


### SPE-Dry™ 96

The SPE Dry 96 utilizes heated gas flow both above and below the collection plate to rapidly dry both aqueous and organic solvents without causing well to well contamination. Additional options on the SPE-Dry 96 allow for compatibility with 24 and 384 well collection plates in addition to PTFE-coated needles for applications that use volatile acids or bases.


### TurboVap® 96

The TurboVap 96 instrument uses patented vortex shearing technology within an enclosed container to allow for efficient evaporation at low (40 °C) temperatures. The small footprint and the rear vent tube enable the use of the TurboVap 96 in the open laboratory provided the vent tube is vented to a suitable waste stream flow. For larger sample volumes (up to 500 mL) other TurboVap options are available. Please contact Biotage for further details.

## Ordering Information

### EVOLUTE EXPRESS Plates

| Part number | Description | Qty |
|---|---|---|
| **EVOLUTE ABN** | | |
| 600-0010-PX01 | EVOLUTE EXPRESS ABN 10 mg Fixed Well Plate | 1 |
| 600-0030-PX01 | EVOLUTE EXPRESS ABN 30 mg Fixed Well Plate | 1 |
| **EVOLUTE CX** | | |
| 601-0010-PX01 | EVOLUTE EXPRESS CX 10 mg Fixed Well Plate | 1 |
| 601-0030-PX01 | EVOLUTE EXPRESS CX 30 mg Fixed Well Plate | 1 |
| **EVOLUTE WCX** | | |
| 602-0010-PX01 | EVOLUTE EXPRESS WCX 10mg Fixed Well Plate | 1 |
| 602-0030-PX01 | EVOLUTE EXPRESS WCX 30 mg Fixed Well Plate | 1 |
| **EVOLUTE AX** | | |
| 603-0010-PX01 | EVOLUTE EXPRESS AX 10 mg Fixed Well Plate | 1 |
| 603-0030-PX01 | EVOLUTE EXPRESS AX 30 mg Fixed Well Plate | 1 |
| **EVOLUTE WAX** | | |
| 604-0010-PX01 | EVOLUTE EXPRESS WAX 10 mg Fixed Well Plate | 1 |
| 604-0030-PX01 | EVOLUTE EXPRESS WAX 30 mg Fixed Well Plate | 1 |

### EVOLUTE EXPRESS Sorbent Selection Plate

| Part number | Description | Qty |
|---|---|---|
| 650-0010-PX01 | EVOLUTE EXPRESS 10 mg Sorbent Selection Plate | 1 |
| 650-0030-PX01 | EVOLUTE EXPRESS 30 mg Sorbent Selection Plate | 1 |

### EVOLUTE Array Pre-filled 96-well Plates

| Part number | Description | Qty |
|---|---|---|
| **EVOLUTE ABN** | | |
| 600-0010-RP | EVOLUTE Array ABN 10 mg/1 mL Plate | 1 |
| 600-0025-RP | EVOLUTE Array ABN 25 mg/1 mL Plate | 1 |
| **EVOLUTE CX** | | |
| 601-0010-RP | EVOLUTE Array CX 10 mg/1 mL | 1 |
| 601-0025-RP | EVOLUTE Array CX 25 mg/1 mL | 1 |

### EVOLUTE Array Loose Wells

| Part number | Description | Qty |
|---|---|---|
| **EVOLUTE ABN** | | |
| 600-0010-R | EVOLUTE Array ABN 10 mg Wells | 100 |
| 600-0025-R | EVOLUTE Array ABN 25 mg Wells | 100 |
| **EVOLUTE CX** | | |
| 601-0010-R | EVOLUTE Array CX 10 mg/1 mL Wells | 100 |
| 601-0025-R | EVOLUTE Array CX 25 mg/1 mL Wells | 100 |
| **EVOLUTE WCX** | | |
| 602-0025-R | EVOLUTE WCX 25 mg 1 mL Wells | 100 |
| **EVOLUTE AX** | | |
| 603-0025-R | EVOLUTE Array AX 25 mg/1 mL Wells | 100 |
| **EVOLUTE WAX** | | |
| 604-0025-R | EVOLUTE Array WAX 25 mg/1mL Wells | 100 |

## EVOLUTE SPE Columns

| Part number | Description | Qty |
|---|---|---|
| **EVOLUTE ABN** | | |
| 600-0001-A | EVOLUTE ABN 10 mg /1 mL | 100 |
| 600-0002-A | EVOLUTE ABN 25 mg/1 mL | 100 |
| 600-0002-B | EVOLUTE ABN 25 mg /3 mL | 50 |
| 600-0002-H | EVOLUTE ABN 25 mg/10 mL XL | 50 |
| 610-0005-B | EVOLUTE ABN 50 mg/3 mL | 50 |
| 610-0010-B | EVOLUTE ABN 100 mg/3 mL | 50 |
| 610-0010-H | EVOLUTE ABN 100 mg/10 mL XL | 50 |
| 610-0020-B | EVOLUTE ABN 200 mg/3 mL | 50 |
| 610-0020-C | EVOLUTE ABN 200 mg/6 mL | 30 |
| 610-0050-C | EVOLUTE ABN 500 mg/6 mL | 30 |
| **EVOLUTE CX** | | |
| 601-0001-A | EVOLUTE CX 10 mg/1 mL | 100 |
| 601-0002-A | EVOLUTE CX 25 mg/1 mL | 100 |
| 611-0005-B | EVOLUTE CX 50 mg/3 mL | 50 |
| 611-0010-B | EVOLUTE CX 100 mg/3 mL | 50 |
| 611-0010-H | EVOLUTE CX 100 mg /10 mL XL | 50 |
| 611-0020-B | EVOLUTE CX 200 mg /3 mL | 50 |
| 611-0020-C | EVOLUTE CX 200 mg/6 mL | 30 |
| 611-0050-C | EVOLUTE CX 500 mg/6 mL | 30 |
| **EVOLUTE WCX** | | |
| 602-0002-A | EVOLUTE WCX 25 mg /1 mL | 100 |
| 612-0005-B | EVOLUTE WCX 50 mg/3 mL | 50 |
| 612-0010-B | EVOLUTE WCX 100 mg/3 mL | 50 |
| 612-0010-H | EVOLUTE WCX 100 mg/3 mL XL | 50 |
| 612-0020-C | EVOLUTE WCX 200 mg/6 mL | 30 |
| 612-0050-C | EVOLUTE WCX 500 mg/6 mL | 30 |
| **EVOLUTE AX** | | |
| 603-0002-A | EVOLUTE AX 25 mg/1 mL | 100 |
| 613-0005-B | EVOLUTE AX 50 mg/3 mL | 50 |
| 613-0010-B | EVOLUTE AX 100 mg/3 mL | 50 |
| 613-0010-H | EVOLUTE AX 100 mg/10 mL XL | 50 |
| 613-0020-C | EVOLUTE AX 200 mg/6 mL | 30 |
| 613-0050-C | EVOLUTE AX 500 mg/6 mL | 30 |
| **EVOLUTE WAX** | | |
| 604-0002-A | EVOLUTE WAX 25 mg/1 mL | 100 |
| 614-0005-B | EVOLUTE WAX 50 mg/3 mL | 50 |
| 614-0010-B | EVOLUTE WAX 100 mg/3 mL | 50 |
| 614-0010-H | EVOLUTE WAX 100 mg/10 mL XL | 50 |
| 614-0020-C | EVOLUTE WAX 200 mg/6 mL | 30 |
| 614-0050-C | EVOLUTE WAX 500 mg/6 mL | 30 |

## Ordering Information

### Sample Processing Manifolds and Instruments

| Part number | Description |
|---|---|
| **VacMaster-96** | |
| 121-9600 | VacMaster-96 Sample Processing Manifold |
| 121-9601 | Vacuum Control Unit VCU-1 |
| 121-9602 | Vacuum Control and Generation Unit VCU-2 |
| **VacMaster -10 and -20** | |
| 121-1016 | VacMaster-10 Sample Processing Manifold |
| 121-2016 | VacMaster-20 Sample Processing Manifold |
| **Pressure+ 96 and 48** | |
| PPM-96 | PRESSURE + 96 Positive Pressure Manifold |
| PPM-48 | PRESSURE + 48 Positive Pressure Manifold |
| **RapidTrace** | |
| C50000 | RapidTrace+ Workstation (1 mL and 3 mL columns) |
| C125713 | RapidTrace+ Workstation (6 mL columns) |
| **SPE Dry 96 Sample Concentrator System** | |
| SD-9600-DHS-NA | SPE Dry 96 Sample Concentrator System, 110 V, USA |
| SD-9600-DHS-UK | SPE Dry 96 Sample Concentrator System, 220 V UK |
| SD-9600-DHS-EU | SPE Dry 96 Sample Concentrator System, 220 V Europe |
| SD-9600-DHS-JP | SPE Dry 96 Sample Concentrator System, 110 V, JP |
| **TurboVap** | |
| C103263 | TurboVap 96 (120 VAC) |
| C103264 | TurboVap 96 (220 VAC) |
| C103198 | TurboVap LV (120 V) |
| C103199 | TurboVap LV (230 V) |

## Tools for Discovery and Development Chemistry

### Discovery Chemistry
- Microwave Synthesis
- Work-Up and Sample Preparation
- Evaporation
- Flash Purification
- Polymer Supported Reagents

### Process Chemistry
- Silica and Polymer Metal Scavengers
- Genotoxin Removal
- Catalyst Screening
- Purification Scale-Up

### Peptide Synthesis and Purification
- Automated, semi-automated and manual synthesizers
  - Microwave peptide synthesis
  - Room temperature peptide synthesis
  - Solution phase peptide synthesis
- Resins for solid phase peptide synthesis
- HPLC columns

### Analytical Chemistry / Sample Preparation
- Automated SPE Systems
- Evaporation Instrumentation
- Molecularly Imprinted Polymers
- Silica and Resin Based SPE Columns and Plates
- Processing Tools for SPE Columns and Plates
- Supported Liquid extraction columns and plates

### For more information, please contact Biotage

**EUROPE**
Main Office: +46 18 56 5900
Toll Free: +1 800 18 56 57 10
Fax: +46 18 59 1922
Order Tel: +46 18 56 57 10
Order Fax: +46 18 56 57 05
order@biotage.com

**NORTH AMERICA**
Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 704 654 4900
Order Fax: +1 434 296 8217
ordermailbox@biotage.com

**JAPAN**
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com

**CHINA**
Tel: +86 21 2898 6655
Fax: +86 21 2898 6153
cn_order@biotage.com
www.biotage.cn

To locate a distributor please visit our web site at www.biotage.com



**Part Number:**
EVO.UG_2012_rev.1

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