# Extraction of Methylmalonic Acid from Serum Using ISOLUTE® SAX Prior to LC-MS/MS Analysis

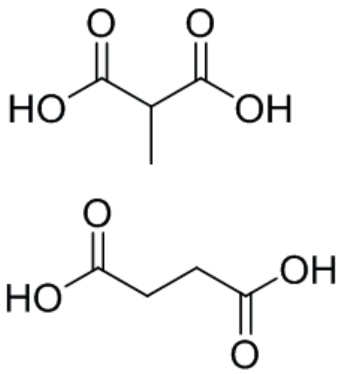



**Figure 1.** Structures of methylmalonic acid (MMA) and succinic acid (SA).

## Introduction

Methylmalonic acid (MMA) in serum is measured to help diagnose a number of disorders, primarily Vitamin B12 deficiency. This application note describes a simple, effective protocol for the extraction of methylmalonic acid (MMA) from serum using ISOLUTE® SAX strong anion exchange solid phase extraction plates, demonstrating high, reproducible analyte recoveries with low protein and phospholipid content in the extracts. The well known isobaric interference, succinic acid, is chromatographically separated to allow accurate quantitation of the MMA.

ISOLUTE SAX is a silica-based strong anion exchange sorbent used to extract acidic analytes, such as MMA, from aqueous samples. Very clean extracts are possible due to the purely anion exchange retention mechanism available using this silica based sorbent. This minimizes the co-extraction of endogenous interferences through hydrophobic (or non-polar) retention.

## Analytes

MMA and MMA-$^{13}C_4$ as internal standard.

## Sample Preparation Procedure

**Format:**
ISOLUTE® SAX 25 mg fixed well plate, part number 500-0025-P01

**Sample Pre-treatment**
To serum (100 µL), add 10 µL of internal standard (10 ng/µL). Allow to stand for ~1 hour to allow binding to occur. Add HPLC grade water (190 µL) and vortex.

**Conditioning**
Condition each well with methanol (500 µL).

**Equilibration**
Equilibrate each well with HPLC grade water (500 µL).

**Sample Loading**
Load 300 µL of pre-treated sample.

**Wash 1**
Elute interferences with HPLC grade water (500 µL).

**Wash 2**
Elute interferences with methanol (500 µL).

**Analyte Elution**
Elute analytes with 2% formic acid in acetonitrile (600 µL) into a collection plate.

**Post Extraction**
Dry the extract in a stream of air or nitrogen using a SPE Dry (40 °C at 40 L/min) or TurboVap (40 °C at 1.0 bar).

**Reconstitution**
Add 100 µL of 0.4% formic acid (aq), seal with a plate mat and vortex for 30 seconds.

## UPLC Conditions

### Instrument
Waters ACQUITY I Class UPLC equipped with a flow through needle (15 µL)

### Column
Gemini 3 µm C18 (100 x 3 mm id)

### Mobile Phase
A: 0.4% formic acid (aq)

B: 0.4% formic acid in methanol

### Flow Rate
0.6 mL/min

**Table 1.** Gradient Conditions - numerical.

| Step | %A | %B | Curve |
|---|---|---|---|
| 0 | 100 | 0 | 1 |
| 1 | 100 | 0 | 6 |
| 2.5 | 98 | 2 | 6 |
| 3 | 100 | 0 | 11 |

**Curve 6:** Linear Gradient

**Curve 11:** Conditions in line initiated immediately once time reached. i.e. 0% B resumed at 3 minutes.

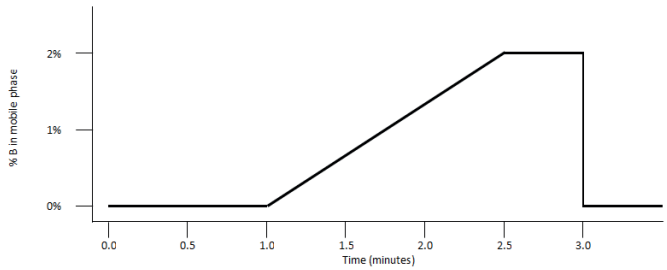



**Figure 2.** Gradient Conditions - graphical

### Injection Volume
10 µL

### Sample Temperature
20 °C

### Column Temperature
50 °C

## MS Conditions

### Instrument
Waters XEVO TQS triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.

### Desolvation Temperature:
500 °C

### Ion Source Temperature:
150 °C

Negative ions were acquired in the multiple reaction monitoring (MRM) mode:

**Table 2.** MRM Conditions.

| Compound | MRM Transition | Cone Voltage (V) | Collision Energy (eV) |
|---|---|---|---|
| MMA | 116.9 > 72.9 | 30 | 9 |
| MMA-$^{13}C_4$ | 121.0 > 76.0 | 30 | 9 |

## Results

### Chromatography
Good separation was achieved between MMA and the isobaric interference succinic acid. **Figure 3**. shows a chromatogram of serum spiked with 10 ng/mL MMA and the baseline raised to 10:1 signal:noise, indicating an approximate lower limit of quantitation.

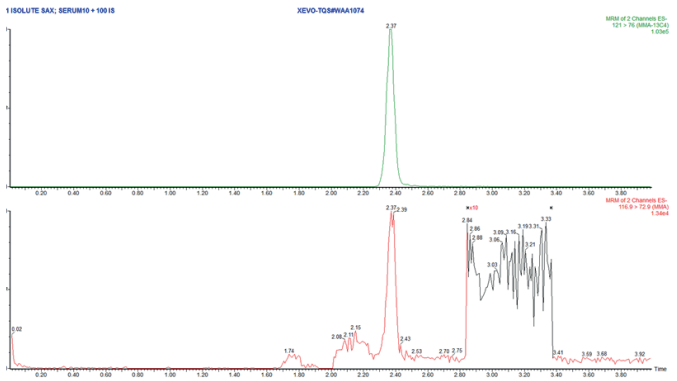


**Figure 3.** Chromatogram of $^{13}C_4$ MMA (top) at 100 ng/mL and MMA (bottom) at 10 ng/mL (~0.085 µMol/L) with x10 signal:noise indicator for the latter.

## Recovery

Serum free of MMA was spiked at 250 ng/mL (~2.11 µMol/L). High reproducible recoveries >90% and corresponding RSDs of <10% were demonstrated. Typical recovery data is shown in **Figure 4.**

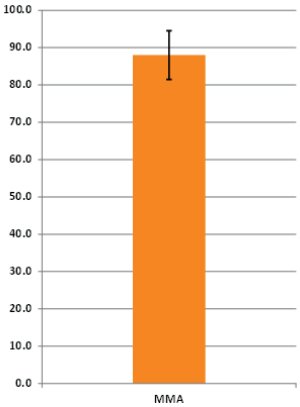



**Figure 4.** Chart demonstrating typical MMA recovery using the optimized ISOLUTE® SAX method.

### Calibration Curves

Good linearity was observed over the range 10 – 2000 ng/mL (~0.085 - ~16.949 µMol/L). **Figure 5.** shows the coefficient of determination $r^2$ for the optimized method. In addition,

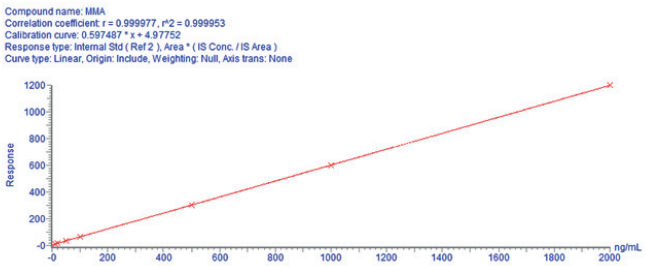



**Figure 5.** Calibration line of spiked serum extracted with the optimized protocol.

**Table 3.** Calculated MMA concentrations.

| Chromsystems Calibration Level | Set Value (ng/mL) | Calculated Value (ng/mL) |
|---|---|---|
| Calibrator 1 | 13.7 | 12.4 |
| Calibrator 2 | 28.4 | 27.0 |
| Calibrator 3 | 51.3 | 52.8 |

## Additional Notes

### Processing Guidelines

- 96-well SPE plates were processed using a Biotage® PRESSURE+96 Positive Pressure Manifold at a pressure of 1–2 psi

### Solvent Composition and Preparation Instructions

- All solvents were HPLC grade.
- 2% formic acid in acetonitrile: Add 200 µL concentrated formic acid to 9.8 mL of HPLC grade acetonitrile. Mix.
- 0.4% formic acid (aq): Add 200 µL concentrated formic acid to 49.8 mL of HPLC grade water. Mix.
- 0.4% formic acid in methanol: Add 200 µL concentrated formic acid to 49.8 mL of HPLC grade methanol. Mix.

## Ordering Information

| Part Number | Description | Quantity |
|---|---|---|
| 500-0025-P01 | ISOLUTE® SAX 25 mg Fixed Well Plate | 1 |
| 121-5203 | Collection plate, 2 mL, square | 50 |
| PPM-96 | Biotage® PRESSURE+ 96 Positive Pressure Manifold | 1 |
| SD-9600-DHS-EU | Biotage® SPE Dry Sample Concentrator System 220/240 V | 1 |
| SD-9600-DHS-NA | Biotage® SPE Dry Sample Concentrator System 100/120 V | 1 |
| C103264 | TurboVap® 96, Evaporator 220/240V | 1 |
| C103263 | TurboVap® 96, Evaporator 100/120V | 1 |

# Appendix

## Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage® Extrahera™, using ISOLUTE® SAX 25 mg SPE plates. Total time taken to process a full 96-well plate was 43 minutes. Method performance was comparable.

This appendix contains the software settings required to configure Extrahera to run this method. An importable electronic copy of this method for Extrahera can be downloaded from www.biotage.com

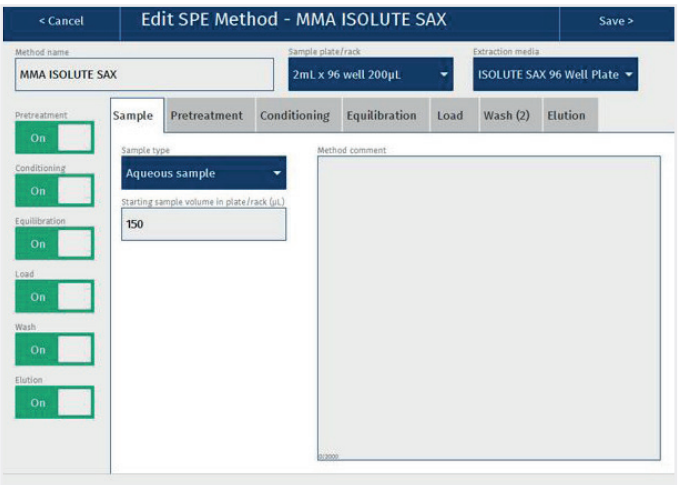

**Biotage® Extrahera™ Data**

| Analyte | Methylmalonic Acid |
|---|---|
| Recovery (n=8) at 100 ng/mL | 92.4% |
| %RSD | 2.5 |
| Linearity ($r^2$) | 0.999* |
| LLOQ | <10 ng/ mL |

*Note: Linearity experiments on Extrahera were run using 3PLUS1® Multilevel Plasma Calibrator Set Methylmalonic acid (Chromsystems Instruments and Chemicals GmbH). Manual processing using these standards gave linearity ($r^2$) of 0.999.

Data (manual processing) in the application note was generated using 'in house' spiked MMA free serum from Golden West Biologicals, Inc.

**Method Name:** MMA ISOLUTE® SAX
**Sample Plate/Rack:** 2 mL x 96 well 200 µL
**Extraction Media:** ISOLUTE SAX 96 Well Plate

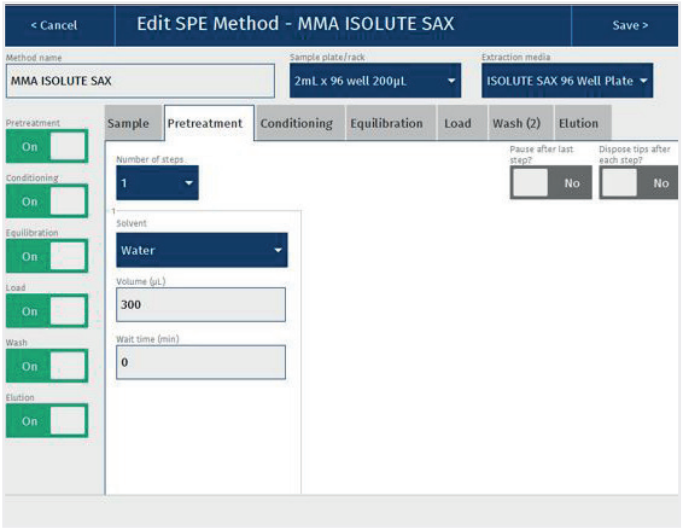



## Settings

**"Sample" Tab**

| | |
|---|---|
| **Sample Type:** | Aqueous Sample |
| **Starting Sample Volume (µL):** | 150 |
| **Method Comment:** | |

## Screenshot

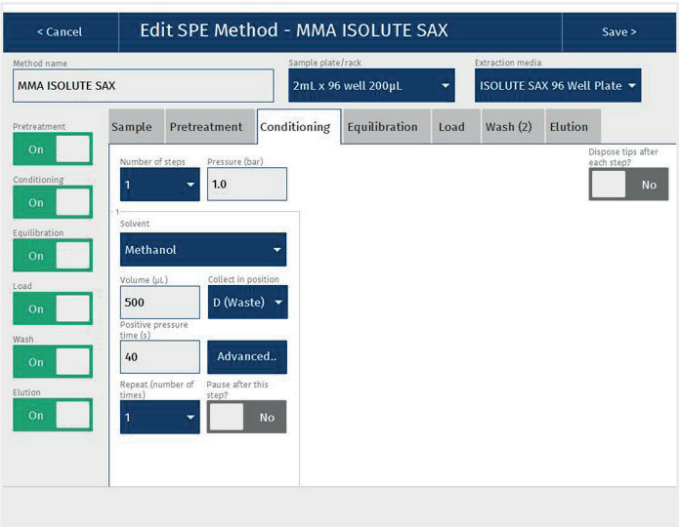



## Settings

| | Pre-treatment | Activated |
|---|---|---|
| | No. of steps | 1 |
| | Pause after last step | No |
| | Dispose tips after last step | No |

| | Solvent |
|---|---|
| 1 | Water |
| 2 | |
| 3 | |
| 4 | |

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Volume (µL) | 300 | | | |
| Wait Time (min) | 0 | | | |

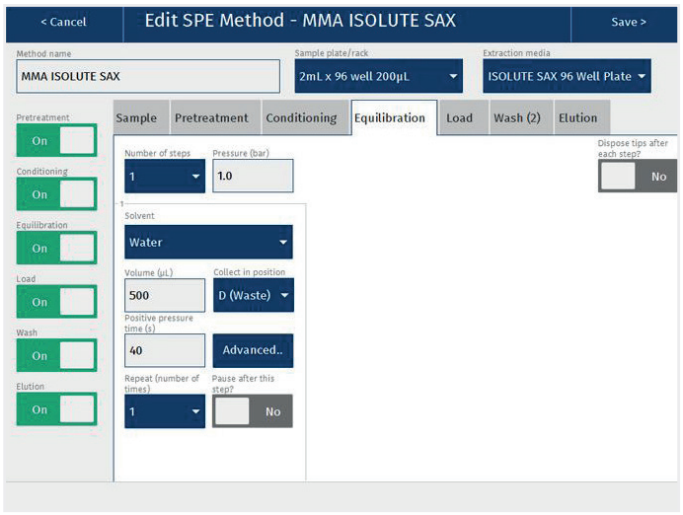



| | Conditioning | Activated |
|---|---|---|
| | No. of steps | 1 |
| | Pressure (Bar) | 1.0 |
| | Dispose tips after this step | No |

| | Solvent |
|---|---|
| 1 | Methanol |
| 2 | |
| 3 | |
| 4 | |

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Volume (µL) | 500 | | | |
| Position | D | | | |
| Positive pressure time (s) | 40 | | | |
| Repeat | 1 | | | |
| Pause after this step | No | | | |

| Advanced Settings |
|---|
| |

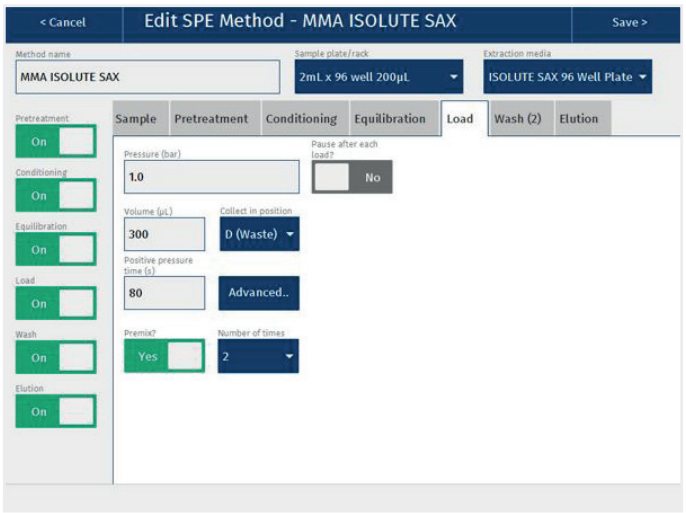



| Equilibration | Not Activated |
|---|---|
| No. of steps | 1 |
| Pressure (Bar) | 1.0 |
| Dispose tips after this step | No |

| Solvent | |
|---|---|
| 1 | Water |
| 2 | |
| 3 | |
| 4 | |

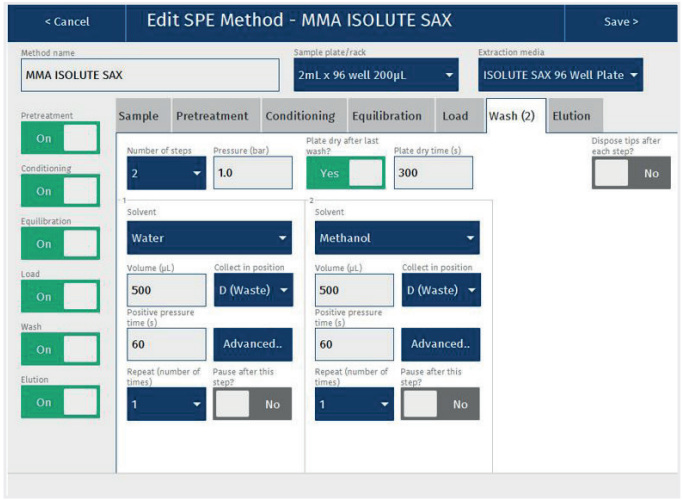
| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Volume (µL) | 500 | | | |
| Position | D | | | |
| Positive Pressure time (s) | 40 | | | |
| Repeat | 1 | | | |
| Pause after this step | No | | | |

| Advanced Settings |
|---|
| |




| Load | |
|---|---|
| Pressure (Bar) | 1.0 |
| Pause after each load | No |
| Volume (µL) | 300 |
| Collect in position | D |
| Positive pressure time (s) | 80 |
| Premix | Yes |
| Number of times | 2 |

| Advanced Settings |
|---|
| |

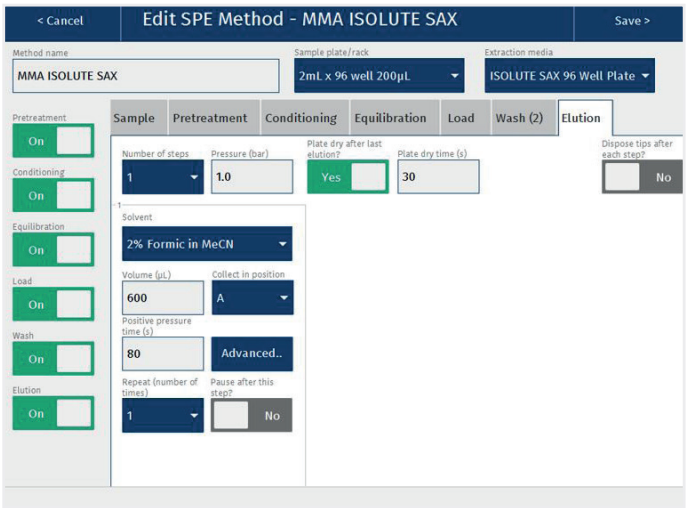



| | Wash | Activated |
|---|---|---|
| | No. of steps | 2 |
| | Pressure (Bar) | 1.0 |
| | Plate dry after last wash | Yes |
| | Plate dry time (s) | 300 |
| | Dispose tips after last step | No |

| | Solvent |
|---|---|
| 1 | Water |
| 2 | Methanol |
| 3 | |
| 4 | |

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Volume (µL) | 500 | 500 | | |
| Position | D | D | | |
| Positive pressure time (s) | 60 | 60 | | |
| Repeat | 1 | 1 | | |
| Pause after this step | No | No | | |

| | Advanced Settings |
|---|---|




| | Elution | Activated |
|---|---|---|
| | No. of steps | 1 |
| | Pressure (Bar) | 1.0 |
| | Plate dry after last elution | Yes |
| | Plate dry time (s) | 30 |
| | Dispose tips after each step | No |

| | Solvent |
|---|---|
| 1 | 2% Formic in MeCN |
| 2 | |
| 3 | |
| 4 | |

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Volume (µL) | 600 | | | |
| Position | A | | | |
| Positive pressure time (s) | 80 | | | |
| Repeat | 1 | | | |
| Pause after this step | No | | | |

| | Advanced Settings |
|---|---|



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## Solvent Properties

| | Solvent Description |
|---|---|
| 1 | Water |
| 2 | Methanol |
| 3 | 2% Formic in MeCN |
| 4 | |
| 5 | |
| 6 | |
| 7 | |
| 8 | |
| 9 | |
| 10 | |

| Solvent | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Reservoir Type** | | **Refillable** | | | | | | **Non Refillable** | | |
| Capacity | N/A | N/A | N/A | | | | | | | |
| Aspiration flow rate (mL/min) | 10 | 10 | 10 | | | | | | | |
| Dispense flow rate (mL/min) | 20 | 20 | 20 | | | | | | | |
| Lower air gap flow rate (mL/min) | 20 | 20 | 20 | | | | | | | |
| Lower air gap volume (µL) | 5 | 5 | 5 | | | | | | | |
| Upper air gap flow rate (mL/min) | 20 | 120 | 120 | | | | | | | |
| Upper air gap volume (µL) | 100 | 100 | 100 | | | | | | | |
| Upper air gap dispense pause | 300 | 300 | 300 | | | | | | | |
| Conditioning? | Yes | Yes | Yes | | | | | | | |
| Conditioning number of times | 2 | 3 | 3 | | | | | | | |
| Conditioning flow rate (mL/min) | 20 | 20 | 20 | | | | | | | |
| Chlorinated | No | No | No | | | | | | | |
| Serial dispense | No | No | No | | | | | | | |



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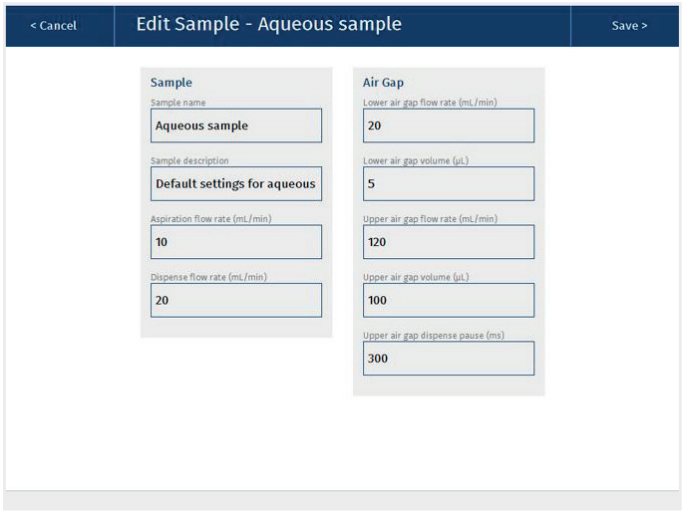
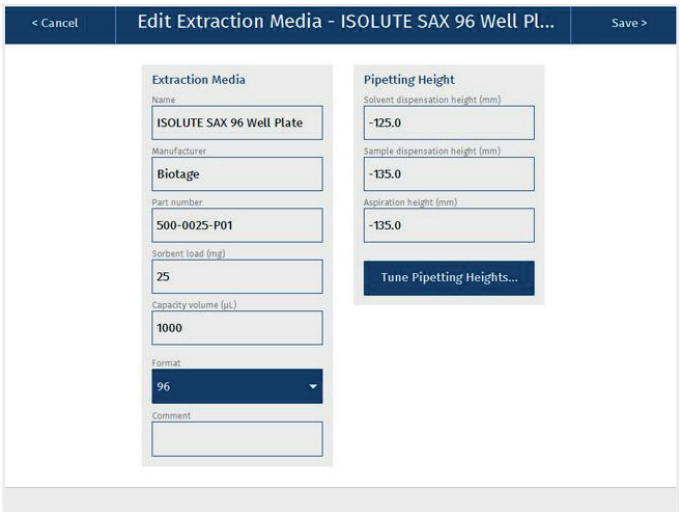
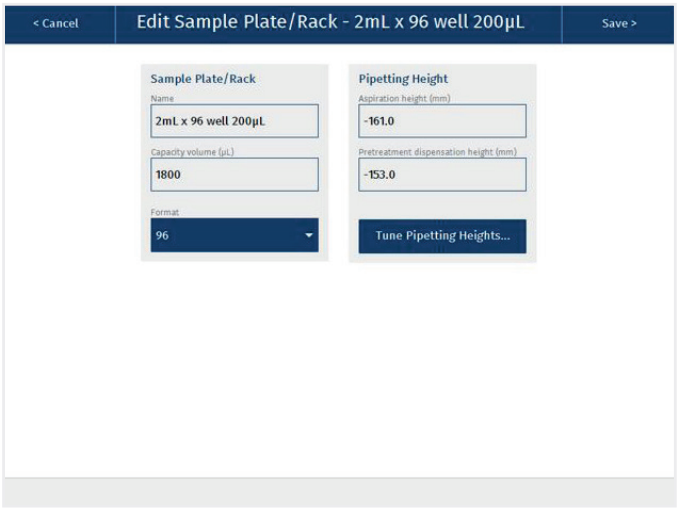


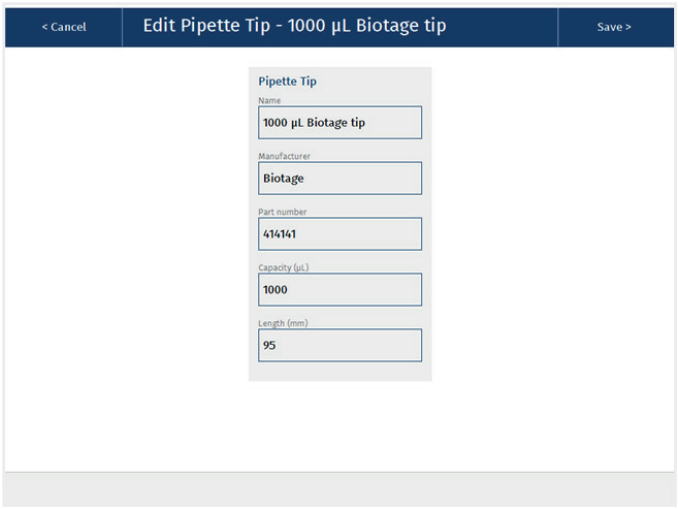

| "Sample" Screen | |
|---|---|
| Sample name | Aqueous sample |
| Sample description | Aqueous sample |
| Aspiration flow rate (mL/min) | 10 |
| Dispense flow rate (mL/min) | 20 |
| Lower air gap flow rate (mL/min) | 20 |
| Lower air gap volume (µL) | 5 |
| Upper air gap flow rate (mL/min) | 120 |
| Upper air gap volume (µL) | 100 |
| Upper air gap dispense pause | 300 |




| "Extraction Media" Screen | |
|---|---|
| Name | ISOLUTE SAX |
| Manufacturer | Biotage |
| Part number | 500-0025-P01 |
| Sorbent load (mg) | 25 |
| Capacity volume (µL) | 1000 |
| Format | 96 |
| Comment | |
| Solvent dispensation height (mm) | -125.0 |
| Sample dispensation height (mm) | -135.0 |
| Aspiration height (mm) | -135.0 |




| "Sample Plate/Rack" Screen | |
|---|---|
| Name | 2 mL Sample x 96 well 200 µL |
| Capacity volume (µL) | 1800 |
| Format | 96 |
| Aspiration height (mm) | -161.0 |
| Pre-treatment dispensation height (mm) | -153.0 |




| "Pipette tip" Screen | |
|---|---|
| Name | 1000 µL Biotage Tip |
| Manufacturer | Biotage |
| Part number | 414141 |
| Capacity (µL) | 1000 |
| Length (mm) | 95 |

## Additional Information

In this automated method, 150 µL of pre-spiked (IS) serum sample is mixed with 300 µL of water during the pre-treatment step. This gives a total volume of 450 µL, from which 300 µL is loaded.

**EUROPE**
Main Office: +46 18 565900
Toll Free: +800 18 565710
Fax: +46 18 591922
Order Tel: +46 18 565710
Order Fax: +46 18 565705
order@biotage.com
Support Tel: +46 18 56 59 11
Support Fax: + 46 18 56 57 11
eu-1-pointsupport@biotage.com

**NORTH & LATIN 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
Support Tel: +1 800 446 4752
Outside US: +1 704 654 4900
us-1-pointsupport@biotage.com

**JAPAN**
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com
jp-1-pointsupport@biotage.com

**CHINA**
Tel: +86 21 2898 6655
Fax: +86 21 2898 6153
cn_order@biotage.com
cn-1-pointsupport@biotage.com

To locate a distributor,
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