Ultra-Sensitive Method for the Determination of 1,25 di-OH Vitamin D2 and 1,25 di-OH Vitamin D3 in Serum Using Supported Liquid Extraction Prior to LC-MS/MS

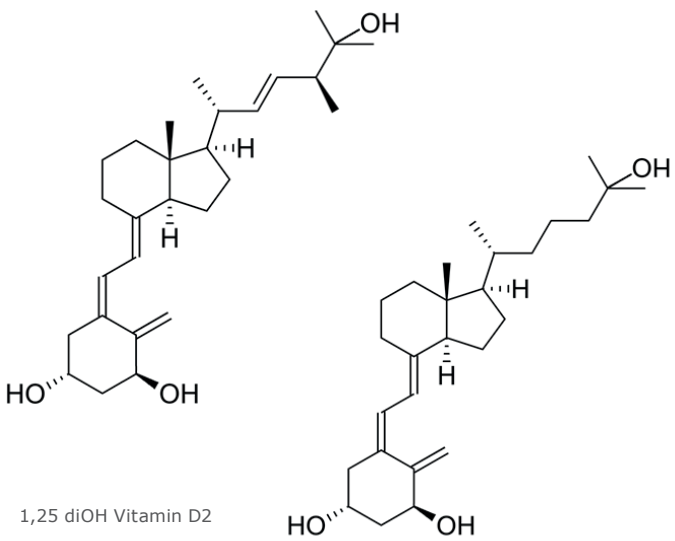

Figure 1. Analyte structures

Introduction

This application note describes a protocol for the extraction of 1,25 di-OH Vitamin D2 and 1,25 di-OH Vitamin D3 metabolites from serum using supported liquid extraction prior to LC-MS/MS detection. The method described in this application achieves highly reproducible recoveries for 1,25 diOH Vitamins D2 and D3. A calibration range between 5 and 500 pg/mL is demonstrated using a starting volume of 0.25 mL of serum. Sensitivity is maximized through the use of a simple PTAD derivatization and formation of a methylamine complex.

ISOLUTE® SLE+ Supported Liquid Extraction plates and columns offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation.

Analytes

1,25 diOH Vitamin D2 and 1,25 diOH Vitamin D3 with deuterated 1,25 diOH Vitamin D3 as internal standard (all purchased from Sigma Aldrich Chemical Co., Poole, UK).

Sample Preparation Procedure

Format:

ISOLUTE® SLE+ 400 µL Supported Liquid Extraction plate, Part number 820-0400-P01

Sample Pre-treatment

Combine a 10 µL volume of internal standard working solution with 250 µL of serum. Mix and leave to stand for at least 30 minutes to allow the protein binding of the analytes and internal standard to reach equilibrium.

Combine the resulting serum and internal standard mixture (260 µL) with a 250 µL volume of propan-2-ol : water (50:50, v/v) solution. Cap the plate and shake for 15 minutes

Sample Loading

Load pre-treated serum (300 µL) under gravity. Allow the sample to absorb for 5 minutes.

Analyte Extraction and Derivatization

Ensure a collection plate containing 200 µL of derivatization solution (0.25 mg/mL PTAD in ethyl acetate : heptane (8:92, v/v)) in each well is in position.

Apply heptane (2 x 700 µL) and allow to flow under gravity. Apply a pulse of positive pressure to pull through any remaining extraction solvent into the collection plate.

Cap the collection plate and shake for between 30 minutes and 1 hour.

Evaporation

Evaporate the extracts to dryness at approximately 40 °C and reconstitute in 70% methanol (aq) containing 50 µL/L methyl-amine (150 µL).

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UPLC Conditions

Instrument
Shimadzu Nexera UHPLC system

Column
ACE Ultracore 2.5 Super C18 50 x 2.1 mm

Mobile Phase:
Acetonitrile : Water (50:50) containing 30 µL methylamine per 500 mL

Flow Rate
0.5 mL min^{-1}

Injection
20 µL

Gradient
Isocratic

Column Temperature
40 °C

Sample Temperature
20 °C

Table 1. Typical retention times for analytes.

Compound	Retention time (min)
1,25 diOH Vitamin D2	1.5
1,25 diOH Vitamin D3	1.2
D_3 1,25 diOH Vitamin D3 (Internal standard)	1.2

Switch Valve Settings

Switch to MS
0.8–2.0 min

Switch to Waste
Initial setting to 0.8 min, 2.0 min to 5.0 min

MS Conditions

Ions were selected in order to achieve maximum sensitivity using multiple reaction monitoring.

Instrument
AB Sciex 5500

Curtain Gas
25 psi

Collision Gas
7 psi

IonSpray Voltage
5500 V

Temperature
550 °C

Ion Source Gas 1 (GS1)
50 psi

Ion Source Gas 1 (GS1)
50 psi

Setting Time
0 ms

Pause Between Mass Ranges
5.007 ms

Table 2. Mass Spectrometer conditions for analytes and internal standard.

Analyte	Transition	DP (V)	EP (V)	CE (V)	CXP (V)	Dwell (ms)
1,25 diOH Vitamin D2 – PTAD – Methylamine complex	635.4 to 314.1	50	8	24	16	130
1,25 diOH Vitamin D3 – PTAD – Methylamine complex	623.4 to 314.1	50	8	29	13	130
D_3 1,25 diOH Vitamin D3 – PTAD – Methylamine complex	626.4 to 317.1	50	8	29	13	130

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Results

Recovery and Reproducibility

Extraction recoveries were calculated by comparing peak areas of extracted samples (spiked at a concentration of 250 pg/mL) with a blank sample fortified with a quantity of analyte equivalent to a recovery of 100%.

Table 3. Extraction recoveries.

Analyte	Average Recovery (n=4)	% RSD
1,25 diOH Vitamin D2	104.3	3.2
1,25 diOH Vitamin D3	99.3	3.6

Linearity

Linearity was checked from 5 to 500 pg/mL. Charcoal stripped serum was used for the production of calibration lines. Calibration lines are constructed using the peak area ratio of 1,25 diOH Vitamin D2 to D_3 1,25 diOH Vitamin D3 and 1,25 diOH Vitamin D3 to D_3 1,25 diOH Vitamin D3 using a linear regression with 1/x weighting. In the diagrams below the blanks have been added as standards but unselected for accurate correlation coefficient calculation.

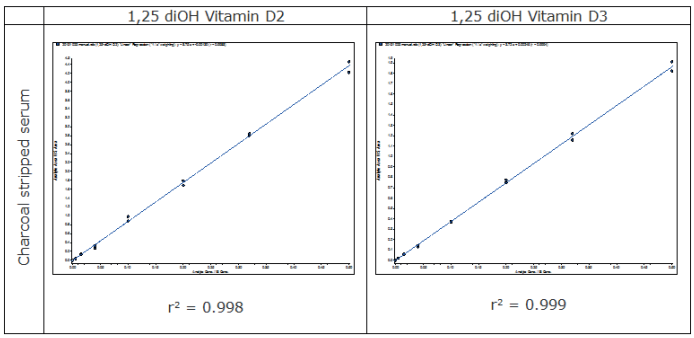

Figure 2. Representative calibration plots of 1,25 diOH metabolites.

Sensitivity

Charcoal stripped serum was used for the production of calibration lines. Sensitivity was demonstrated by comparing the chromatograms of remaining endogenous levels and those over-spiked at a concentration of 5 pg/mL (displayed on the same scale).

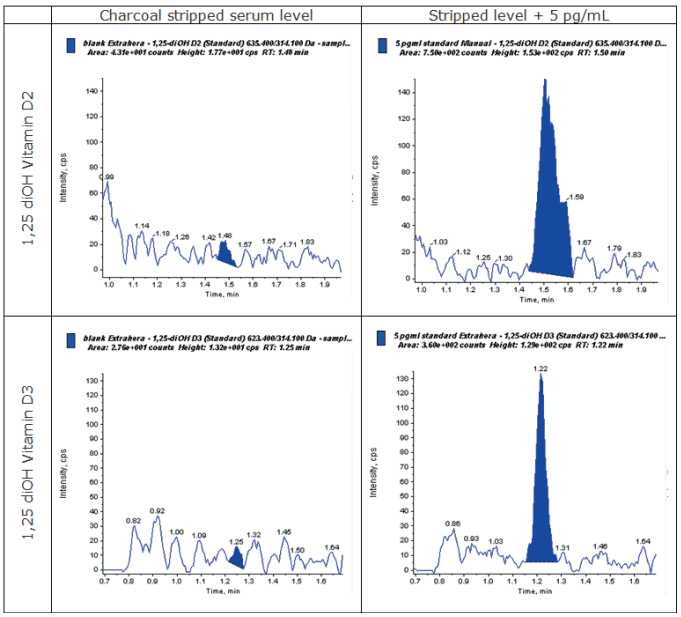

Figure 3. Signal at the lower limit of quantitation.

Selectivity

No peak could be seen from extracting and derivitizing a spiked sample of 24,25 diOH Vitamin D3 suggesting that this cannot form the PTAD methylamine complex. No effect on 1,25 diOH measurement was observed by adding 24,25 diOH Vitamin D3 at a high concentration as are shown in the full scale traces below (all displayed with identical scales).

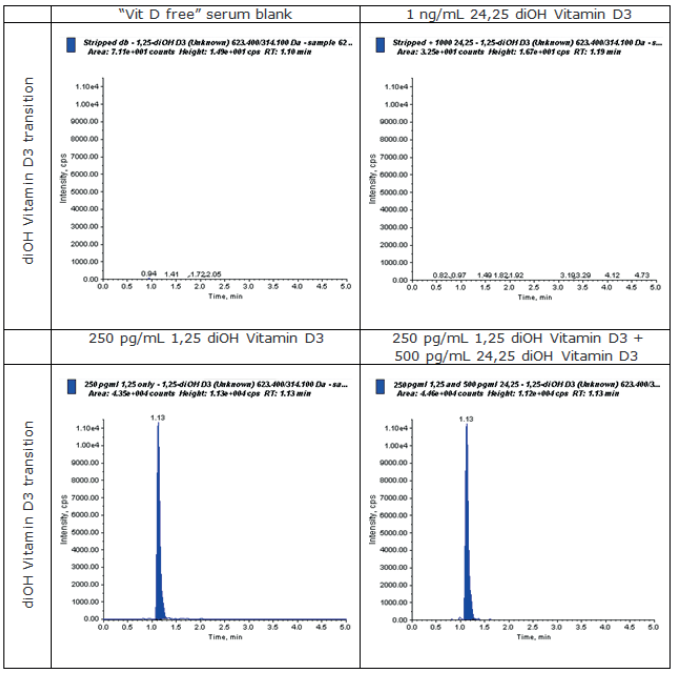

Figure 4. Absence of 24,25 diOH Vitamin D3 cross reactivity.

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Preparation of Standards and Calibrators

1. Prepare separate substock solutions of 1,25 diOH Vitamin D2 and 1,25 diOH Vitamin D3 at concentrations of 0.1 µg/mL in methanol and store at approximately -20 °C when not in use.
2. Prepare internal standard working solution by diluting D3 1,25 diOH Vitamin D3 to a concentration of 6.25 ng/mL in methanol
3. Prepare the top calibration standard by spiking charcoal stripped or Vitamin D free serum with 1,25 diOH Vitamin D2 and 1,25 diOH Vitamin D3 substock solutions such that they each had a concentration of 500 pg/mL. A 2 mL volume of this calibration solution is typically prepared by combining 10 µL of each of the two substock solutions and diluting to a total volume of 2mL with serum.
4. The top calibration standard was either used neat; or aliquots of 6, 18, 48, 120, 240 and 384 µL were transferred into separate vials and each diluted to a total volume of 600 µL with blank serum prior to mixing and allowing to equilibrate. This gave additional calibration standards at concentrations of 5, 15, 40, 100, 200 and 320 pg/mL in a quantity sufficient for duplicate sampling if required.

Reagent Preparation

1. Derivatization solution. Dissolve 5 mg of PTAD in 1.6 mL ethyl acetate before diluting to a total volume of 20 mL with heptane. This volume is sufficient for a theoretical 100 analyses. Prepare the solution daily, immediately prior to use
2. Reconstitution solvent: prepare 100 mL of reconstitution solvent by combining 70 mL methanol with 30 mL water and 5 µL methylamine.
3. UPLC mobile phase. Prepare 1 L of mobile phase by combining 500 mL of water, 500 mL of acetonitrile and 60 µL of methylamine.
4. Water used throughout was 18.2 MΩ.cm drawn from a Direct-Q water purifier (Merck Millipore, Watford, UK).
5. Other reagents
   a. PTAD (4-Phenyl-1,2,4-triazole-3,5-dione) Sigma-Aldrich Chemical Co. (Poole, UK).
   b. Methylamine, Sigma-Aldrich Chemical Co. (Poole, UK)
   c. Propan-2-ol, Methanol, Acetonitrile, Ethyl Acetate, Heptane. Sigma-Aldrich Chemical Co. (Poole, UK)
   d. Vitamin D free Serum, Golden West Biologicals Inc. (Temecula, CA, USA)

Additional Notes

» Sample pre-treatment. 1,25-OH vitamin D metabolites are strongly protein bound. Serum sample is mixed with aqueous 50% (v/v) propan-2-ol to disrupt protein binding. The ratio of serum to 50% propan-2-ol (aq) was optimized at 1:1 (v/v) for maximum analyte recovery and sensitivity.

   a. Higher proportions of serum to aqueous propan-2-ol lead to reduced recoveries due to insufficient binding disruption
   b. Lower proportions of serum to aqueous propan-2-ol lead to decreased sensitivity due to the smaller sample volume that can be loaded
   c. Insufficient mixing at this stage lead to reduced and/or variable analyte recovery.

» Extraction solvent: Other extraction solvents such as MTBE and hexane were evaluated as an alternative to heptane. MTBE gave very low analyte recoveries and should be avoided. Hexane gave an equivalent recovery for the 1,25 diOH Vitamin D3 complex but a reduction of about 25% for 1,25 diOH Vitamin D2 complex, and is not therefore recommended.

» PTAD derivatization.

   a. Derivatization is performed within the extraction procedure for streamlined workflow and increased analyte sensitivity. In tests the derivatization prior to evaporation was found to give a 2–3 times improvement in peak size compared to the more traditional 'final' derivatization.
   b. b.The incubation period of 30 mins to 1 hour shaking before evaporation was optimized. It is not recommended that the plate is evaporated immediately after elution. Allowing the plate to shake for only 15 minutes prior to evaporation resulted in a reduction of peak areas by as much as 10%.
   c. Preparation of 'fortified blank' extracts for recovery calculations can be difficult due to the solvent limitations of the PTAD derivatization step. During development of this method, a fortification solution was prepared of the metabolites in acetonitrile, this was dispensed into a vial, evaporated and then reconstituted in ethyl acetate containing PTAD at 3.125 mg/mL. A 16 µL volume of this solution was added to the wells of a fortification derivitization test and diluted by the addition of 184 µL heptane.
   d. The use of PTAD derivatization to improve sensitivity has little effect on the overall cost of the assay per sample. At the time of analysis and assuming no wastage PTAD costs were approximately $0.0033/€0.0029/£0.0021 per sample.
   e. Concentrations quoted in this report refer to the levels of the vitamin D metabolite and not concentrations of the metabolite-PTAD-methylamine complex itself.

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» A methanol based reconstitution solvent was used as it was found to give an improved peak shape than when reconstituting in the mobile phase.

» UPLC Conditions

a. It is essential that the UPLC column (and guard cartridge, if used) are stable at high pH conditions as the mobile phase (60 µL methylamine per litre) recorded a pH of approximately pH 9 to 10.
b. The nature of PTAD derviatisation gives 2 complex species for each metabolite form. Because of the high performance of the UPLC column used in this application note, compressed separation conditions are used to ensure co-elution of both complex species, and hence accurate quantification of each analyte.
c. The method uses an isocratic mobile phase for a number of reasons. The sample needs to be reconstituted in a relatively strong solvent to solubilize the derivatized complexes. The use of a gradient starting with a more aqueous mobile phase could result in peak fronting.

» Simultaneous extraction and analysis of mono-OH Vitamin D metabolites.

a. This extraction method can be used to simultaneously extract 25-OH Vitamin D2 and 25-OH Vitamin D3. The UPLC method can also separate 25-OH Vitamin D2 and 25-OH Vitamin D3 giving maximum peaks at 3.8 min and 2.9 min respectively. Because of the higher levels of the single hydroxyl metabolites in serum it is recommended that calibration standards of these are spiked approximately 100 times higher. The 25-OH Vitamin metabolites each give two distinct peaks of which the second larger peak can be used for quantification purposes.
b. The run time of 5 minutes is long enough for endogenous 25 OH Vitamin D metabolites that have formed a complex with PTAD and methylamine to pass through the column. If a shorter run time is used it should be checked that these compounds from a previous injection don't interfere or quench the signal from the di-OH metabolites in the next run.

Ordering Information

Part Number	Description	Quantity
820-0400-P01	ISOLUTE® SLE+ 400 µL Supported Liquid Extraction Plate	1
121-5203	Collection plate, 2 mL, square	50
121-5204	Piercable sealing cap	50
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
C103264	TurboVap® 96, Evaporator 220/240V	1
C103263	TurboVap® 96, Evaporator 100/120V	1

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Appendix

Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage® Extrahera™, using ISOLUTE SLE+ 400 µL Supported Liquid Extraction plates. 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

Table 1. Relative Performance of Extrahera vs Manual Method

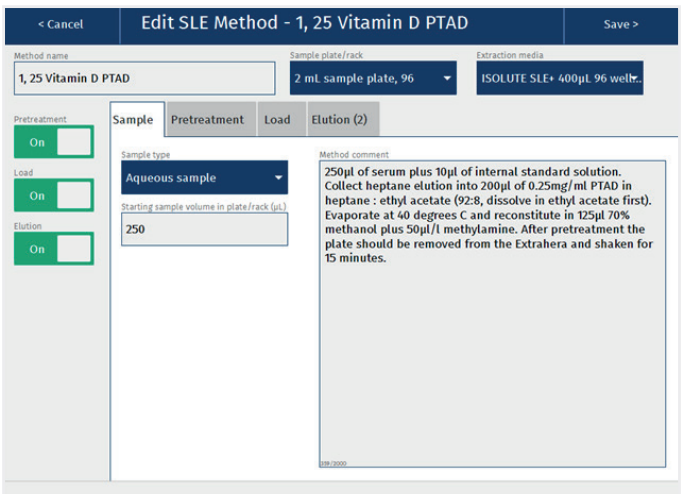
	Biotage® Extrahera™ Method	Manual Method
Average Peak Area 1,25 diOH Vitamin D2	13625	11800
Improvement vs. Manual Method (%)	15.5	
% RSD of Peak Areas (n=8)	4.3	5.5
Average Peak Area 1,25 diOH Vitamin D3	18975	19187
Improvement vs. Manual Method (%)	-1.1	
% RSD of Peak Areas (n=8)	4.6	3.9

Table 2. Biotage® Extrahera™ Performance

Analyte	Linearity (5–500 pg/mL)	LLOQ
1,25 diOH Vitamin D2	0.997	~5 pg/mL
1,25 diOH Vitamin D3	0.994	~5 pg/mL

Method name: 1, 25 Vitamin D PTAD
Sample plate/rack: 2 mL sample plate, 96
Extraction Media: ISOLUTE® SLE+ 400 µL 96 Well Plate

Screenshot



Settings

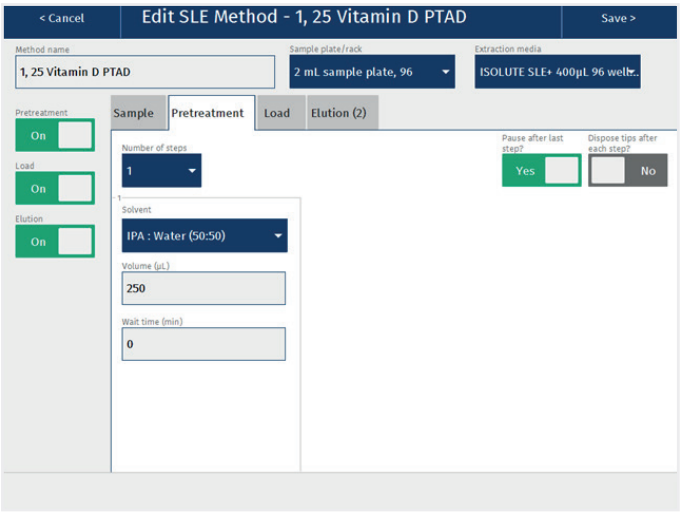
"Sample" Tab

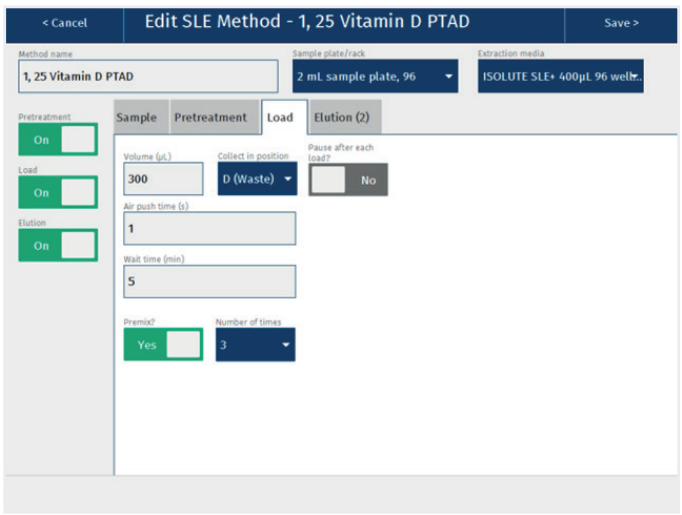
Sample Type: Aqueous Sample
Starting Sample Volume (µL): 250
Method Comment:

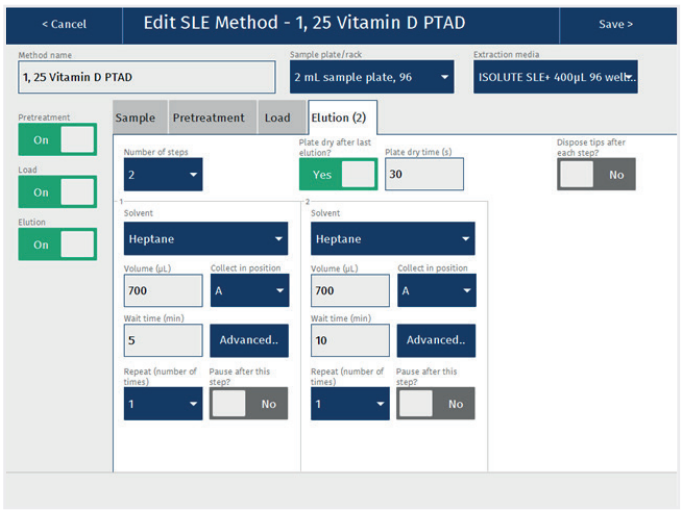
Following pretreatment the plate should be removed from Extrahera and shaken for 15 minutes.

Collect heptane elution into a collection plate containing 200 µL of a 2.5 mg/mL solution of PTAD in heptane:ethyl acetate (92:8, dissolve PTAD in ethyl acetate first). Evaporate at 40 °C and reconstitute in 125 µL of methanol:water (70:30) containing 5 µL/L of methylamine.

Screenshot







Settings

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

	Solvent
1	IPA : Water (50 : 50)
2	
3	
4	

	1	2	3	4
Volume (µL)	250			
Wait Time (min)	0			

	Load	Activated
	Air Push Time (s)	1
	Pause after each load	No
	Volume (µL)	300
	Collect in position	D
	Wait time (min)	5
	Premix	Yes
	Number of times	3

	Elution	Activated
	No. of steps	2
	Pause after last step	No
	Plate dry	Yes
	Plate dry time (s)	30

	Solvent
1	Heptane
2	Heptane
3	
4	

	1	2	3	4
Volume (µL)	700	700		
Collect in position	A	A		
Wait time (min)	5	10		
Repeat	1	1		
Pause	No	No		

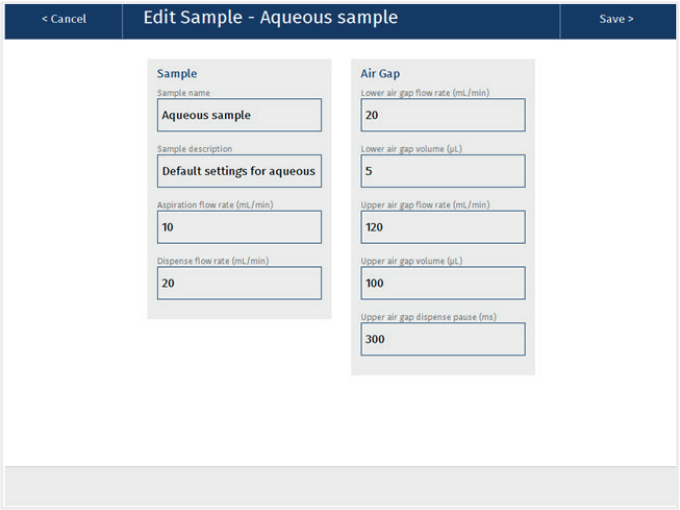
	Advanced Settings
	No

Solvent Properties

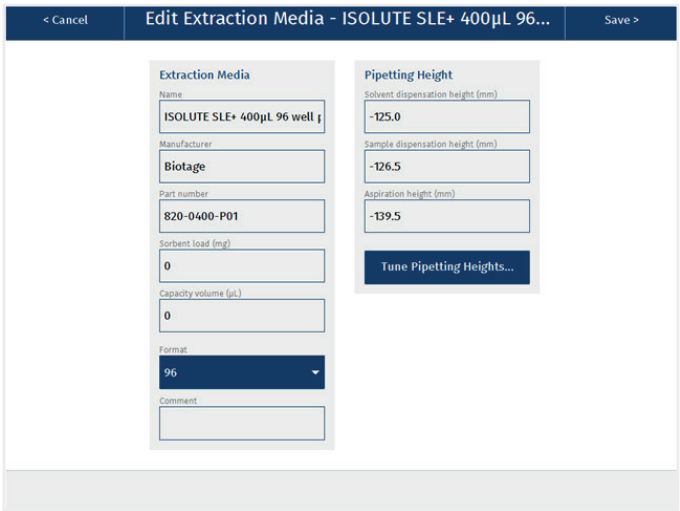
	Solvent Description
1	Heptane
2	
3	
4	
5	
6	
7	
8	
9	
10	

Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir type	Refillable					Non Refillable				
Capacity	N/A									
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									
Conditioning?	Yes									
Conditioning number of times	3									
Conditioning flow rate (mL/min)	20									
Chlorinated	No									
Serial dispense	No									

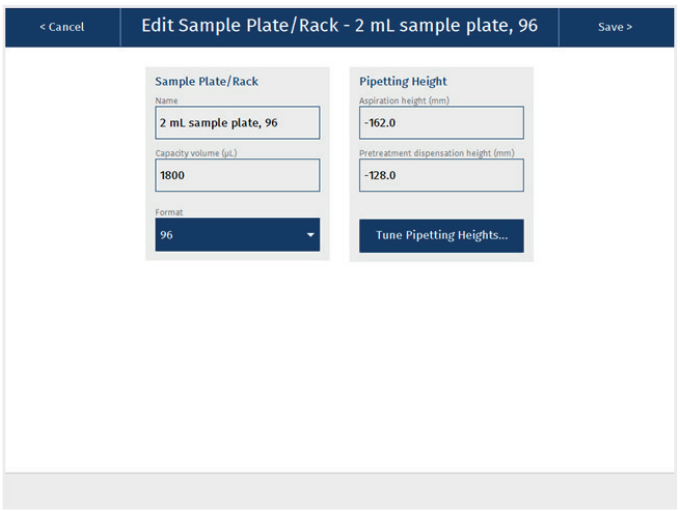
Screenshot



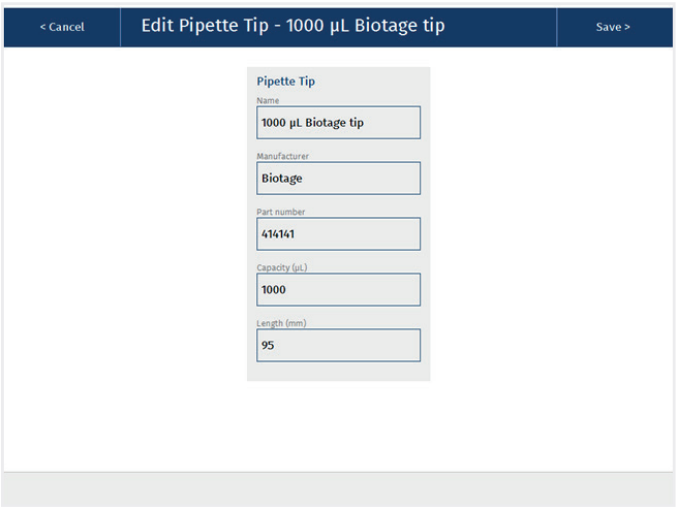
"Sample" Screen	
Sample name	Aqueous sample
Sample description	Aqueous sample
Aspiration flow rate (mL/min)	10
Dispense flow rate (mL/min)	20
Lower air gap flow rate (mL/min)	20
Lower air gap volume (µL)	5
Upper air gap flow rate (mL/min)	120
Upper air gap volume (µL)	100
Upper air gap dispense pause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



"Extraction Media" Screen	
Name	ISOLUTE® SLE+ 400 µL 96-well plate
Manufacturer	Biotage
Part number	820-0400-P01
Sorbent load (mg)	0
Capacity volume (µL)	0
Format	96
Comment	N/A
Solvent dispensation height (mm)	-125.0
Sample dispensation height (mm)	-126.5
Aspiration height (mm)	-139.5



"Sample Plate/Rack" Screen	
Name	2 mL Sample Plate, 96
Capacity volume (µL)	1800
Format	96
Aspiration height (mm)	-162.0
Pre-treatment dispensation height (mm)	-128.0



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

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