Method Optimization for the Low Level Detection of Vitamin B7 from Human Serum Using UPLC-MS/MS Analysis

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Introduction

Part of the B complex group of water soluble vitamins, vitamin B7 is involved in various metabolic processes within the body. It is a small polar carboxylic acid which can present challenges in bioanalysis. This poster discusses the impact of optimization of various parts of the method development process to maximize sensitivity allowing low level analysis of vitamin B7 from human serum. Method parameters: ionization mode polarity, precursor ion selection, MRM transitions, chromatography and LC mobile phase additives along with solid phase extraction protocols were optimized for increased sensitivity.

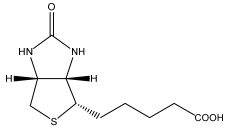

Figure 1. *Structure of Vitamin B7.*

Experimental

Reagents

Vitamin B7 and $^2D_2$ ISTD, formic acid, ammonium hydroxide, ammonium acetate, ammonium fluoride and LC/MS grade solvents were obtained from Sigma-Aldrich Chemical Co. (Poole, UK). Water (18.2 MΩ.cm) was drawn fresh daily from a Direct-Q 5 water purifier (Merck Millipore, Watford, UK). Human serum and stripped serum was purchased from Golden West Biologicals Inc. (Ca, USA.).

Sample Preparation

Extractions were performed using the 10 mg 96-well plate format.

Solid Phase Extraction Optimization: SPE investigated polymer-based non-polar retention with corresponding mixed-mode strong and weak anion exchange mechanisms using the EVOLUTE® EXPRESS sorbent family. The final SPE protocols are detailed in ***Table 1.*** All solvent/buffer steps were optimized to 500 µL while elution solvent volumes were minimized to 200 µL.

Vitamin B7-$^2D_2$ ISTD: 200 µL of serum spiked with ISTD at 250 pg/mL.

Table 1. *Optimized SPE procedures.*

Step	EVOLUTE EXPRESS® ABN	EVOLUTE® EXPRESS AX	EVOLUTE® EXPRESS WAX
Condition	MeOH	MeOH	MeOH
Equilibration	1% Formic acid aq	10 mM NH_4OAc pH8	10 mM NH_4OAc pH6
Pre-treatment	1:1 1% Formic acid aq	1:1 10 mM NH_4OAc pH8	1:1 10 mM NH_4OAc pH6
Sample load	400 µL	400 µL	400 µL
Wash 1	H_2O	10 mM NH_4OAc pH8	10 mM NH_4OAc pH6
Wash 2	95/5 H_2O/MeOH	MeOH	MeOH
Elution	0.1% NH_4OH 90/10 H_2O/MeOH	0.5% Formic acid in MeOH	2% NH_4OH MeOH

Post extraction: All extracts were evaporated to dryness using a SPE Dry unit at 40 °C and reconstituted in 200 µL 90/10 aq/MeCN.

UPLC Conditions

Instrument: Waters ACQUITY I-Class UPLC equipped with a 15 µL flow-through needle (Waters Assoc., Milford, MA, USA)
Column: ACE Excel C18-PFP: 100 mm x 2.1 mm id, 1.7 µm, (ACT, UK)
Mobile Phase: A: 1 mM NH_4F aq **: B:** MeCN
Flow Rate: 0.4 mL/min
Gradient: Linear ramp 10-18 % B over 1.5 min; step to 80 % B; hold 0.5 min; resume initial starting conditions at 2 min
Column Temperature: 40 °C
Injection Volume: 10 µL

Mass Spectrometry

Instrument: Xevo TQ-S triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface. Negative ions were acquired in the multiple reaction monitoring (MRM) mode using the deprotonated precursor ions as shown in ***Table 2***.
Desolvation Temperature: 500 °C: **Ion Source Temperature:** 150 °C
Collision Gas Pressure: 3.6 x 10^{-3} mbar: **Collision energy:** 15 eV

Table 2. *MS conditions.*

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Vitamin B7 Quant	243.1 > 166.0	25	15
Vitamin B7 Qual	243.1 > 200.0	25	15
Vitamin B7-[$^2H_2$]	245.1 > 168.0	25	15

Results

LC/MS Optimization

The majority of work presented in the literature uses + ion mode for vitamin B7. As a small polar carboxylic acid, ionization is possible using both + and – ion. Chromatography demonstrated better retention when using acidic mobile phase additives and higher organic content using + ion mode. However, more selective MRM transitions were obtained using – ion mode. Overall sensitivity and signal to noise was better in – ion mode when using appropriate mobile phases not containing acidic additives. Further modification of mobile phase resulted in selection of 1 mM NH_4F aq and MeCN as the polar aprotic solvent as shown in ***Figure 2.***

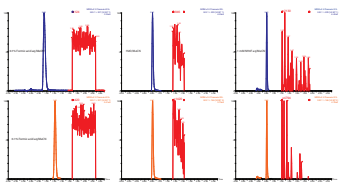

Figure 2. *MRM transition signal to noise comparing polarity mode and solvent selection.*

Extraction Optimization

Initial experiments using generic methodology for each sorbent demonstrated recoveries around 70% using EVOLUTE® EXPRESS WAX while EVOLUTE® EXPRESS AX and the non-polar EVOLUTE® EXPRESS ABN delivered > 80% as shown in ***Figure 3.*** Further method modification using the WAX sorbent demonstrated minimal improvement therefore use was discontinued.

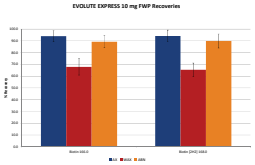

Figure 3. *Extraction recovery profiles for SPE chemistry comparison.*

Further method optimization for recovery and extract cleanliness using EVOLUTE® EXPRESS ABN and AX sorbents are demonstrated in ***Figures 4 and 5***. respectively. Non-polar wash protocols were performed using H_2O and 95/5 H_2O/MeOH. EVOLUTE EXPRESS AX was optimized using 10 mM NH_4OAc buffer at a pH of 8 using a standard generic wash regimen of aq buffer followed by MeOH.

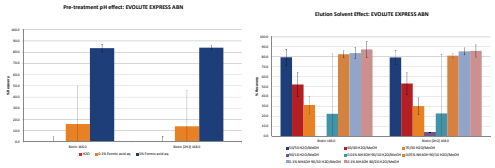

Figure 4. *Extraction recovery profile for pre-treatment (left) and elution solvent (right) optimization using EVOLUTE® EXPRESS ABN.*

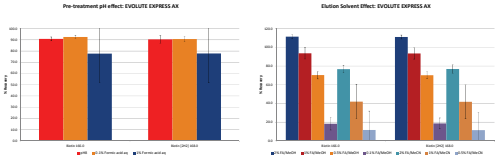

Figure 5. *Extraction recovery profile for pre-treatment (left) and elution solvent (right) optimization using EVOLUTE® EXPRESS AX.*

Final modification to improve extract cleanliness for EVOLUTE® EXPRESS ABN involved pH modification to allow elution in high aq proportions. EVOLUTE® EXPRESS AX was optimized for elution solvent acidity. Due to the presence of water wettable components it was also possible to eliminate plate conditioning steps prior to sample loading. This resulted in a simple load-wash-elute procedure. ***Figure 6.*** demonstrates final extraction recoveries (documented in ***Table 1.)*** along with corresponding L-W-E procedure for EVOLUTE® EXPRESS ABN.

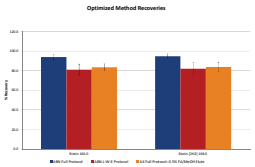

Figure 6. *Extraction recovery profile for pH modification of elution solvent using EVOLUTE® EXPRESS ABN.*

Final extraction protocols were investigated for phospholipid content. ***Figure 7.*** demonstrates the total ion chromatograms (TICs) of typical phospholipid MRMs.

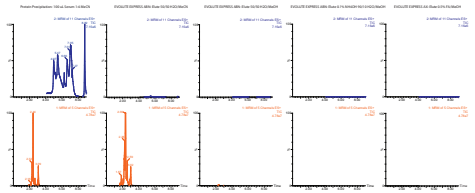

Figure 7. *Phospholipid MRM TICs for final serum extracts for each method.*

Extract cleanliness was also investigated using post-column infusion (PCI) experiments. Blank extracts injected into Vitamin B7 infused mobile phase was used to determine regions of suppression for each technique, as demonstrated in ***Figure 8***.

Figure 8. *PCI baselines of the various optimized extraction protocols, indicating suppression with negative intensity (Left.*

Overall performance was deemed superior using EVOLUTE® EXPRESS ABN. Spiked calibration curves constructed from 25-1000 pg/mL returned excellent coefficients of determination (r^2) and sensitivity. Typical calibration lines are demonstrated in ***Figure 9.***

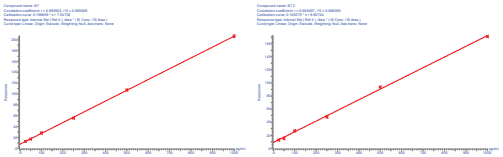

Figure 9. *Serum calibration curves extracted using EVOLUTE EXPRESS® ABN.*

Conclusion

- Better sensitivity was observed using negative ion with the combination of ammonium fluoride (aq) and MeCN as mobile phases.
- SPE optimization resulted in recoveries greater than 90% while demonstrating good removal of matrix components in the form of proteins, phospholipid and sample pigmentation.
- Optimization of various parts of the method development process resulted in a sensitive assay for the analysis of Vitamin B7 from serum using EVOLUTE® EXPRESS ABN.

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