"A high throughput solid phase extraction method for vitamin B3 and related metabolites from pooled human serum using ISOLUTE SCX-3 96 well plates prior to mixed mode LC-MS/MS"

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Introduction

There are 8 water soluble analogues of vitamin B that are relevant to the metabolic health of the human host. Of particular interest to this study is the determination of vitamin B3, a precursor to the synthesis of hormones that cascade through a number of biochemical systems in vivo. The development data presented in this report detailed sample prep workflow strategies to facilitate population screening of the parent compound nicotinic acid (pka(s) = 2.2, 4.8) as well as 2 relevant metabolites niacinamide (pka = 3.54) and nicotinuric acid (pka(s) = 3.1, 3.5) in a single analysis. The structure of these analytes are detailed in Figure 1. The analytes of interest were fortified into pooled mixed gender serum. The samples were loaded on to a 25mg 96 well plate. The reconstituted extracts were measured using a gradient mixed-mode LC-MS/MS method.

Analytes of Interest

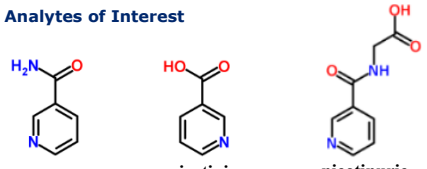

Figure 1: Structures of the analytes of interest

Experimental Procedure

Reagents

HPLC grade water, methanol, acetonitrile, nicotinuric acid, acetic acid and formic acid (FA) were purchased from Sigma-Aldrich Co. (Atlanta, GA.). Nicotinic acid and niacinamide standards were obtained from Cerilliant Corp (Round Rock, TX). The biological fluids were obtained from BioChemEd services.

Table 1*: Positive ion mode transitions (+)*

Otimized solid phase extraction methods

Samples were optimized using a Biotage ISOLUTE SCX-3, 25 mg 96 well plate. The sorbent chemistry is a ethylbenzene sulfonic acid functionalized silica (non-end-capped). The sorbent chemistry is further detailed in Figure 2. The optimized method is detailed in Table 1.

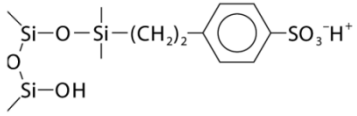

Figure 2: *ISOLUTE SCX-3 sorbent chemistry*

Table 1: *Sample preparation method*

Step	25 mg, ISOLUTE SCX-3, 96 well plate method
Sample	50µL serum + 150µL 2% acetic acid(aq)
Condition	1 mL MeOH
Equilibration	1 mL 2% acetic acid (aq)
Load	200µL pretreated sample
Wash 1	2 x 1 mL 68/30/2 ; H_2O/MeOH/acetic acid
Wash 2	2 x 1 mL 98/2 ; MeOH/acetic acid
Elute	2 x 400µL ; 95/5 MeOH/NH_4OH
Evap / Recon	100µL 0.1% formic acid (aq)

Chromatography

Chromatographic separation was accomplished using the Agilent 1200 Liquid Handling System (Agilent Technologies, Berkshire, UK). Optimized gradient chromatographic conditions (Table 2) were identified using a IMTAKT Scherzo SM-C18 column (2 mm x 150mm, 3.0 µm). The injection volume was 20 µL. The gradient conditions were given in Table 2. The mobile phase A and B were 5mM ammonium formate / 0.1% FA (aq) and MeCN respectively.

Table 2: *LC Gradient conditions*

Step #	Time (min)	Flow Rate (µL/min)	A (%)	B (%)
1	0.5	200	80	20
2	3.0	200	70	30
3	4.0	200	70	30
4	5.0	200	80	20
5	8.0	200	80	20

Mass Spectrometry

Detection of the target analytes was optimized using an Applied Biosystems /MDS Sciex 4000 Q-Trap hybrid triple quadrapole / linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA.) equipped with a Turbo Ionspray® interface operated in positive ion mode. The MRM transitions used in this study were detailed in Table 3. Data acquisition can stop collecting after **4.5 min**. (to facilitate multi-plexed column switching methods).

Table 3*: MS/MS parameters*

Analyte	g /mole	MRM transition (m/z)	DP	CE	Dwell time (ms)
niacin	123.1	124.1 → 80.1	125	35	300
niacinamide	122.1	123.1 → 80.0	125	25	300
nicotinuric acid	180.0	181.0 → 79.0	40	25	300

Results

The sorbent selection for this study proved interesting as nicotinic acid and nicotinuric acid have both acid and basic pKa values. A series of polymeric based cation and anion ion exchange sorbents were evaluated; however, the ethylbenzene sulfonic acid functionalized silica-based sorbent demonstrated the best choice inclusive of all three analytes. A representative chromatogram for a 20 ng/mL fortified serum sample is detailed in Figure 3.

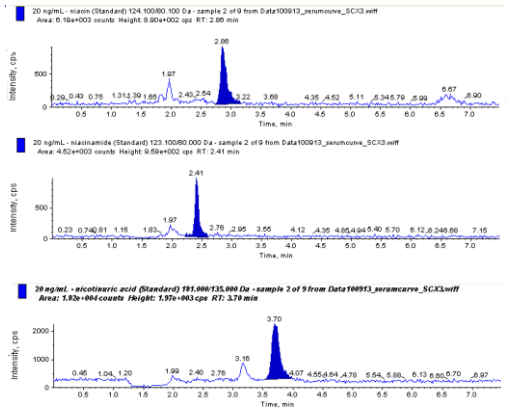

Figure 3: Relative recovery (%) and method repeatability for the extraction of vitamin B3 and related metabolites from serum.

A set of fortified serum specimens was also prepared at 40 ng/mL levels (n=6). The relative recovery plot is detailed in Figure 4. The method repeatability as %RSD was determined <15% for all analytes.

The optimization of the sample conditioning step compared the following variables: 2% formic, 2% acetic, 4% acetic, 50mM NH4Ac (pH=6), 50 mM NH_4Ac (pH=7). The peak area response for 2% acetic acid provided the best results inclusive of all three analytes.

A gradient mix of water and methanol was evaluated for the wash 1 step to target the endogenous polar interferences (e.g. organic and inorganic salts). It was determined that incorporating 2% acetic acid into the optimized 70/30 water/MeOH solution was helpful in maintaining analyte recoveries. Acetonitrile did not offer any advantage as wash solvent or an elution solvent. Evaluation of the wash 2 solvent did not show analyte response as breakthrough in the wash.

There was a significant benefit in the quality of the data obtained when increasing the wash volumes in replicate aliquots. The analyte suppression values can be optimized as < 20% for each (Figure 4). A loss of analyte recovery was observed after 3x cycles so a balance of analyte recovery vs sample cleanliness should be considered when targeting clinically relevant LOQ values.

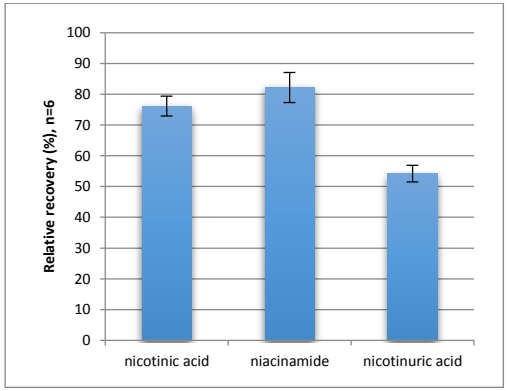

Figure 4: Relative recovery (%) and method repeatability (%RSD)

Conclusions

The ISOLUTE SCX-3 96 well plate format demonstrated as a viable option for serum measurements over a relevant concentration range in clinical diagnostics.

It is anticipated that strategies presented in this poster would be of broad-based interest to clinical laboratories focused on **combining parent/metabolite assays into a single analysis**

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