Automated analysis of a Panel of Fat Soluble Vitamins A, D, E and K in Serum by Supported Liquid Extraction and LC-MS/MS Analysis

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Introduction

Simultaneous fat-soluble vitamin testing can be challenging for a variety of reasons: solubility limitations of the analytes, high levels of protein binding and wide variation of concentration ranges from one vitamin to another. This poster investigates extraction optimization strategies to provide a method for the simultaneous analysis of Vitamin A (Retinol and Beta Carotene), Vitamin D (25-OH D2 and D3), Vitamin E (Alpha Tocopherol) and Vitamin K (Phylloquinone K1, and Menaquinone K2). Samples could be processed manually or on the automated Extrahera™ platform.

Experimental

Reagents

Standards, ISTDs, BHT, ammonium acetate and formic acid were purchased from Sigma-Aldrich Company ltd. (Gillingham, UK). LC/MS grade solvents were from Honeywell Research Chemicals (Bucharest, Romania). 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 were purchased from Golden West Biologicals Inc. (Ca, USA.) and serum calibrators from Chromsystems (Munich, Germany).

Sample Preparation

Extractions were developed using supported liquid extraction. ISOLUTE® SLE+ was used in the 400 µL capacity 96-well plate format (P/N 820-0400-P01) following a load-wait-elute procedure (***Figure 1***).

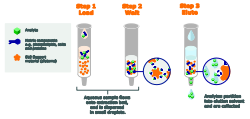

Figure 1. *Schematic of ISOLUTE® SLE+ Supported Liquid Extraction Procedure.*

Sample Pre-treatment: Serum (100 µL) or substitute matrix was spiked with 10 µL of standards and ISTD solution in IPA. Solutions were left to stand in the dark for an hour to equilibrate. Samples were pre-treated with 400 µL of IPA : Heptane (1:3) containing BHT at 1 mg/mL and mixed thoroughly.

Sample Application: A 500 µL aliquot of the pretreated matrix was transferred onto the ISOLUTE® SLE+ plate. A pulse of positive pressure at 5 psi (3-5 seconds) was applied to initiate flow and the samples left to absorb for 5 minutes.

Analyte Extraction: Heptane (500 µL) was applied and allowed to flow under gravity for 5 minutes. A second aliquot of heptane (500 µL) was applied and allowed to flow under gravity for 5 minutes. A pulse of positive pressure at 10 psi (10-20 seconds) allowed complete removal of the final aliquot.

Post extraction: Extracts were evaporated at room temperatures and reconstituted in 150 µL of IPA prior to injection.

UPLC Conditions

Instrument: Waters Acquity UPLC (Waters Assoc., Milford, MA, USA)
Column: Restek Raptor Biphenyl 2.7 µm, 100 x 2.1 mm + EXP guard (Restek, USA)
Mobile phase: A, 5 mM NH_4OAc, 0.1% formic acid (aq);
B, 5 mM NH_4OAc, 0.1% formic acid in 75/25 MeOH/IPA
Flow rate: 0.4 mL/min
Gradient: Initial 60/40; linear to 100% B, 3 min and hold; resume initial conditions, 5.2 min.
Column / Sample temp: 40 / 10 °C **Injection volume:** 10 µL

Mass Spectrometry

Instrument: Waters Quattro Premier XE triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK). Positive ions were acquired in the multiple reaction monitoring (MRM) mode (***Table 2***).
Desolvation Temp: 450 °C **Ion Source Temp:** 150 °C
Collision Gas Pressure: 3.5 x 10^{-3} mbar:

Table 1. **MRM Parameters**

Analyte	Transition	Cone, V	Collision Energy, V
α-tocopherol (E) *	433.3 > 165.9	25	22
Retinol (A)	269.3 > 92.9	18	20
ß-carotene (A)	536.3 > 444.4	30	15
25-OH-vitamin D2	395.5 > 269.5	30	30
25-OH-vitamin D3	383.5 > 257.5	30	17
d6 25-OH-vitamin D3	389.5 > 263.5	30	16
Phylloquinone (K1)	445.3 > 186.9	22	20
Menaquinone-4 (K2)	451.4 > 187.0	30	23

* Detuned with unoptimized transition due to high concentration response

Results

Evaporation Optimization

Evaporation losses were measured in Retinol, Alpha Tocopherol and Beta Carotene using Biotage 2 mL collection plates with room temperature and no Ethylene Glycol giving optimal results. This is demonstrated in **figure 2** (the error bars represent the RSDs above and below the extraction recovery bars to illustrate precision).

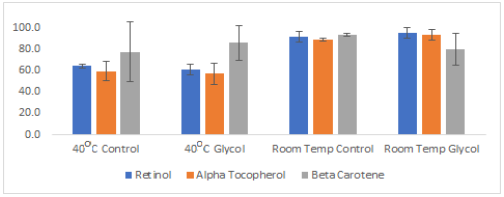

Figure 2. *Evaporation Investigation (with and without 2 µL ethylene glycol at room temperature and at 40°C).*

Analyte handling challenges

Fat soluble vitamins may be light sensitive so amber tubes are used throughout. Alpha Tocopherol exists as a thick oil making stock solutions difficult to prepare to desired concentrations. Beta Carotene has limited solubility but was soluble in MTBE at concentrations of 0.1 mg/mL or below. The antioxidant BHT (Butylated Hydroxy Toluene) was used at a level of 1 mg/mL in all lab prepared stocks, sub-stocks and pre-treatment solvents.

Both Vitamin Ks exist in serum at very low concentrations, likely to be well below 10 ng/mL and less than 0.05% of the concentrations typically measured of Alpha Tocopherol which required 'de-tuning'.

Sample precipitation challenges

Fat soluble vitamins are very highly protein bound in serum. In order to accurately quantify these they must extract in a consistent manner to calibration standards which are manually spiked to specified levels in vitro. Performing a precipitation step is necessary however it creates challenges. When using the precipitation method from our 25 OH Vitamin D method the majority of the fat soluble vitamins were found to either stick to the polypropylene walls of the sample container or stay in the precipitate. Having too strong a precipitate can make sample mixing difficult. Propan-2-ol was used as a precipitation solvent as this led to a finer precipitate that could be mixed more easily in a pipette tip. Heptane was added during the load step as the presence of the immiscible solvent encouraged the separation of the analytes from the protein precipitate and encouraged the analyes to stay in solution. **Figure 3** shows that when rapidly mixed the sample can form a temporary suspension.

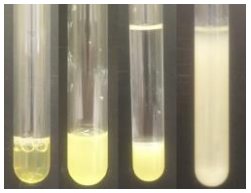

Figure 3. *Illustration of sample (left to right) untreated, treated prior to 1 hr incubation, pretreated and pretreated with mixing to demonstrate the temporary suspension.*

Samples were processed manually or using the Biotage® automated workstation Extrahera™ Classic. Due to the inconsistent sample appearance the entire sample is transferred to the SLE. Despite the large loading volume only about 200 µL is aqueous or aqueous soluble. Following evaporation samples needed to be reconstituted in 100% IPA as the presence of water within this solvent can result in analytes coming out of solution over the course of an analytical run. To improve the solubility of Beta Carotene in the mobile phase and it's chromatography IPA was added to mobile phase B at a level of 25% v/v.

ISOLUTE® SLE+ Optimization

Initial work focused on the use of Ethyl acetate as this gave the greatest recoveries of the analytes compared to heptane or MTBE. Tests found that this method extracted phospholipids and so heptane was used instead based on its non polarity and it's successful use for the Vitamin D method. SLE optimization was then generally directed by solubility challenges of the analytes.

Results in **figure 4** demonstrate a significant improvement in recoveries when 100µL sample was precipitated and loaded without Heptane, with 100 µL heptane added and finally as 400 µL of a suspension of predominantly Heptane and IPA (final method).

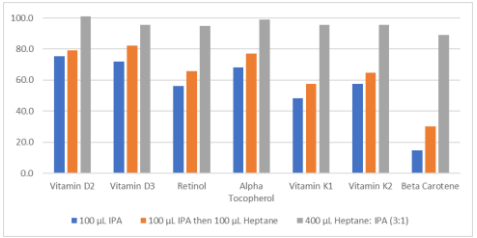

Figure 4. *Development of pretreatment to 100 µL serum. Addition of 100 µL IPA only, IPA followed by 100 µL Heptane and then addition of 400 µL of a Heptane : IPA (3:1) solution.*

Representative calibration lines in **figure 5** show a combination of linear and logarithmic plots. 25 OH Vitamin D2 (a) 25 OH Vitamin D3 (b) and Retinol (c) used a ratio with d6 25 OH Vitamin D3. Alpha Tocopherol (d), Vitamin K1 (e), Vitamin K2d4 (f) and beta carotene (g) were based on peak areas only. A Vitamin K specific internal standard will be necessary for accurate K1 and K2d4 measurement.

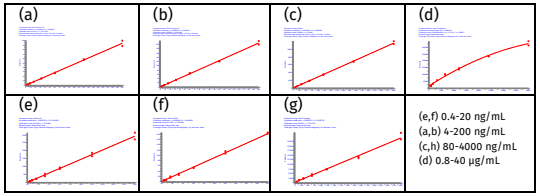

Figure 5. *Stripped serum calibration lines of vitamins A, D, E and K*

Extracts were phospholipid free as is demonstrated in **figure 6** where the extracts are compared alongside acetonitrile protein precipitation of the same volume of serum.

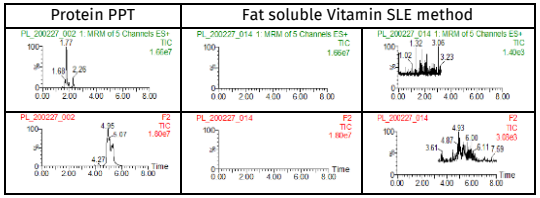

Figure 6. *Phospholipid profile of extract displayed 'full scale' (right) and on the same scale as an equivalent protein precipitation (left).*

Conclusion

A method has been developed for the simultaneous determination of vitamins A, D, E and K in human serum.

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