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Simultaneous Analysis of Vitamins A and E in Serum by UPLC-MS/MS for Clinical Research

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Abstract

This Application note demonstrates a fast-clinical research method for the simultaneous analysis of vitamins A and E in human serum.

Benefits

- Sample requirement of only 100 µL

- Removal of phospholipids using Oasis PRiME HLB µElution Plate

- Fast analysis time, three minutes

Introduction

Vitamins A and E are fat soluble and they each have several forms. Retinol is the major form of vitamin A and alpha-tocopherol is the major form of vitamin E.

Extraction and analysis of vitamins A and E by LC-MS/MS has historically involved laborious sample preparation and lengthy analysis time with high solvent consumption. Currently, the majority of vitamin A and E analysis is performed by HPLC with UV detection, however, some of these methods still suffer time-consuming steps.

As a result, a clinical research method has been developed for the simultaneous extraction and analysis of vitamins A and E by UPLC-MS/MS. The method uses low sample volume and low mobile phase flow rates to perform the analysis in a three-minute run time. Chromatographic separation of extracted samples was performed on an ACQUITY UPLC I-Class System using an ACQUITY UPLC HSS PFP Column, followed by mass detection on a Xevo TQD Tandem Quadrupole Mass Spectrometer (Figure 1).

Figure 1. Waters ACQUITY UPLC I-Class/Xevo TQD System.

Experimental

UPLC conditions

System:	ACQUITY UPLC I-Class (FTN) with Column Manager
Needle:	30 µL
Column:	ACQUITY UPLC HSS PFP, 1.8 µm, 2.1 × 50 mm (p/n: 186005965)
Mobile phase A:	Water with 2 mM ammonium acetate and 0.1% formic acid

Mobile phase B:	Methanol with 2 mM ammonium acetate and 0.1% formic acid
Needle wash solvent:	90% methanol$_{(aq)}$
Purge solvent:	20% methanol$_{(aq)}$
Column temp.:	40 °C
Injection volume:	20 µL
Flow rate:	0.4 mL/min
Gradient:	See Table 1
Run time:	3.0 min (approximately 3.5 min injection-to-injection)

MS conditions

System:	Xevo TQD
Resolution:	MS1 (0.75 FWHM) MS2 (0.75 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (see Table 2 for details)
Polarity:	ESI positive
Capillary:	0.6 kV
Source temp.:	150 °C
Desolvation temp.:	500 °C

Dwell time:	0.03 sec

Data management

MassLynx v4.2 with TargetLynx XS Application Manager

Method conditions

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.40	35	65	Initial
2.00	0.40	2	98	6
2.55	0.40	35	65	11

Table 1. Gradient table.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Vitamin A (Quan)	269.15	93.0	22	22
Vitamin A (Qual)	269.15	83.0	22	22
[^{2}H$_8$]-Vitamin A	277.3	98.0	30	20
Vitamin E (Quan)	431.4	165.1	30	30
Vitamin E (Qual)	431.4	83.0	30	35
[^{2}H$_6$]-Vitamin E	437.4	171.1	30	30

Table 2. MRM parameters of vitamin A and vitamin E quantifier ions, qualifier ions, and their internal standards.

Sample preparation

Vitamin A (retinol) and vitamin E (alpha-tocopherol) certified reference solutions were purchased from Sigma Aldrich (Poole, UK) and the stable labeled internal standards 2H8-vitamin A and ^{2}H$_6$-vitamin E, were purchased from Buchem B.V. (Apeldoorn, Netherlands) and Sigma Aldrich, respectively. Calibrators were prepared in a surrogate matrix of MSG2000 stripped human serum

purchased from Golden West Biologicals (Temecula, CA). The calibration range for vitamin A was 100–2000 ng/mL and 2.1–21.1 µg/mL for vitamin E. The QCs were also prepared in MSG2000 stripped human serum at 300, 600, and 1500 ng/mL for vitamin A and 4.1, 7.1, and 16.1 µg/mL for vitamin E.

Note: The MSG2000 contained endogenous levels of vitamin E (1.1 µg/mL), therefore, standards and QC were prepared by standard addition.

Sample extraction

Fifty µL of internal standard (~20 µg/mL of $^2H_8$-vitamin A and $^2H_6$-vitamin E) was added to 100 µL of sample. The samples were precipitated with 450 µL of ethanol and diluted with 200 µL of water. Samples were mixed and centrifuged. The supernatant was diluted prior to solid-phase extraction, 100 µL supernatant was added to 900 µL of ethanol: water (5:3 v/v). Following dilution, 650 µL was transferred to an Oasis PRiME HLB 96-well µElution Plate (p/n: 186008052). The samples were washed with 25% acetonitrile$_{(aq)}$ and eluted into a 96-well plate containing 1 mL glass inserts (p/n: 186000855) with 2 × 65 µL of acetonitrile. Water (70 µL) was added to the 96-well plate and mixed before analysis.

Results and Discussion

No system carryover was observed from high concentration samples into subsequent blank injections. A 1:4 dilution was successfully employed on high concentration samples with accuracies ranging from 86% to 102%.

Precision was assessed by extracting and measuring five replicates of samples across five days (n=25). Total precision and repeatability were ≤6.9% CV at concentrations of 300, 600, and 1500 ng/mL for vitamin A and 4.1, 7.1, and 16.1 µg/mL for vitamin E (Table 3).

	Vitamin A		Vitamin E	
	Repeatability (%CV)	Total (%CV)	Repeatability (%CV)	Total (%CV)
Low QC	4.8	5.4	3.9	6.8
Mid QC	3.4	3.4	3.1	5.8
High QC	3.9	4.7	3.1	6.9

Table 3. Precision performance summary for vitamin A and vitamin E.

Analytical sensitivity was assessed by extracting and quantifying 10 replicates of low-level vitamin A and E samples prepared in stripped serum over five days. The limit of quantification (LOQ) was determined to be the lowest concentration at which precision (repeatability) was ≤20% CV and S:N (ptp) was ≥10:1. The LOQ was determined to be 50 ng/mL for vitamin A and 1.1 μg/mL for vitamin E (Table 4).

Compound	Spiked concentration (ng/mL)	Precision (%CV)
Vitamin A	100 ng/mL	7.3
	50 ng/mL	9.6
	10 ng/mL	40.0
Vitamin E	2.1 μg/mL	6.2
	1.6 μg/mL	4.3
	1.3 μg/mL	14.2
	1.1 μg/mL	14.7

Table 4. Analytical sensitivity summary for vitamin A and vitamin E.

The method was shown to be linear across the range of 28–4800 ng/mL for vitamin A and 2–51.6 μg/mL for vitamin E, when low and high pools were mixed in known ratios over the range. All calibration lines in spiked stripped serum were linear with a coefficient of determination (r^2) ≥0.997 for vitamin A and ≥0.991 for vitamin E across 14 separate occasions.

Typical endogenous interferences (albumin, bilirubin, and uric acid) and exogenous compounds (retinyl palmitate, retinoic acid, retinal, and vitamin E-acetate) were tested and recoveries of the test samples compared to controls were all within ±15%. In addition, chromatographic separation was achieved from structurally similar compounds of vitamins A and E, as shown in Figures 2 and 3, respectively.
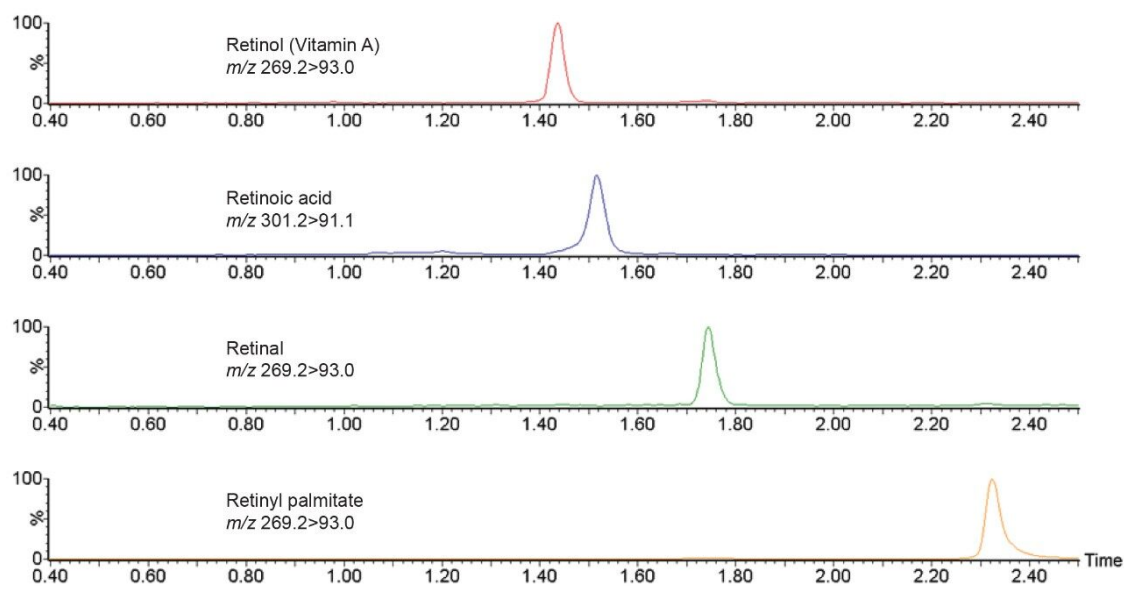
Figure 2. Chromatographic separation of vitamin A from its metabolites and structurally similar compounds.
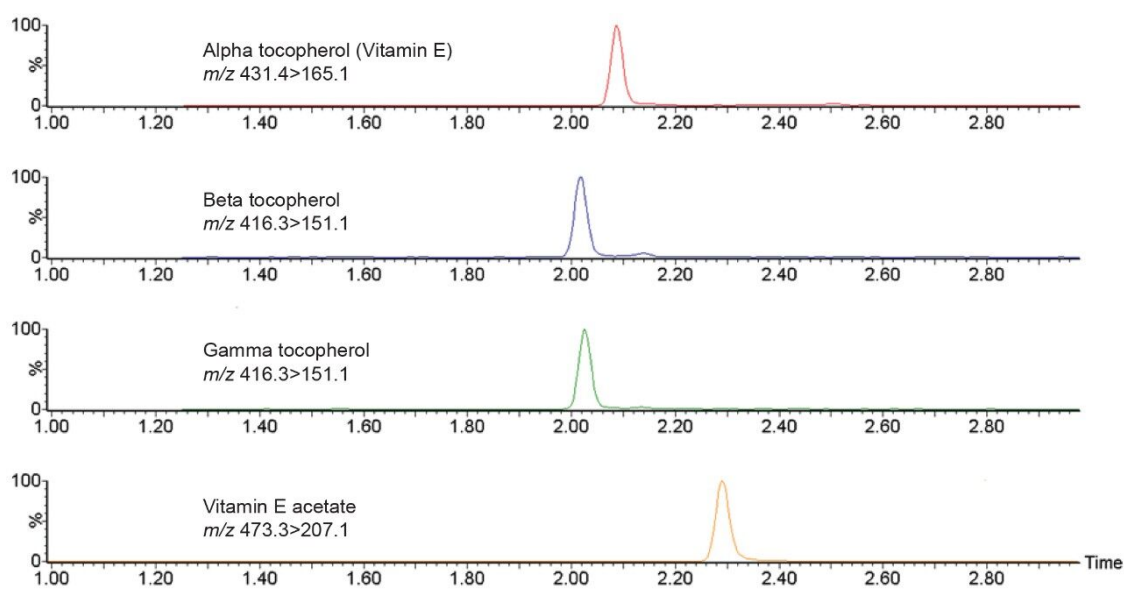

Figure 3. Chromatographic separation of vitamin E from structurally similar compounds.

Matrix factor investigations were performed using donor serum samples from six individuals. For vitamin E, the endogenous peak areas were separately quantified and post-spiked samples at low- and high-concentration levels were adjusted using the mean peak areas to enable comparison to

solvent spiked samples (Table 5).

Compound	Spiked concentration (ng/mL)	Matrix factor based on peak area mean (Range)	Matrix factor based on response mean (Range)
Vitamin A	300	1.01 (0.96–1.04)	1.03 (1.00–1.09)
	1500	1.01 (0.89–1.10)	1.08 (0.99–1.13)
Vitamin E	4100	0.94 (0.85–0.98)	1.00 (0.93–1.04)
	161000	0.93 (0.87–0.98)	1.03 (0.94–1.09)

Table 5. Matrix factor summary for vitamin A and vitamin E.

The results of matrix factor investigations show that the method developed does not suffer from significant matrix effects. The use of Oasis PRiME HLB µElution for the sample preparation helps minimize interferences from the matrix through the removal of phospholipids. Using the method detailed, Oasis PRiME HLB reduced the total number of phospholipids detected in a serum sample by >96% when compared to the same sample extracted by protein precipitation (Figure 4).
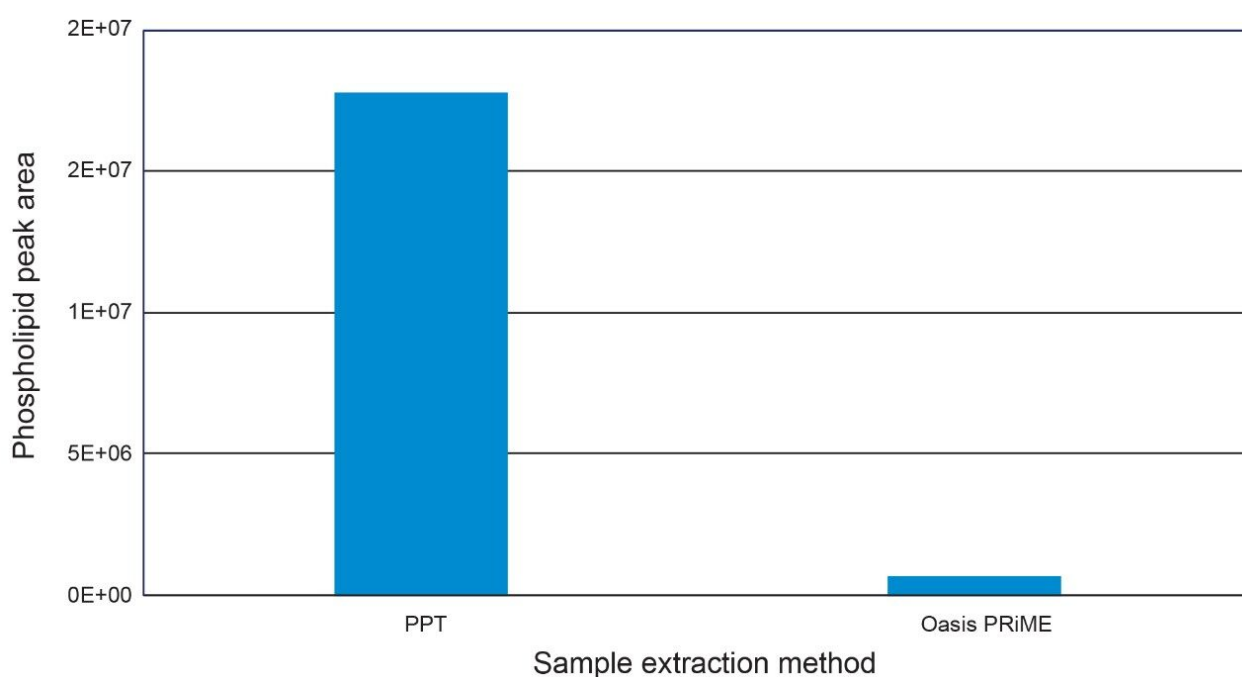

Figure 4. The bar graph shows the mean residual phospholipid traces showing the significant difference between protein precipitation and Oasis PRiME HLB.

Accuracy was assessed by analyzing 30 UK NEQAS vitamin A and E samples with calculated concentrations compared to the ALTM (All Laboratory Trimmed Mean). The correlation for vitamins A and E can be seen in Table 6, showing good agreement with the EQA scheme.

Analyte	Deming regression			Linear fit (r)	Bland-Altman bias
	Equation	Proportional bias?	Constant bias?		
Vitamin A	y=0.11+0.90x	Y (p=0.0003)	Y (p=0.0040)	0.91	-7.0%
Vitamin E	y=-1.63+0.97x	N (p=0.3753)	Y (p=0.0270)	0.98	-10.9%

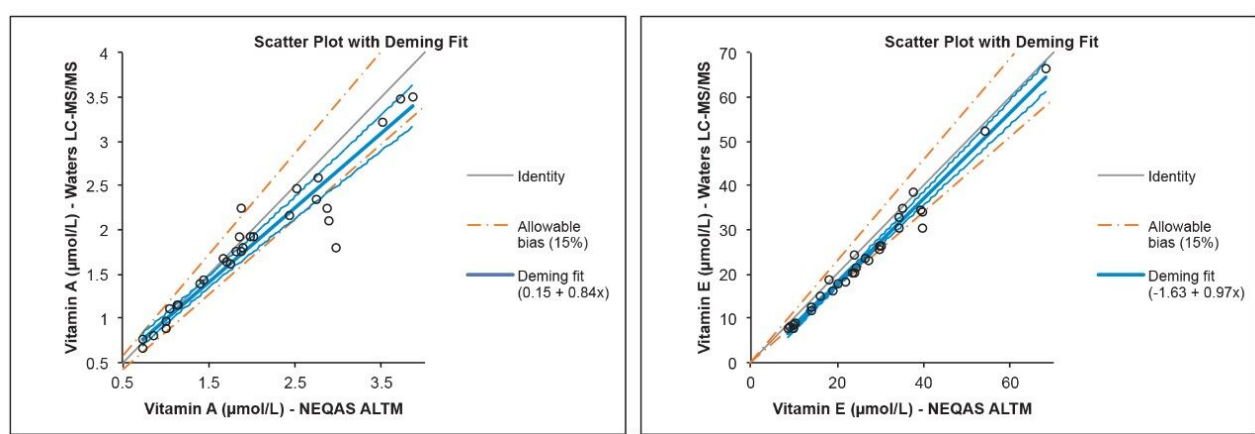*Table 6. Accuracy summary for vitamins A and E.*

Figure 5. Deming regression plots comparing the UK NEQAS all laboratories trimmed mean values to the Waters LC-MS/MS method.

Conclusion

A fast-clinical research method has been developed for the simultaneous analysis of vitamins A and E in serum. Using only 100 µL of serum in combination with a short run time, this method allows for high throughput of samples. In addition, the use of Oasis PRiME HLB µElution Plates minimizes phospholipid interferences. The assay described demonstrates excellent precision over five days and linearity across the required range with no significant carryover and no endogenous or exogenous interferences observed. The method demonstrates good agreement with UK NEQAS samples.

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

Xevo TQD Triple Quadrupole Mass Spectrometry <https://www.waters.com/134608730>

MassLynx MS Software <https://www.waters.com/513662>

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