Nele Peersman^a, Jan Van Elslande^a, Yannick Lepage, Samira De Amicis, Koenraad Desmet and Pieter Vermeersch*

UPLC-MS/MS method for determination of retinol and α -tocopherol in serum using a simple sample pretreatment and UniSpray as ionization technique to reduce matrix effects

<https://doi.org/10.1515/cclm-2019-1237>

Received November 29, 2019; accepted February 4, 2020; previously published online March 2, 2020

Abstract

Background: Our goal was to develop a simple, rapid and precise ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the determination of retinol and α -tocopherol in serum. Currently published LC-MS/MS methods either require complex extraction procedures (liquid-liquid or solid-phase) or do not meet desirable specifications for imprecision in serum (coefficient of variation [CV] <6.8% and 6.9%, respectively).

Methods: Sample preparation consisted of a simple protein precipitation with ethanol and acetonitrile. Stable isotope-labeled internal standards (IS) and a homemade calibration curve were used for quantification. The analysis was performed using an Acquity I-class Xevo TQ XS LC-MS/MS. Chromatographic runtime was 6.0 min using a reversed phase gradient elution. UniSpray (US) as an ionization technique was compared to electrospray ionization (ESI). Analytical validation included matrix effect, recovery and trueness compared to National Institute of Standards and Technology (NIST) standards and United Kingdom National External Quality Assessment Service (UK NEQAS) samples.

Results: Intra- and inter-run CVs were <4.9% for retinol and <1.7% for α -tocopherol, both complying with desirable specifications for imprecision. Bias compared to NIST standards was <3.1% for both compounds. The method was linear over the entire tested range. The lower limit of quantification (LLOQ) with US was lower than with ESI for both retinol (0.022 vs. 0.043 mg/L) and α -tocopherol (0.22 vs. 0.87 mg/L). Matrix effects were not significant (<15%)

for retinol. However, for α -tocopherol matrix effects of on average 54.0% were noted using ESI, but not with US.

Conclusions: We developed a fast, precise and accurate UPLC-MS/MS method for the determination of retinol and α -tocopherol in human serum using a single-step sample pretreatment. Ionization using US eliminated the matrix effects for α -tocopherol.

Keywords: α -tocopherol; liquid chromatography; retinol; tandem mass spectrometry; UniSpray.

Introduction

Vitamin A and vitamin E are small fat-soluble organic compounds which are obtained from the diet and are essential in trace amounts for a range of physiological functions including growth, reproductive functions, vision, neurologic function and immunity [1, 2]. Vitamin A is defined as all-trans retinol, which can be formed out of three provitamins A (α - and β -carotene and β -cryptoxanthin). Retinol is converted into retinyl esters for storage and two bioactive molecules, retinal and retinoic acid, which function as a chromophore in the eye and as a transcription factor, respectively [1, 3]. There are eight naturally occurring vitamin E members (α -, β -, γ -, δ -tocopherol and α -, β -, γ -, δ -tocotrienol). α -Tocopherol is the most predominant form of vitamin E in blood and tissues and is the most widely studied. Vitamin E acts mainly as anti-oxidant, next to many other functions [2].

Determinations of serum retinol and α -tocopherol are used in clinical practice to assess vitamin A and E status, respectively [4]. Besides clinical deficiency and hypervitaminosis, vitamin A and E and their related compounds have been implicated in immunity [5, 6], cancer [7] and age-related diseases [8, 9]. An increased interest in these molecules in the past years has led to a significant increase in the number of laboratory requests for the determination of these vitamins. Fast, high-throughput and affordable analytical methods using small volumes of serum or plasma are therefore needed.

Multiple methods have been published for the determination of retinol and/or α -tocopherol in serum or

^aNele Peersman and Jan Van Elslande contributed equally to this work.

*Corresponding author: Pieter Vermeersch, Clinical Department of Laboratory Medicine, University Hospitals Leuven, Herestraat 49, 3000 Leuven, Belgium, E-mail: pieter.vermeersch@uzleuven.be
Nele Peersman, Jan Van Elslande, Yannick Lepage, Samira De Amicis and Koenraad Desmet: Clinical Department of Laboratory Medicine, University Hospitals Leuven, Leuven, Belgium

plasma [10–12]. Liquid-chromatography (LC) coupled to tandem mass-spectrometry (MS/MS) is the current state-of the art technique for measuring retinol and α -tocopherol. Despite the lower sensitivity and selectivity, diode array detection (DAD) is still widely used [11]. In the United Kingdom National External Quality Assessment Service (UK NEQAS) program for vitamin assays of September 2019, for example, 87% of laboratories still use DAD and only eight of the 62 participants use MS/MS for the determination of retinol and α -tocopherol.

Ultra-performance (UP)LC-MS/MS requires the ionization of compounds eluting from the LC system. UniSpray (US) is a new atmospheric pressure ionization technique which nebulizes and ionizes the sample using a cylindrical metal rod placed at high voltage, which is located between the sprayer and the ion inlet orifice of the MS/MS [13] (Figure 1). This geometry is considered to result in a better ionization efficiency for some molecules and could be less prone to matrix effects [13–15]. The use of US has only been described in a handful of articles for the determination of prostaglandines and thromboxanes [15], pharmaceutical compounds [13] and pesticides [14].

Electrospray ionization (ESI), in contrast, is known to be particularly prone to matrix effects leading to variable ionization efficiency [15–17].

Retinol and α -tocopherol have limited stability in solution as these compounds are susceptible to photo-degradation, as well as artifactual oxidation and temperature dependent degradation [10, 18]. Therefore, a fast and simple sample preparation is preferred [12]. Most methods do, however, require a complex sample pretreatment, such as protein precipitation followed by liquid-liquid extraction or solid phase extraction [12]. Such pretreatment steps are laborious, increase the complexity of the method (which increases the risk for errors) and reduce throughput.

Only two published methods for retinol and α -tocopherol determination use a simple one step protein precipitation consisting of the addition of organic solvent spiked with internal standards (IS) followed by centrifugation [19, 20]. The first method by Lazzarino et al. [20] uses DAD which can be considered inferior to MS/MS [20]. The second method for plasma by Le et al. [19] uses LC-MS/MS and stable-isotope labeled IS [19], but this method

Figure 1: Schematic representation of both ionization techniques.

(A) Schematic representation of an ESI source. The high voltage (HV) applied to the spray tip of the heated capillary results in the formation of an aerosol of charged droplets. As the solvent evaporates from the droplets, charged gas-phase ions are formed. (B) Schematic representation of a US source. The liquid sample is nebulized using a grounded capillary positioned closely to a stainless steel cylindrical target rod at high voltage. When the gas flow comes into contact with the target rod, small charged droplets are formed. Charged gas-phase ions are subsequently formed via desolvation. The downstream gas flow follows the curvature of the surface of the rod towards the inlet orifice of the sample cone. This effect is known as the Coandă effect (see insert for 3D visualization). LC, liquid chromatograph; MS, mass spectrometer.

does not comply with desirable specifications for analytical imprecision for retinol and α -tocopherol in serum (relative standard deviation or coefficient of variation [CV] <6.8% and <6.9%, respectively) or retinol in plasma (<3.1%) [21–23]. There is no criterion for α -tocopherol in plasma. The desirable specifications for analytical precision are calculated based on biological variation data [24–26] using the formulas as described by Ricós et al. in 1999 [23]. As the concept that analytical specifications should be based on biological variation characteristics is widely recommended, we considered these desirable criteria as an important benchmark for analytical precision throughout this article [27, 28].

The goal of this study was to develop and validate a fast, simple and precise UPLC-MS/MS method for the simultaneous determination of retinol and α -tocopherol in serum, with a performance complying with the desirable criteria for imprecision and trueness. In an attempt to reduce matrix effects, we tested the novel Waters US source and compared its performance with a standard ESI source. The method was validated based on the European Medicines Agency (EMA) guideline on Bioanalytical method validation [29] and a review on validation of LC-MS/MS methods by Honour [30].

Materials and methods

Reagents

Stable isotope-labeled IS were α -tocopherol-trimethylphenyl- $^{13}\text{C}_9$, molecular weight (molecular weight [MW] 439.64 g/mol, enrichment $\geq 99.0\%$), purchased from Sigma Aldrich (Overijse, Belgium) and retinol-12,12,20- $^{13}\text{C}_3$ (MW 289.34 g/mol, enrichment $\geq 99.0\%$) purchased from Buchem BV (Minden, The Netherlands). The water used throughout the study was purified by a Milli-Q apparatus (Millipore, Bedford, MA, USA). Methanol, ethanol and acetonitrile (LC-MS grade) were bought from Biosolve (Valkenswaard, The Netherlands). LC-MS grade ammonium acetate and formic acid were purchased from Fluka Analytical (Sigma Aldrich, Overijse, Belgium).

Stock solutions, calibration curves and quality controls

The IS working solution was prepared by diluting the stock solutions with ethanol to obtain a final IS concentration of 0.200 mg/L for retinol-12,12,20- $^{13}\text{C}_3$ and 1.00 mg/L for α -tocopherol-trimethylphenyl- $^{13}\text{C}_9$. The precipitation reagent (with IS) contained ethanol/acetonitrile (44/56), with final concentrations of 0.110 mg/L for retinol- $^{13}\text{C}_3$ and 0.60 mg/L for α -tocopherol- $^{13}\text{C}_9$, respectively.

Calibration standards were prepared using commercially available National Institute of Standards and Technology (NIST)-traceable standards. The standard for retinol (Standard V-011; 100 mg/L in ethanol) was purchased from Sigma-Aldrich (St. Gallen, Switzerland)

and the standard for α -tocopherol (CERV-020, 1000 mg/L in ethanol) was purchased from LGC (Molsheim, France). Nine different calibration standard solutions were prepared in a bovine serum albumin (BSA) solution. The BSA solution was prepared by dissolving 60 g/L BSA ($\geq 98\%$ pure, Sigma Aldrich, Overijse, Belgium) in 0.9% NaCl and adding 0.2% human serum. Final concentrations ranged from 0.022 mg/L to 5.556 mg/L for retinol, and from 0.22 mg/L to 55.56 mg/L for α -tocopherol. The blank was an unspiked BSA solution. Levels of retinol and α -tocopherol were below the limit of detection in this solution, defined as a 3 times the signal-to-noise ratio.

Internal quality control materials (iQC-L and iQC-H) were purchased from Chromsystems (Vitamins A and E Serum Control level I+II, Chromsystems Instruments and Chemicals, Grafelfing, Germany). The lyophilized iQC materials were reconstituted in 2 mL ultrapure water (Milli-Q, 18 $\mu\Omega$), following the manufacturer's instructions. For the assessment of trueness, an NIST standard reference material (968f) containing retinol and α -tocopherol was purchased from LCG standards (Molsheim, France).

Sample preparation

Patient blood samples were collected in 5 mL serum separator tubes (Vacutainer™ SST5, Becton Dickinson, Temse, Belgium). After centrifugation, serum was stored at -20°C until analysis. At the time of analysis, 15 μL of serum was pipetted in polystyrene tubes and the tubes were immediately vortexed after adding 300 μL of precipitation reagent. After storage in a fridge for 1 h at 4°C (protected from light), samples were again vortexed and subsequently centrifuged (10 min at 16,100g). The supernatant was transferred to 1 mL glass vials (Tru-View maximum recovery vials, Waters Corporation, Asse, Belgium) and samples were placed in the autosampler of the LC system at 4°C .

UPLC-MS/MS conditions

For each sample, 0.5 μL of supernatant was injected. Reverse-phase chromatographic separation was carried out on an Acquity I-class LC system (Waters LTD, Watford, UK) using a Kinetex F5 analytical column (2.6 μm , 100 mm $\times$ 3.0 mm, Part. Nr. 00D-4723-YO, Phenomenex, Utrecht, The Netherlands) maintained at 40°C . The flow rate was set at 0.5 mL/min with a maximum pressure of 6000 PSI. Mobile phase A was a mixture of methanol containing 2 mM ammoniumacetate and 0.1% of formic acid. Mobile phase B consisted of water containing 2 mM of ammoniumacetate and 0.1% of formic acid. The gradient started at 85% mobile phase A, increasing linearly to 98% of mobile phase A after 3 min. After 1 min at 98% of mobile phase A, initial conditions were restored and kept for 2 min until the next injection. The total runtime (injection to injection) was 6 min. Under these chromatographic conditions retinol eluted at a retention time of 1.45 min and α -tocopherol at a retention time of 3.34 min. A chromatogram of the low level iQC is shown in Figure 2.

Mass spectrometry analysis was performed using a triple quadrupole mass spectrometer (Xevo TQ-XS, Waters Ltd., Watford, UK). During method development an ESI source in positive mode and a US source were used. The following ESI parameters were used: source and desolvation temperature of 150°C and 600°C , nitrogen gas at a flow rate of 1000 L/h (as desolvation gas) and capillary voltage set at 0.5 kV. The source and desolvation temperature of the US source were set at 150°C and 500°C . Impactor voltage was set at 3.1 kV. For each

Figure 2: Representative chromatograms using UniSpray ionization.

(A) Chromatogram of the low internal quality control sample for retinol (approximately 0.500 mg/L) and (B) α -tocopherol (approximately 6.80 mg/L). The captions show the retention time (minutes) and peak area. IS, internal standard; MRM, multiple reaction monitoring; US+, UniSpray positive mode.

parameter, one quantifier and one qualifier were used. Further details on the settings used for the MS are found in Table 1. For ESI and US experiments identical cone voltage and collision energy was used. All results described in the article were generated using the US source unless otherwise indicated. Data acquisition and processing were performed using MassLynx™ software, v.4.2 (Waters Corporation).

Linearity and lower limit of quantification (LLOQ)

Linearity was assessed by evaluating the coefficient of determination (R^2) of the regression equation proposed by the MassLynx software based on the measured concentrations of the calibrators (nine points). For each calibration point, we considered a deviation from

Table 1: Optimized mass spectrometry settings for retinol, α -tocopherol and their stable isotope-labeled IS.

Analyte	MRM-transition	Dwell time, ms	Cone voltage	Collision energy, eV	Retention time, min
Retinol quantifier	269.2 > 93.8	30	24	20	1.45
Retinol qualifier	269.2 > 107.0	30	24	18	1.45
Retinol IS	272.2 > 96.1	30	24	18	1.45
α -Tocopherol quantifier	430.2 > 165.1	30	44	24	3.34
α -Tocopherol qualifier	430.2 > 121.1	30	44	42	3.34
α -Tocopherol IS	439.2 > 174.1	30	44	24	3.34

IS, internal standard; MRM, multiple reaction monitoring; eV, electron volt.

the nominal spiked concentration of <15% as acceptable. The aim was to obtain a value of $R^2 > 0.99$ using a regression equation with an order as low as possible (preferably first order). Linearity of this regression equation was tested using Analyse-it for Excel, v4.90 (Analyse-it Software Ltd., Leeds, UK). Linearity was confirmed by mixing a patient sample with a low and a high concentration, at ratios of 1/0, 3/1, 1/1, 1/3 and 0/1.

The LLOQ was defined as the lowest calibration standard with an acceptable precision (CV <20%) and accuracy (<20% deviation from the nominal value) and a signal-to-noise ratio greater than 10, based on the EMA guideline [29, 30]. Precision at the LLOQ was also investigated by determining retinol and α -tocopherol in a patient sample with a concentration close to the LLOQ.

Precision and trueness

Intra-run precision was determined by injecting a single sample of iQC-L, a single sample of iQC-H, a single patient sample with normal concentrations, and a single specially selected patient sample close to the LLOQ 10 times (from a single vial) in one run. Inter-run precision was determined by analyzing the iQC-L, iQC-H during 10 consecutive working days. An inter-run CV of <6.8% for retinol and <6.9% for α -tocopherol were considered acceptable based on the Westgard desirable biological database [21–23].

Method trueness was evaluated by measuring the 968f NIST standard, and subsequently assessing the % deviation from its target mean value. In addition, we also reanalyzed 27 samples from the UK NEQAS which were stored at -20°C for a maximum of 24 months (rounds 186–194). An average bias of <15% compared to the target concentration for both analytes was considered acceptable based on the EMA guideline [29]. We also considered compliance with the Westgard desirable criteria for trueness (bias of <5.8% and <5.1% for retinol and α -tocopherol, respectively) [21–23].

Selectivity

The retention times of 17 structurally-related compounds were identified using the MS-scan mode and multiple reaction monitoring. The transitions were based on literature sources [31, 32]. A mixture containing tocotrienols and tocopherols was also used to determine the retention times of α -tocopherol related compounds. The NIST 968f standard was used to determine the retention time of six retinol related compounds. Retinol acetate and tocopherol acetate were spiked in a serum sample and ethanol at a concentration level of 20,000 mg/L. See online Data Supplement for additional information.

Carry-over

Carry-over was determined by injecting 3 times the highest calibration standard (H), followed by 3 times a blank (B) [30]. Carry-over in the first blank sample (B1) must be <20% of the LLOQ and <5% for the IS area according to EMA [29]. Additionally, the percentage of carryover calculated with the following formula must be <0.1% [29]: % carry over = $100 \cdot ((\text{area B1} - \text{area B3}) / (\text{area H3} - \text{area B3}))$, with B3 being the third blank and H3 being the third high standard.

Extraction recovery and matrix effect

The matrix effect and recovery were evaluated using the post-extraction spike method as described by Matuszewski et al. [33]. Nine routine serum samples (including one hemolytic and two lipemic samples) and one specially selected sample close to the LLOQ were processed 3 times. First unspiked, by adding 20 μL of ethanol to 180 μL of serum (unspiked), to determine the endogenous concentration. Then routinely the samples were spiked by adding 20 μL of stock solution containing 5,000 mg/L of retinol and 25,00 mg/L of α -tocopherol to 180 μL of serum. The sample close to the LLOQ was spiked with 20 μL of stock solution containing 0,700 mg/L of retinol and 700 mg/L of α -tocopherol. This was done before extraction (pre-extraction spiked) and after extraction (post-extraction spiked). The same amount of stock solution was also added to 180 μL of pure ethanol (spiking in solvent). Using the following formulas, the matrix effect, extraction recovery and process efficiency (PE) were calculated [33]: matrix effect (%) = $(\text{area}^{\text{post-extraction spiked}} - \text{area}^{\text{unspiked}}) / \text{area}^{\text{spiking in solvent}}$; recovery (%) = $\text{area}^{\text{post-extraction spiked}} / \text{area}^{\text{pre-extraction spiked}}$; PE (%) = $(\text{concentration}^{\text{pre-extraction spiked}} - \text{concentration}^{\text{unspiked}}) / \text{concentration}^{\text{spiking in solvent}}$.

According to EMA, the CV of the IS-normalized matrix factor (PE) should not be greater than 15% [29].

Comparison ESI and US ionization source

Intra-run precision, linearity and LLOQ for ESI were determined as described for US, using the same calibration curve, iQCs and the same patient samples. The matrix effect, recovery and PE were determined in four routine serum samples (no hemolytic or lipemic samples) and in one specially selected sample close to the LLOQ as described above. Each of the samples was processed 3 times, and the CV of the concentration in the unspiked condition was compared to the CV obtained with the US source in the same condition (cfr. supra).

A method correlation for retinol and α -tocopherol was performed by analyzing 10 external quality control samples (UK NEQAS) and 20 patient samples with both ionization techniques. Correlation parameters were calculated using Passing-Bablok regression analysis using Microsoft Excel, Analyse-it v4.90. The areas of the IS used in the analyses of these 30 samples were also assessed. The average IS area and the variation (as CV) was compared between ESI and US.

Ethical approval

This study was approved by the Institutional Review Board of the University Hospitals Leuven.

Results

Linearity and LLOQ

The US method was linear over the entire range of the calibration curve between 0.022 mg/L and 5.556 mg/L for

retinol ($R^2 > 0.99$) and 0.22–55.56 mg/L for α -tocopherol ($R^2 > 0.99$). The lowest calibration point was considered the LLOQ, 0.022 mg/L for retinol and 0.22 mg/L for α -tocopherol. At the LLOQ, the CV was $<5\%$ and the average bias compared to the theoretical concentration was $<3\%$ for both retinol and α -tocopherol. The average bias for the other calibration points was $<7\%$ for both analytes. The linearity was confirmed in serum by mixing a low and a high patient sample for retinol (range 0.045–1.003 mg/L) and α -tocopherol (range 0.64–38.0 mg/L), with an observed bias of $<5\%$ for all points and a $R^2 > 0.99$, for both components. Intra-run precision for a specially selected patient sample close to the LLOQ (retinol and α -tocopherol concentration estimated at 0.025 mg/L and 0.64 mg/L, respectively) was 15.9% for retinol and 2.2% for α -tocopherol, also within the 20% criterion of EMA [29]. The observed LLOQ was well below the lower limit of the reference range for retinol and α -tocopherol (0.300–0.800 mg/L and 5.00–18.00 mg/L, respectively) [34].

Precision and trueness

Results of the intra-run and inter-run precision experiments are shown in Table 2. The intra- and inter-run CVs were $<4.9\%$ for retinol and $<1.7\%$ for α -tocopherol for iQC-L and iQC-H. These results are within with the desirable criteria for precision, namely $<6.8\%$ for retinol and $<6.9\%$ for α -tocopherol. The intra-run CV for the patient sample with normal concentrations was 2.4% and 0.2% for retinol and α -tocopherol, respectively, comparable to the intra-run precision of iQC-L and iQC-H. The results for trueness are shown in Table 3. Bias for both retinol and α -tocopherol was within the desirable criteria for trueness ($<5.8\%$ and $<5.1\%$, respectively) [21–23].

Table 2: Results for precision (each sample was injected 10 times).

	Target value (range), mg/L	Mean, mg/L	Quantifier US		Quantifier ESI
			CV _{intra}	CV _{inter}	CV _{intra}
Retinol					
iQC L	0.43 (0.34–0.51)	0.445	2.8%	4.9%	2.9%
iQC H	1.64 (1.31–2.00)	1.373	3.0%	4.1%	2.7%
Patient sample	/	0.571	2.4%	/	2.7%
α -tocopherol					
iQC L	7.85 (6.28–9.43)	7.39	0.7%	1.7%	0.7%
iQC H	17.5 (14.0–21.0)	16.36	0.5%	1.6%	0.5%
Patient sample	/	10.63	0.2%	/	0.8%

CV, coefficient of variation or relative standard deviation; CV_{intra}, intra-run CV; CV_{inter}, inter-run CV; iQC, internal quality control sample; L, low; H, high; /, not determined; LLOQ_{ESI}, lower limit of quantification for ESI. ESI, electrospray ionization; US, Unispray.

Selectivity and carry-over

The retention times (RTs) of seven α -tocopherol related compounds are shown in Figure 3A and the RT of three retinol-related compounds are shown in Figure 3B. Retinol acetate and tocopherol acetate have a RT of 2.3 and 3.8 min (not shown in Figure). We also confirmed that α -carotene, β -carotene and lycopene elute after 4 min. No significant carry-over was detected ($<0.1\%$) for retinol and α -tocopherol. See online Data Supplement for additional information on selectivity.

Recovery and matrix effects with US

The results of the experiments regarding recovery and matrix effects are shown in Table 4. PE was within the EMA criterion of 15% for all nine routine samples. For the specially selected sample close the LLOQ, PE was 111% for retinol and 117% for α -tocopherol, just above the EMA criterion for α -tocopherol.

Table 3: Results for trueness compared to NIST standards (level 1 and 2) and UK NEQAS samples.

	Target, mg/L	%Bias	Average Z-score
Retinol			
NIST L1 (n = 1)	0.327	0.2%	/
NIST L2 (n = 1)	0.658	2.7%	/
UK NEQAS (n = 27)	0.328–1.202	2.0%	−0.2
α -tocopherol			
NIST L1 (n = 1)	5.15	2.2%	/
NIST L2 (n = 1)	11.95	3.1%	/
UK NEQAS (n = 27)	3.93–30.40	2.0%	−0.3

NIST, National Institute of Standards and Technology; UK NEQAS, United Kingdom National External Quality Assessment Services.

Figure 3: Overlay chromatograms with retention times. (A) Chromatogram of the tocotrienol-and tocopherol mixture. (1): δ-Tocotrienol, (2 and 3): β- and γ-tocotrienol, (4): α-tocotrienol, (5): δ-tocopherol, (6 and 7): β- and γ-tocopherol, and (8): α-tocopherol. (B) Chromatogram of the NIST 968f standard. (1) retinol, (2 and 3) lutein and zeaxanthin, and (4): cryptoxanthin.

Table 4: Recovery, matrix effect and process efficiency for routine serum samples with US and ESI.

Ionization technique	US (n=9)		ESI (n=4)	
	Retinol (0.191–0.658)	α-Tocopherol (6.35–20.73)	Retinol (0.238–0.472)	α-Tocopherol (6.41–10.39)
Average recovery	104.3%	106.6%	97.9%	93.8%
%CV of recovery	4.5%	4.5%	4.5%	5.9%
Average matrix effect	103.3%	90.7%	105.4%	54.0%
%CV of matrix effect	4.3%	9.0%	7.3%	9.2%
Average process efficiency	101.3%	88.4%	104.7%	90.4%
%CV of process efficiency	6.4%	2.6%	5.5%	8.5%

CV, coefficient of variation; ESI, electrospray ionization; US, UniSpray.

Comparison of US and ESI

The ESI method for retinol was linear between 0.043 mg/L and 5.556 mg/L ($R^2 > 0.99$). The LLOQ for retinol with ESI was 0.043 mg/L with a bias 1% at 0.043 mg/L, compared to 0.022 mg/L for US. This was confirmed with a patient sample with an estimated concentration of 0.026 mg/L using US which was not measureable with ESI, while a sample with a value of 0.045 mg/L gave a CV of 7.9% and 11.0% with US and ESI, respectively.

The ESI method for α-tocopherol was linear between 0.87 and 55.56 mg/L ($R^2 > 0.99$), compared to 0.22–55.56 mg/L for US. The bias at the LLOQ for ESI of 0.87 mg/L for α-tocopherol was 18%, close to the acceptable bias of 20% according to EMA, while the bias was 50% at a theoretical concentration of 0.43 mg/L.

The correlation of the results of 10 external quality control samples and 20 patient samples is shown in

Figure 4A. The correlation coefficient was 0.98 for retinol and 0.96 for α-tocopherol. The average CV of the unspiked routine plasma samples (n=4, each sample processed 3 times) in the matrix effect experiments was 6.2% for retinol and 6.8% for α-tocopherol using ESI, just within the desirable specifications. For US, the average CVs of the unspiked plasma samples (n=9) were lower: 5.4% and 1.5% for retinol and α-tocopherol, respectively.

The IS areas of the correlation experiment (with box-plots) are shown in Figure 4B. The CV of the IS areas for retinol was comparable for ESI (CV 3.7%) and US (CV 3.6%). On the contrary, for α-tocopherol, the CV of the IS areas was significantly higher ($p < 0.001$) with ESI (CV 12.0%) compared to US (CV 2.4%).

PE with ESI was within the EMA criterion of 15% for the four routine patient samples. For the specially selected sample close to the LLOQ of US, which was below the LLOQ of ESI for both parameters, the PE and matrix

Figure 4: Comparison of ESI and US.

(A) Comparison of ionization techniques using Passing-Bablok regression analysis ($n = 30$) for measuring of retinol and α -tocopherol. (B) IS areas for retinol and α -tocopherol of the samples used in the correlation experiment ($n = 30$). ESI, electrospray ionization; US, Unispray; IS, internal standard; CI, confidence interval.

effects were 117% and 105.4% for retinol and 231% and 54% for α -tocopherol, respectively.

Discussion

Our method is the first published method for α -tocopherol and retinol determination in serum using a simple one step protein precipitation that meets the desirable specifications for imprecision. Our method is also the first LC-MS/MS method for a routine clinical laboratory parameter with US, a novel ionization technique.

The use of US has only been described in a handful of articles. In 2016, Riches et al. and Lubin et al. reported that US resulted in higher signal intensities for several compounds (oil components, cosmetics and pharmaceutical products) by infusion experiments [13, 35]. In 2017, Lubin

et al. showed that the higher signal intensity resulted in a lower LLOQ for US compared to ESI for three of five studied prostaglandins/thromboxanes in human plasma [15]. In addition, the use of US was also reported to result in a larger dynamic range (low concentrations) as well as a small but statistically significant decrease in matrix effects compared to ESI for most of the 24 pharmaceutical compounds studied in human plasma [13]. Finally, Galani et al. showed an improved LLOQ and a higher PE (a factor 3.5 on average) with US compared to ESI for several pesticides in coffee, apple and water [14].

The LLOQ in our study with US was 2 times lower for retinol and 4 times lower for α -tocopherol compared to ESI. The lower LLOQ could be due to an improved ionization efficiency created by the unique geometry of the US source. In US, molecules are ionized through impactor ionization, which might promote droplet breakup

and desolvation by inducing several hydrodynamic and aerodynamic phenomena, such as the Coandă effect and microvortices at the fluid stagnation site on the metallic surface of the cylindrical rod (Figure 1) [13]. No relation could, however, be established between the physico-chemical properties of certain molecules and whether or not US would result in better signal intensity [13, 14].

Other methods with an LLOQ similar to our method that also meet the desirable specifications for imprecision have been published. Zhang et al. [36], for example, described an UPLC-MS/MS method (using both an ESI and APCI ionization source) with a linear dynamic range starting at 0.14 mg/L and 0.006 mg/L for retinol and α -tocopherol, respectively. The precision of this method was comparable to our numbers as well, with an intra-run CV of <3.5%, and <2.5% for retinol and α -tocopherol, and an inter-run CV of <2.0% and <1.4% [36]. Other published LC-MS/MS methods using ESI that complied with the desirable criteria for imprecision include Middtun and Ueland [37], Konieczna et al. [38] and Hinchliffe et al. [39]. All these methods do, however, require a complex sample preparation, suggesting that an extraction step is necessary to achieve suitable performance with ESI to reduce matrix effects.

In our study matrix effects were acceptable for retinol (90–110%), independent of the ionization technique used. However, for α -tocopherol, a significant matrix effect was observed with ESI but not with US. Middtun and Ueland observed similar matrix effects of 52% for α -tocopherol using ESI [37]. Of note, we calculated matrix effects according to the EMA guideline, which defines matrix effects as the ratio of the peak area of the analyte spiked in the presence of matrix to the peak area of the analyte spiked in the absence of matrix. Many authors including Le et al., however, use the term “matrix effects” to describe recovery (i.e. $\text{area}^{\text{pre-extraction spiked}} / \text{area}^{\text{post-extraction spiked}}$). None of the other articles found in our literature search [19, 20, 36–44] calculated matrix effects according to the EMA guideline for α -tocopherol [37]. Matrix effects can occur when constituents of the sample matrix elute from the LC together with the analyte of interest and interfere with ionization [30]. Ionization can be enhanced or suppressed in a variable and unpredictable way leading to imprecision, even when deuterated IS are used [16].

The simple sample preparation, combined with a short injection to injection time of 6.0 min, makes our method suitable for high-throughput analysis. In the literature, the analysis time of methods for retinol and α -tocopherol varies from 4 to 15 min for UPLC methods [19, 36, 37, 39, 41–43] compared to 30–50 min with standard HPLC methods [20, 38, 40, 44]. Despite the short injection

to injection time, we were able to separate all eight molecules in the vitamin E family. We also demonstrated that seven retinol-related compounds do not co-elute with retinol.

In summary, we developed and validated a fast, precise and accurate UPLC-MS/MS method for the determination of retinol and α -tocopherol in serum using US. Our method is to the best of our knowledge the first published method with a simple single-step protein precipitation which meets the desirable criteria for imprecision and trueness. Matrix effects for α -tocopherol were significantly lower using US compared to ESI, confirming earlier reports that US results in an improved ionization efficiency and lower matrix effects for some compounds, leading to a higher signal intensity and a lower LLOQ.

Acknowledgment: We thank Thomas Van Den Bossche for designing the 3D insert in Figure 1.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Employment or leadership: PV is a senior clinical investigator of the Research Foundation-Flanders (FWO).

Research funding: The research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

References

1. Widjaja-Adhi MA, Golczak M. The molecular aspects of absorption and metabolism of carotenoids and retinoids in vertebrates. *Biochim Biophys Acta – Mol Cell Biol Lipids* 2019. <https://www.sciencedirect.com/science/article/pii/S1388198119302227>.
2. Jiang Q. Natural forms of vitamin E: Metabolism, antioxidant, and anti-inflammatory activities and their role in disease prevention and therapy. *Free Radic Biol Med* 2014;72:76–90.
3. O'Byrne SM, Blaner WS. Retinol and retinyl esters: biochemistry and physiology: thematic review series: fat-soluble vitamins: vitamin A. *J Lipid Res* 2013;54:1731–43.
4. Höller U, Bakker SJL, Düsterloh A, Frei B, Köhrle J, Konz T, et al. Micronutrient status assessment in humans: current methods of analysis and future trends. *Trends Anal Chem* 2018;102:110–22.

5. Polcz ME, Barbul A. The role of vitamin A in wound healing. *Nutr Clin Pract* 2019;34:695–700.
6. Zinder R, Cooley R, Vlad LG, Molnar JA. Vitamin A and wound healing. *Nutr Clin Pract* 2019;34:839–49.
7. Jiang Q. Natural forms of vitamin E and metabolites – regulation of cancer cell death and underlying mechanisms. *IUBMB Life* 2019;71:495–506.
8. Tan BL, Norhaizan ME. Carotenoids: how effective are they to prevent age-related diseases? *Molecules* 2019;24. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6539799/>.
9. Mocchegiani E, Costarelli L, Giacconi R, Malavolta M, Basso A, Piacenza F, et al. Vitamin E-gene interactions in aging and inflammatory age-related diseases: implications for treatment. A systematic review. *Ageing Res Rev* 2014;14:81–101.
10. Lai JF, Franke AA, States U. Analysis of circulating lipid-phase micronutrients in humans by HPLC: review and overview of new developments. *J Chromatogr B Anal Technol Biomed Life Sci* 2013;931:23–41.
11. Albahrani AA, Greaves RF. Fat-soluble vitamins: clinical indications and current challenges for chromatographic measurement. *Clin Biochem Rev* 2016;37:27–47.
12. Karaźniewicz-Łada M, Głowska A. A review of chromatographic methods for the determination of water- and fat-soluble vitamins in biological fluids. *J Sep Sci* 2016;39:132–48.
13. Lubin A, Bajic S, Cabooter D, Augustijns P, Cuyckens F. Atmospheric pressure ionization using a high voltage target compared to electrospray ionization. *J Am Soc Mass Spectrom* 2017;28:286–93.
14. Galani JH, Houbraken M, Van Hulle M, Spanoghe P. Comparison of electrospray and UniSpray, a novel atmospheric pressure ionization interface, for LC-MS/MS analysis of 81 pesticide residues in food and water matrices. *Anal Bioanal Chem* 2019;411:5099–113.
15. Lubin A, Geerinckx S, Bajic S, Cabooter D, Augustijns P, Cuyckens F, et al. Enhanced performance for the analysis of prostaglandins and thromboxanes by liquid chromatography-tandem mass spectrometry using a new atmospheric pressure ionization source. *J Chromatogr A* 2016;1440:260–5.
16. Stahnke H, Kittlaus S, Kempe G, Hemmerling C, Alder L. The influence of electrospray ion source design on matrix effects. *J Mass Spectrom* 2012;47:875–84.
17. Loos G, Van Schepdael A, Cabooter D. Quantitative mass spectrometry methods for pharmaceutical analysis. *Philos Trans R Soc A Math Phys Eng Sci* 2016;374. <https://www.ncbi.nlm.nih.gov/pubmed/27644982>.
18. Greaves RF, Woollard GA, Hoad KE, Walmsley TA, Johnson LA, Briscoe S, et al. Laboratory medicine best practice guideline: vitamins A, E and the carotenoids in blood. *Clin Biochem Rev* 2014;35:81–113.
19. Le J, Yuan TF, Zhang Y, Wang ST, Li Y. New LC-MS/MS method with single-step pretreatment analyzes fat-soluble vitamins in plasma and amniotic fluid. *J Lipid Res* 2018;59:1783–90.
20. Lazzarino G, Longo S, Amorini AM, Di Pietro V, D'Urso S, Lazzarino G, et al. Single-step preparation of selected biological fluids for the high performance liquid chromatographic analysis of fat-soluble vitamins and antioxidants. *J Chromatogr A* 2017;1527:43–52.
21. Ricos V, Alvarez F, Garcia-Laria A, Hernandez C, Jimenez J, Minchinela C, et al. Desirable Biological Variation Database specifications – Westgard 2014. <https://www.westgard.com/biodatabase1.htm> (accessed October 28, 2019).
22. EFLM Biological Variation n.d. <https://biologicalvariation.eu/> (accessed December 27, 2019).
23. Ricós C, Alvarez V, Cava F, García-Lario JV, Hernández A, Jiménez CV, et al. Current databases on biological variation: pros, cons and progress. *Scand J Clin Lab Invest* 1999;59:491–500.
24. Talwar DK, Azharuddin MK, Williamson C, Teoh YP, McMillan DC, O'Reilly DS. Biological variation of vitamins in blood of healthy individuals. *Clin Chem* 2005;51:2145–50.
25. Gallagher SK, Johnson LK, Milne DB. Short- and long-term variability of selected indices related to nutritional status. II. Vitamins, lipids, and protein indices. *Clin Chem* 1992;38:1449–53.
26. Olmedilla B, Granado F, Blanco I, Rojas-Hidalgo E. Seasonal and sex-related variations in six serum carotenoids, retinol, and α -tocopherol. *Am J Clin Nutr* 1994;60:106–10.
27. Sandberg S, Fraser CG, Rita Horvath A, Jansen R, Jones G, Vincent S, et al. Defining analytical performance specifications: consensus statement from the 1st Strategic Conference of the European Federation of Clinical Chemistry and Laboratory Medicine Consensus statement Analytical performance specifications. *Clin Chem Lab Med* 2015;53:833–5.
28. Ricos C, Perich C, Minchinela J, Alvarez V, Simon M, Biosca C, et al. Application of biological variation – a review. *Biochem Medica* 2009;19:250–9.
29. Committee for Medicinal Products for Human Use (CHMP). Guideline on bioanalytical method validation. 2012.
30. Honour JW. Development and validation of a quantitative assay based on tandem mass spectrometry. *Ann Clin Biochem* 2011;48:97–111.
31. Li H, Tyndale ST, Heath DD, Letcher RJ. Determination of carotenoids and all-trans-retinol in fish eggs by liquid chromatography-electrospray ionization-tandem mass spectrometry. *J Chromatogr B Anal Technol Biomed Life Sci* 2005;816:49–56.
32. Jiang Q, Xu T, Huang J, Jannasch AS, Cooper B, Yang C. Analysis of vitamin E metabolites including carboxychromanol and sulfated derivatives using LC/MS/MS. *J Lipid Res* 2015;56:2217–25.
33. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem* 2003;75:3019–30.
34. Rifai N, Horvath AR, Wittwer C. Tietz textbook of clinical chemistry and molecular diagnostics, 6th ed. Amsterdam, The Netherlands: Elsevier, 2018.
35. Riches E, Bajic S, Elia E, Langley GJ, Herniman JM. MS atmospheric pressure ionisation sources: their use and applicability. *Chromatogr Today Artic* 2017. <https://www.chromatographytoday.com/article/ion-chromatography-ic/58/waters-corporation/pms-atmospheric-pressure-ionisation-sources-their-use-and-applicability/2240>.
36. Zhang H, Quan L, Pei P, Lin Y, Feng C, Guan H, et al. Simultaneous determination of Vitamin A, 25-hydroxyl vitamin D 3 α -tocopherol in small biological fluids by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Anal Technol Biomed Life Sci* 2018;1079:1–8.
37. Midttun Ø, Ueland PM. Determination of vitamins A, D and E in a small volume of human plasma by a high-throughput method based on liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2011;25:1942–8.
38. Konieczna L, Kaźmierska K, Roszkowska A, Szlagatys-Sidorkiewicz A, Bączek T. The LC–MS method for the

- simultaneous analysis of selected fat-soluble vitamins and their metabolites in serum samples obtained from pediatric patients with cystic fibrosis. *J Pharm Biomed Anal* 2016;124:374–81.
39. Hinchliffe E, Rudge J, Reed P. A novel high-throughput method for supported liquid extraction of retinol and alpha-tocopherol from human serum and simultaneous quantitation by liquid chromatography tandem mass spectrometry. *Ann Clin Biochem* 2015;53:434–45.
40. Albahrani AA, Rotarou V, Roche PJ, Greaves RF. A simultaneous quantitative method for vitamins A, D and E in human serum using liquid chromatography-tandem mass spectrometry. *J Steroid Biochem Mol Biol* 2016;159:41–53.
41. Granado-Lorencio F, Herrero-Barbudo C, Blanco-Navarro I, Pérez-Sacristán B. Suitability of ultra-high performance liquid chromatography for the determination of fat-soluble nutritional status (vitamins A, E, D, and individual carotenoids). *Anal Bioanal Chem* 2010;397:1389–93.
42. Bell EC, John M, Hughes RJ, Pham T. Ultra-performance liquid chromatographic determination of tocopherols and retinol in human plasma. *J Chromatogr Sci* 2014;52:1065–70.
43. Midttun Ø, McCann A, Aarseth O, Krokeide M, Kvalheim G, Meyer K, et al. Combined measurement of 6 fat-soluble vitamins and 26 water-soluble functional vitamin markers and amino acids in 50 μ l of serum or plasma by high-throughput mass spectrometry. *Anal Chem* 2016;88:10427–36.
44. Hrvolová B, Martínez-Huélamo M, Colmán-Martínez M, Hurtado-Barroso S, Lamuela-Raventós RM, Kalina J. Development of an advanced HPLC–MS/MS method for the determination of carotenoids and fat-soluble vitamins in human plasma. *Int J Mol Sci* 2016;17. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5085750/>.
-
- Supplementary Material:** The online version of this article offers supplementary material (<https://doi.org/10.1515/cclm-2019-1237>).