Detection and Quantification of Vitamins in Microliter Volumes of Biological Samples by LC-MS for Clinical Screening

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Abstract

A method for simultaneous determination of water-soluble vitamins B_1, B_2, B_3 (nicotinamide), B_5, B_6 (pyridoxamine), B_9 and fat-soluble vitamins E (α-tocopherol) and K_1 in tears, and B_1, B_2, B_3, B_5, B_6, B_9, A (retinol) and E in blood serum is described. Liquid chromatography-mass spectrometry (LC-MS) was used with a ternary mobile phase of water and acetonitrile containing

0.1% formic acid and methanol containing 5 mM ammonium formate. Vitamins were quantified using an internal standard method. Using 25 μL injection volumes, the limits of detection were in the range of 0.066-5.3 ng in tear, and 0.087-1.1 ng in serum with linear responses for all vitamins. Intra- and inter-day precision and recoveries were satisfactory. This work is the first to demonstrate simultaneous vitamin detections in microliter of biological samples which has distinct advantages in many diagnostic applications with limited available fluids (e.g., tears; elderly anemic blood) or sampling small subjects (e.g., rodents).

Keywords: tear, blood serum, LC-MS, water-soluble vitamins, fat-soluble vitamins[1]

[1] ACN: Acetonitrile, BHT: Butylated Hydroxytoluene, D_2O: Deuterium Oxide, DMSO: Dimethyl Sulfoxide, ESI: Electrospray Ionization, FA: Formic Acid, IS: Internal Standard, LC: Liquid Chromatography, LOD: Limits of Detection, LOQ: Limits of Quantification, MeOH: Methanol, MS: Mass Spectrometry, rcf: Relative Centrifugal Force, RSD: Relative Standard Deviation, RT: Retention Time.

Introduction

Essential biochemical functions of vitamins in the human body include important roles in protein metabolism, maintenance of blood glucose levels, regulation of cell growth and cell differentiation [1]. Thirteen different vitamins are currently recognized by the USDA [2] and are classified into two groups according to their solubility; water-soluble vitamins include all B vitamins and vitamin C while the fat-soluble vitamins include vitamins A, D, E and K. Water-soluble vitamin deficiencies can cause permanent tissue damage and debilitating effects in humans while fat-soluble vitamins assist in anabolic and catabolic pathways in the body and are a current source of interest to nutritionists and clinicians.

All living tissues require vitamins and nutrients. The cornea is the outermost, transparent layer of living cells in the eye that helps focus light and protect the complex network of nerves and tissues in the eye [3]. The metabolism of the cornea requires a constant supply of amino acids, vitamins and other nutrients; no blood vessels extend to the cornea [3,4], so tears likely supply these nutrients. Previously we demonstrated the determination of water-soluble vitamins B_1, B_2, B_3, B_5 and B_9 and fat-soluble vitamin E in tears and blood serum via two separate LC-MS methods [5,6].

Water-soluble and fat-soluble vitamins have diverse chemical structures and properties, making their determination from a single chromatography assay challenging [7]. Previously reported vitamin detection methods predominantly focused on determining individual vitamins or a subset

of vitamins with similar polarities. For example, HPLC methods exist for a subset of water-soluble vitamins in blood serum, multivitamin tablets and food [8-11]. HPLC assays for several fat-soluble vitamins in blood serum, tablets and daily products have been reported as well [12-17]. There are also methods for simultaneous extraction of water-soluble and fat-soluble vitamins while they eventually used separate analytical methods for analysis [18,19]. Although simultaneous detection of water-soluble and fat-soluble vitamins in a single chromatography run from a single aliquot of sample has been reported, these methods are only reported to determine vitamin contents in uncomplicated, non-biological matrices such as pharmaceutical preparations [20-23], foodstuff [20,22,24,25] and beverages [26]. To date, no simultaneous vitamin detection method is available for extraction and detection of multiple vitamins with different polarities from biological samples. The method by Ferreiro-Vera and colleagues [27] was able to determine only two vitamins with different polarities in the blood serum while unified supercritical fluid and liquid chromatography method by Taguchi *et al.* [28] was only validated with standard solutions. Determination of tear vitamins A [29,30], D [31] and C [32,33] are also separately reported in the literature.

In this paper, we report a new, ternary solvent LC method using electrospray ionization (ESI) mass spectrometry for the identification and quantification of water-soluble and fat-soluble vitamins. The present LC-MS method is able to capture all 7 B vitamins and 5 fat-soluble vitamins (including two forms of vitamins D); while B_1, B_2, B_3 (nicotinamide), B_5, B_6 (pyridoxamine), B_9, A (retinol) and E (α-tocopherol) were successfully detected in blood serum

and B_1, B_2, B_3 (nicotinamide), B_5, B_9, E (α-tocopherol) and K_1 were successfully detected in human tear samples.

To improve upon assays that only measure a subset of vitamins, this combined method was developed using commonly available and robust analytical tools to provide a more complete nutritional status with reduced material and chemical demands, reduced instrument preparation and run times, smaller sample volumes and shortened active technician time. Further, small sample requirements improve the ability to detect vitamins in infants, sample limited situations (e.g. tears), elderly patients, or those with anemia. This paper describes the methodology used in a larger clinical study. Results from an extension of this method applied to tears and blood serum of 45 infant/parent pairs will be published in a subsequent paper. Here, we describe the technical aspects of the combined analytical strategy, which enables simultaneous determination of most water-soluble and fat-soluble vitamins without the need for separate assays.

Material and Methods

Materials and Chemicals

The purchased standard water-soluble and fat-soluble vitamins were: thiamine hydrochloride (reagent grade, ≥99%, HPLC), (-)-riboflavin (≥98%), nicotinamide (≥98%,TLC), D-pantothenic acid hemicalcium salt (≥98%, TLC), pyridoxamine dihydrochloride (≥98%), biotin (≥99%, TLC), folic acid (≥97%), retinol (synthetic, ≥95% (HPLC), crystalline), cholecalciferol (pharmaceutical secondary standard), 25-hydroxycholecalciferol (≥98%, HPLC), (±α)-

tocopherol (pharmaceutical secondary standard) and phylloquinone (analytical standard) from Sigma-Aldrich (St. Louis, MO, USA). Vitamin metabolites were selected based on the clinical assays [34,35] and availability in biological samples [36,37] (details in Supplemental Information, Section 2).

Stable isotope internal standards (IS) of vitamins B_1, thiamine-[4-methyl-^{13}C-thiazol-5-yl-$^{13}C_3$] hydrochloride, E, α-tocopherol-[ring-5,7-dimethyl-d_6] and K-[5,6,7,8-d_4, 2-methyl-d_3] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stable isotopes of vitamins B_2, riboflavin-[$^{13}C_4$,$^{5}N_2$], B_5, calcium pantothenate-[$^{13}C_3$,^{15}N], biotin-[d_2], and D_3-[6,19,19-d_3] were purchased from Isosciences (Trevose, PA, USA) and B_3, nicotinamide-[2,4,5,6,-d_4] and A, retinol-[d_5] were purchased from C/D/N isotopes (Pointe-Claire, Quebec, Canada) and ALSACHIM (Illkirch Graffenstaden, France), respectively. LC-MS grade methanol (MeOH), acetonitrile (ACN), acetone, formic acid (FA), ammonium formate, dimethyl sulfoxide (DMSO), butylated hydroxy toluene (BHT) and 99.99% deuterium oxide (D_2O) were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was purified using a Thermo UV/UF ultrapure water system (Waltham, MA, USA).

Standard Solutions for Calibration Curves

Stock solutions of 5 mM thiamine, nicotinamide, pantothenic acid and pyridoxamine in water and riboflavin, biotin and folic acid in DMSO were prepared in glass containers and stored at -20˚C. Solutions of 50 mM retinol, cholecalciferol and α-tocopherol, 5 mM 25-

hydroxycholecalciferol and 25 mM of phylloquinone in MeOH were stored in liquid nitrogen at -196˚C. IS stock solutions were 10 mM thiamine-[4-methyl-^{13}C-thiazol-5-yl-$^{13}C_3$] hydrochloride, 2 mM riboflavin-[$^{13}C_4$,$^{5}N_2$], 25 mM nicotinamide-[2,4,5,6,-d_4], 25 mM calcium pantothenate-[$^{13}C_3$,^{15}N], 25 mM biotin-[d_2], 3.4 mM retinol-[d_5], 5 mM α-tocopherol-[ring-5,7-dimethyl-d_6], and 2.5 mM vitamin K-[5,6,7,8-d_4, 2-methyl-d_3] in MeOH and 0.28 mM vitamin D_3-[6,19,19-d_3] in ethanol and stored in liquid nitrogen (-196˚C).

Mixtures containing all water-soluble vitamins in H_2O and all fat-soluble vitamins in MeOH were prepared with concentrations at 200 μM, and mixtures of IS with concentrations at 100 μM for water-soluble vitamins in D_2O and for fat-soluble vitamins in MeOH were prepared periodically and stored at -20˚C. 1.5 g/L solution of BHT in MeOH was also prepared and stored at -20˚C. Finally, six calibration levels were prepared by diluting mixture solutions of water-soluble and fat-soluble vitamins in MeOH to give final concentrations in the range of 0.3-10 μM for all analytes. 4 μM water-soluble and fat-soluble vitamin IS and 0.75 g/L BHT (for vitamins stabilizations) were also added. All solutions were protected from UV light during preparation, use, and storage.

Blood and Tear Preparation

Blood and tear samples were obtained from individuals with informed, documented consent by a phlebotomist in a local clinic following IRB protocols (M0934, [336669-5]), approved by Michigan Tech and UP Portage Health review boards. 70 μL tears were collected from each

individual by placement of two Schirmer strips (each marked to 35 μL), one inside each of the patient's lower eyelids. Strips were stored in a 1.5 mL eppendorf tube at -20˚C. Blood samples were collected in no-additive tubes (red top) and centrifuged for 10 min at 896 relative centrifugal force (rcf) to separate plasma. The plasma was removed and stored at -20˚C in a glass container.

For extraction of water-soluble and fat-soluble vitamins simultaneously from blood, 800 μL of MeOH/ACN/Acetone, 1:1:1 (v/v/v), containing 1 μM of each water-soluble and fat-soluble vitamin IS and 200 μL of 1.5 g/L BHT solution were added to 200 μL plasma and vortexed. The mixture was incubated at 4˚C for 10 min to precipitate proteins, then vortexed and centrifuged for 10 min at 896 rcf. The supernatant (serum) was dried under nitrogen and analytes were reconstituted in 100 μL of 0.1% FA in water/MeOH, 9:1 (v/v).

Simultaneous extraction of water-soluble and fat-soluble vitamins from tears was accomplished from the two tear strips (70 μL tears) via addition of 400 μL MeOH, ACN, acetone (1:1:1 by volume). 2 μM water-soluble and fat-soluble IS was added along with 70 μL BHT solution for vitamin stabilization. The vial containing sample and solvents was incubated at 4˚C for 10 min, then centrifuged at 896 rcf for 10 min. Supernatant was dried under a gentle stream of nitrogen and reconstituted in 100 μL of 0.1% FA in water/MeOH, 9:1 (v/v).

LC-MS/MS Analysis

LC-MS/MS was performed using an Accela LC quaternary pump coupled with an autosampler and an LCQ Fleet MS with an electrospray (ESI) probe (Thermo Scientific, Waltham, MA, USA). Separation was performed using a Waters (Milford, MA, USA) Atlantis T3 column, 2.1 mm x 150 mm, packed with 3 μm C_{18} silica and 100 Å pore size coupled with a guard column (Atlantis T3 Sentry, 2.1 mm x 10 mm). A 1:5 ratio post column binary fixed flow splitter (20% to MS, 80% to waste) was used to increase the analyte ionization efficiency (This is unnecessary with a heated ESI probe.).

The ternary mobile phases were A) 0.1% FA in water, B) 0.1% FA in ACN and C) 5 mM ammonium formate in MeOH. The gradient was 0 min, 100% A; 7 min, 100% A; 12 min, 50% A/50% B; 16 min, 20% A/80% B; 16.01 min, 100% C; 34 min, 100% C. ESI and MS parameters were optimized over six segments with 4 kV spray voltage and 275˚C capillary temperature for all vitamins. The capillary and tube lens voltages were optimized over time and after instrument maintenance and were in the range of 1 - 46 V and 60 - 115 V, respectively. The electrospray probe was operated in the positive ion mode in segments 1 - 6 and the sheath gas flow rate was set to 10 and 20 (arbitrary units), respectively for water-soluble and fat-soluble vitamins. A 25 μL sample with full loop injection was introduced at a flow rate of 0.2 mL/min for the first 16 min and 0.4 mL/min for the last 18 min. The column was re-equilibrated between runs with 20 column volumes of mobile phase A for 20 min. Autosampler and column temperatures were fixed at 5 and 25˚C, respectively. Nitrogen was used as a nebulizing gas. All data acquisition was done with Xcalibur 2.3 (version C).

Linearity and Limit of Detection

For vitamin quantifications, signals from triplicate analysis of the six calibration solutions were measured and calibration curves were built by plotting the ratio of analyte peak area to the area of IS versus concentration using the least-squares regression method.

Limits of detection (LOD) and limits of quantification (LOQ) were determined based on the standard deviation method [38]. The LOD and LOQ were respectively defined as 3 and 10 times the S/m ratio, where S is the signal standard deviation from the replicate injections (n = 7) of a low-level sample (standard solution, tear or blood serum) and m is the slope of the linear calibration curve. The standard deviations were calculated for concentrations lower than the LOQ and the RSD were < 20%.

Results and Discussion

In our related publication [6], we developed separate HPLC methods for the determination of water-soluble and fat-soluble vitamins in tears and blood serum. These methods required 18 min for the water-soluble vitamins separation and 25 min for the fat-soluble vitamins separation and each method needed 28 min for column re-equilibrium. Further, separate extractions were required and the total blood serum volume was 500 μL. We used these methods as a starting point for development of a combined method, which is described here. Original implementations of the combined method took > 60 min, but flow rate and gradient optimization enabled run times to be cut in half. The combined method described herein can detect 12 water-soluble and

fat-soluble vitamins in < 34 min using three mobile phases which reduced the total sample preparation and analysis time by 42% compared to our separate methods [6]. Eight of these vitamins were successfully extracted and detected from tears and blood serum using a single extraction step in < 30 min with a total sample volume of 200 μL blood serum and 70 μL tears. This method therefore enables detection of a majority of water-soluble and fat-soluble vitamins simultaneously from limited sample volumes.

Optimization of LC-MS/MS Conditions

Using the ternary gradient elution and ESI-MS/MS conditions described in section 2.4, water-soluble and fat-soluble vitamins eluted from the LC column in < 34 min as shown in Figure 1. The chromatography run was divided into six segments with respect to the analyte retention times to allow the ion trap mass analyzer to scan the precursor ions (listed in Table 1) using selected ion scanning mode. For quantifications, MS/MS specifications were used with selected reaction monitoring. Thus, specific fragment ions of each precursor ion were captured in order to increase the resolution and selectivity.

Figure 1 shows the chromatograms achieved by a standard mixture solution containing the 12 water-soluble and fat-soluble vitamins under described LC-ESI-MS/MS conditions. Chromatograms were generated from the signals for the MS/MS fragment ions listed in Table 1. Peak areas were used for quantification.

The separation mechanism selected for detection of water-soluble and fat-soluble vitamins was based on the structure of the compounds. An extensive literature review was conducted to minimize the time and cost for the method development. Water-soluble vitamins are polar compounds while fat-soluble vitamins are relatively less polar than water-soluble vitamins. Thus, a reversed phase C_{18} column was selected because it is known to be an appropriate method for separation of compounds that differ by polarity. Reverse phase separations start with high aqueous mobile phase while increasing the solvent composition throughout the gradient. Acetonitrile and methanol are common solvents used in reversed phase separations. The LC separation was optimized by changing compositions of the mobile phases in order to achieve the best and fastest separation (Supplemental Information, Figure 1S). For the mass spectrometry detection, the polar functional groups in water-soluble vitamin structures enable easy ionization by protonation. Ion formation was enhanced by 0.1% FA added to the mobile phases A and B (mass spectra are shown in Figure 2). Fat-soluble vitamins are considerably less polar than water-soluble vitamins and lack functional groups that readily accept or donate electrons, so mobile phase additives were necessary to facilitate their ionization with adduct formation using the hydroxyl (vitamin A, D_3, 25(OH)D_3 and E) or oxygen (vitamin K_1) groups in their structures. Mobile phase additives that have been used to promote ion formation for fat-soluble vitamins include formic acid [7], silver perchlorate [39], ammonium acetate [40], ammonium formate [41] or cesium acetate [42]. We systematically tested these additives at different concentrations. Cationic adducts from ammonium acetate, silver perchlorate and cesium acetate did not yield sufficient

spectral intensity (Figure 3). However, the hydrogen adduct peak height generated by 5 mM ammonium formate demonstrated sufficiently enhanced ionization concurrent with increasing mobile phase C pH to ~6 (below pKa of 9.25 for ammonium ion) and was thus chosen as the third mobile phase modifier that elutes fat-soluble vitamins. The mass spectra for all 12 vitamins and IS are shown in Figure 2 with the base peaks representing the protonated vitamins (see Table 1).

Analysis of Standard Vitamins with Optimized LC-MS

Using the identified LC-MS parameters, the water-soluble and fat-soluble vitamin retention times were determined from 3 replicate injections of the 0.3 to 10 μM concentrations as documented in Table 1. A summary of the chromatography and mass spectrometry parameters for detection of water-soluble and fat-soluble vitamins simultaneously is provided in Table 1. All 12 vitamins were detected in positive ESI mode. Vitamin B_1 and its corresponding IS (thiamine-[$^{13}C_4$]) precursor ions were observed as molecular ions, $[M]^+$. The precursor ions of vitamin A and its IS (retinol-[d_5]) resulted from the dehydration of the protonated molecule, $[M+H-H_2O]^+$. Peaks at *m/z* 429 and 435 were observed for vitamin E and its IS (tocopherol-[d_6]), which are produced from dehydrogenation of the protonated molecule to yield $[M+H-H_2]^+$ [43,44]. For all other vitamins and their IS, precursor ions were generated from the protonated molecule, $[M+H]^+$. The time periods of the six segments are also shown in Table 1. For quality assurance, Table 1 also lists some fragment ions produced with the optimized collision energies along with ones used for quantification.

Linearity, LOD, LOQ and Precision

The LOD and LOQ were calculated by the method described in section 2.5 and were determined in standard solutions for all 12 vitamins, and in tears and serum samples for detectable vitamins. Table 2a reports the standard curves and R^2 values for each of the 12 vitamins. The instrument response was linear for all vitamins with correlation coefficients > 0.99. The ranges of linearity for vitamins B_5 and B_6 were up to 200 μM, B_2, B_3, B_9, 25(OH)D_3, D_3, E and K_1 were up to 100 μM, B_1 and A were up to 50 μM and B_7 up to 10 μM. These values indicate sufficiently high reliability that is consistent with other published techniques [13].

Inter-day (n = 7) and intra-day (n = 6) precision were evaluated with replicate injections of samples. RSD values were in the range of 1.6-12% except for tear vitamin B_9 (Table 2b). Tear B_9 precision was low (29% and 57% RSD) and as such, calculated B_9 concentrations in tears may not be reliable. Recoveries of vitamins were estimated by spiking the tear and serum samples and calculating the extracted amounts, which were 84.8 - 102 % for all detectable vitamins except for serum B_9. Serum B_9 recovery was as low as 36.1%, which caused the calculated amounts in serum samples to be less reliable than other vitamins. Precisions, except for vitamin B_9 were sufficient for use in subsequent assays and consistent with other vitamin techniques [13,22]. B_9 recovery was also tested using a stable B_9 isotope, B_9-[$^{13}C_5$], as an internal standard. Low recoveries of ~10% were still obtained from the serum. This result is likely due to the combination of instability and low concentrations for this tested form of vitamin B_9 in serum samples. According to the May Clinic [45], more sensitive methods such as competitive binding

assays are required for reliable detection of vitamin B_9. In these assays, folate is measured as an indicator of all folic acid derivatives, which in serum is almost entirely present as N-(5)-methyl tetrahydrofolate [46].

Tears and Serum Analysis

To test the combined method performance on complex biological samples, vitamins were extracted from tears and blood serum of five human subjects under procedures described in section 2.3, and analyzed with the LC-MS/MS combined detection method for water-soluble and fat-soluble vitamins. Vitamin concentrations resulting from the five individuals are summarized in Table 3. The combined method detected vitamins B_1, B_2, B_3, B_5, B_6, B_9, A and E in blood serum and vitamins B_1, B_2, B_3, B_5, B_6, B_9 and E in tears. Serum B_9 recovery and tear B_9 precision were low as described in section 3.3, thus the detected amounts are not reported in Table 3. This method was also tested on newborn tears and resulted detection of vitamin K_1. The detectable vitamin K_1 concentrations in newborns can be explained by the vitamin K shot that they receive after birth. This data will be reported in a future paper. Figure 4 shows the vitamin chromatograms achieved from analysis of a tear sample (Figure 4a) and a serum sample (Figure 4b) under the described combined method. Our combined method, compared to our previously published method, is capable of detecting most water-soluble and fat-soluble vitamins simultaneously in human blood and it is also the first demonstration of simultaneous detection of these two groups of vitamins in human tears. A longitudinal study will be published with the application of this combined method for vitamin detections in infants and parents.

Our combined method was also tested using a triple quadrupole MS with higher sensitivity at our co-author's laboratory in the Kellogg Eye Institute and the same vitamins remained undetectable. Undetected K_1 in serum was attributed to the very low sample concentrations (0.0004 - 0.002 μM [47]) which were lower than our method LOD. K_1 was also undetectable in serum of newborns which lead us to hypothesize that vitamin K is probably higher in tears than serum of newborns. The undetected B_7 and vitamin D metabolites were tested for potential matrix effects. Standard solutions of these vitamins were spiked into pooled serum and tear samples and precision and recoveries were calculated. B_7 and D vitamins were recovered 91.6 - 103% with inter- and intra-day precision of 3.4 - 9.0%. Thus, the undetected vitamins were not due to matrix effects. This is not surprising since the presence of IS would elucidate matrix effects. However, B_7 and vitamin D are both protein-bound vitamins [48,49], such that a proteolysis step is required to break the protein bond and release the vitamins prior to protein precipitation. The body requires extremely small concentrations of B_7 which can efficiently be recycled, and food sources with B_7 are abundant [50], thus the inability to detect B_7 is not a major concern. Deficiency of vitamin K is also quite rare because intestinal bacteria produce this vitamin, which is also abundant in many foods [51]. The biologically active form of vitamin D is the 25(OH)D considered for nutritional health diagnosis [52]. Thus, only one of the critical vitamins for nutritional health, 25(OH)D, was undetectable in the time and resource-efficient combined method presented herein.

Comparisons of results with the literature revealed additional efficiencies with our combined method. Liquid-liquid extractions (LLE) with hazardous organic solvents (e.g., hexane) or solid-

phase extractions were primarily used to pre-concentrate vitamin analytes prior to HPLC [10,27,53]. Our previously published separate water-soluble and fat-soluble vitamin methods [5] included 3 LLE steps with hexane were used for fat-soluble vitamins. In the combined method herein, both groups of vitamins were extracted under a single extraction step. Chatzimichalakis *et al.* [10] published a simultaneous method for determination of only B-complexes (thiamine, riboflavin, nicotinic acid and nicotinamide, pyridoxine, folic acid, and cyanocobalamin) in blood serum and pharmaceuticals with solid-phase extraction and total analysis time of 27 min. While in our combined method, 6 different B vitamins were extracted and eluted in less than 15 min with a flow rate of 4 times less and lower detection limits along with two fat-soluble vitamins.

In separate LC-MS methods, which are perceived to be the rapid standard, analysis takes 5 min with at least 15 min of active technician time for sample preparation for each vitamin. In the method published by Papadoyannis *et al.* [53], solid-phase extraction cartridges were necessary to separate water-soluble and fat-soluble vitamins from 500 μL of blood plasma. Further, vitamin detection required two different HPLC columns. Our combined method analyzed 8 vitamins in < 30 min with 3 min or less active technician time. Thus, our total assay time was 33 min for 8 vitamins compared to 20 min per samples for a total time of 160 min for 8 vitamins. Further, since our combined method requires only one column cleaning and stabilization cycle, while the separate assays require one each, solvent utilization decreases and instrument utilization time can increase by 42 %. Other sensitive quantification methods, like Enzyme Linked ImmunoSorbent Assay (ELISA), require 2 to 24 hours' assay time and ~30 min active technician time per vitamin

assay. When compared to the combined method presented herein, active technician time is reduced by a factor of ~5. Thus, the method presented herein demonstrates advantages beyond prior protocols for determination of water-soluble and fat-soluble vitamins in serum samples. To the best of our knowledge, simultaneous detection of water-soluble and fat-soluble vitamins in complex biological samples has not been previously reported in the literature. This combined method provides a resource-lean and efficient means to simultaneously detect most water-soluble and fat-soluble vitamins in complex biological samples.

Conclusions

This paper describes the first demonstration of simultaneous determination and quantification of 8 water-soluble and fat-soluble vitamins from clinically-obtained human tears and blood serum samples. Our simultaneous protocol was able to capture 12 water-soluble and fat-soluble vitamins in < 34 min from standard solutions, while among these vitamins, B_1, B_2, B_3 (nicotinamide), B_5, B_6 (pyridoxamine), B_9, E (α-tocopherol) and K_1 were simultaneously extracted and detected in tears and B_1, B_2, B_3 (nicotinamide), B_5, B_6 (pyridoxamine), B_9, A (retinol) and E (α-tocopherol) were simultaneously extracted and detected in blood serum in < 30 min. Previously published methods have not demonstrated simultaneous extraction and detection of these vitamins from biological samples. The combined method presented herein optimized extraction solvents combined with tuned LC properties such as column, ternary mobile phase and eluent modifiers to produce sufficient sensitivity and peak resolutions that are consistent or better

than separate methods. Also, isotopically labeled versions of the target analytes utilized as IS reduced sample preparation errors and compensated for matrix effects and recoveries.

Compared to separate methods for water-soluble and fat-soluble vitamins, our combined method decreased instrument preparation and run time, reduced active technician time by a factor of 5, reduced material and chemical demands, and reduced sample demands. Sample preparation time was also shortened since a single extraction step efficiently extracted both water-soluble and fat-soluble vitamins. This combined method is highly beneficial for applications with limited availability of samples (e.g., infant tears; elderly anemic blood) or sampling small subjects (e.g., rodents). Further, this combined method detects all but one of the critical water-soluble and fat-soluble vitamins for clinical screening and provides substantial time and resource savings for nutritional assessments from biofluids.

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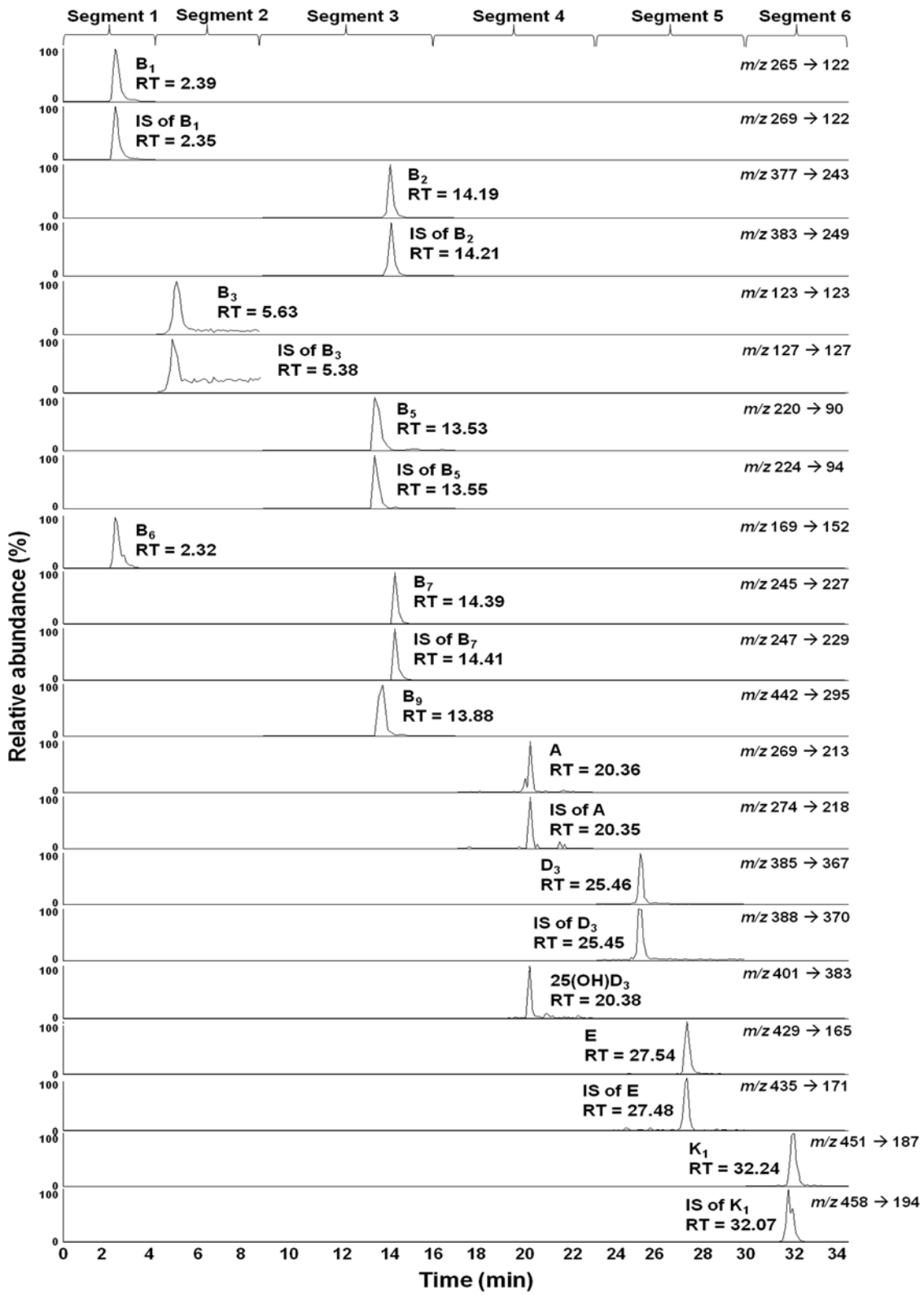


Figure 1. Chromatograms of 12 water-soluble and fat-soluble vitamins detected simultaneously with the LC-ESI-MS/MS method using a standard vitamin solution. Total analysis time was 34 min. Peaks illustrate the selected fragment ions of the precursor ions generated under selected reaction monitoring mode and include the analyte names and retention times (RT). For quantification of vitamins B_6 and B_9, the peak area of vitamin B_2 internal standard (IS) was used; and for quantification of 25(OH)D_3, the peak area of the vitamin D_3 IS was used.

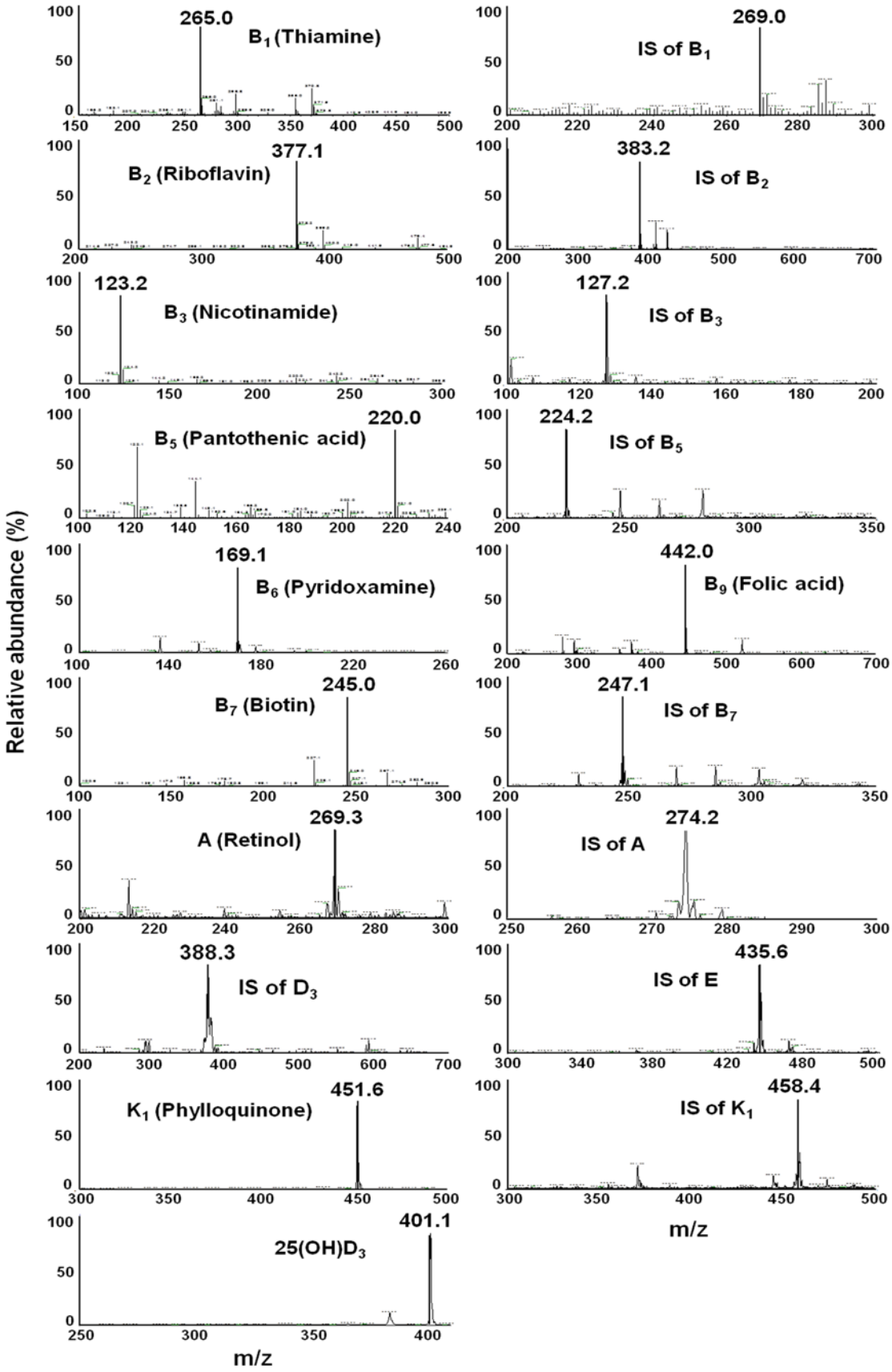


Figure 2. ESI-mass spectra for water-soluble vitamins (with 0.1% formic acid in mobile A and B) and fat-soluble vitamins (with 5 mM ammonium formate in mobile phase C) and their corresponding internal standards (stable isotope substituted analytes) generated from a vitamin standard solution. Precursor ions of the analytes are labeled on the spectra. The fragment ions for each of these precursor ions are reported in Table 1 and were used for quantification. The mass spectra of vitamin D_3 and E are shown in Figure 3.

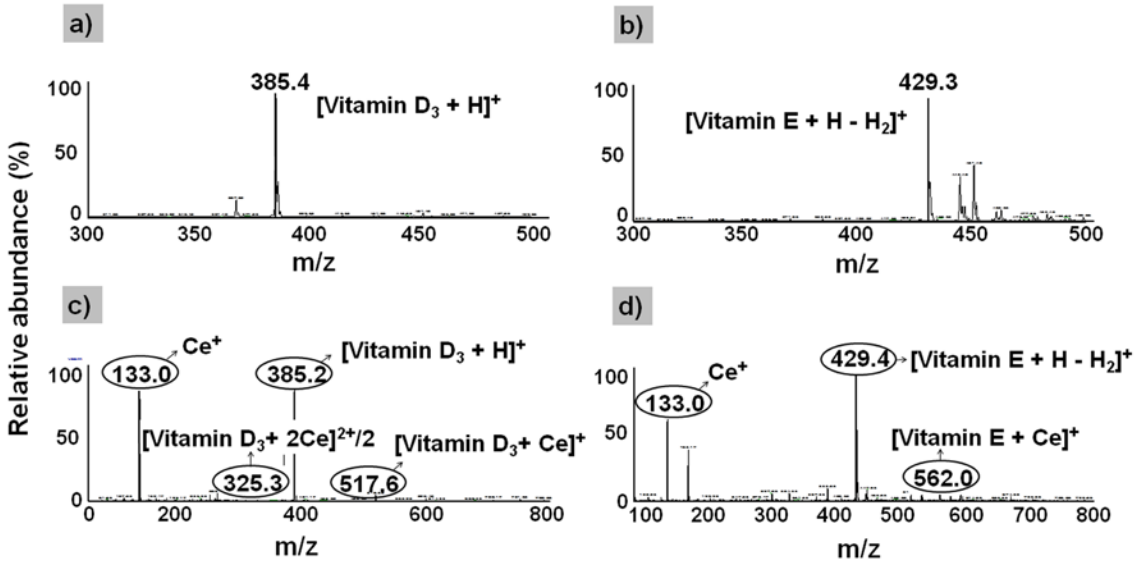


Figure 3. Mass spectra of a) vitamin D_3 and b) vitamin E with ammonium formate compared to c) vitamin D_3 and d) vitamin E with cesium acetate. Cesium cation (Ce^+) adducts were not sufficiently intense for reliable detection, while the hydrogen adduct peak height generated by ammonium formate demonstrated sufficiently enhanced ionization and was chosen as the preferred mobile phase C modifier.

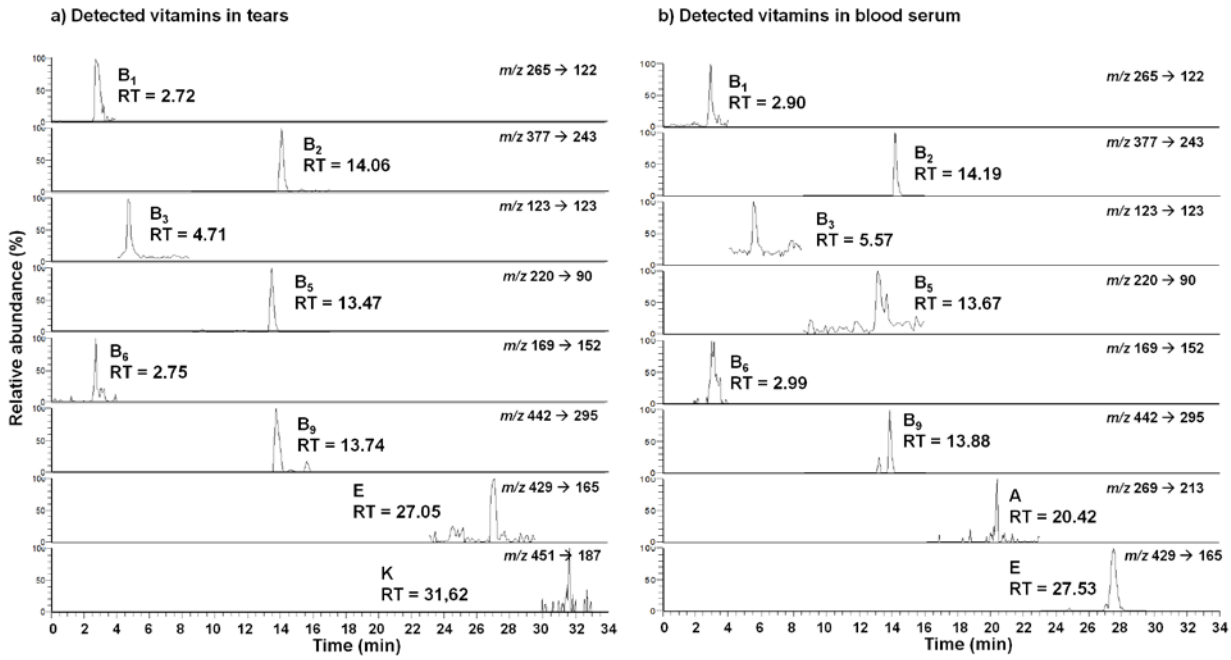


Figure 4. LC-MS/MS chromatograms of water-soluble and fat-soluble vitamins detected in a) a tear sample and b) a blood serum sample using the < 30 min, reduced materials/chemicals combined vitamin method presented herein.

List of Tables

Table 1. Chromatography and Mass Spectrometry Results for Simultaneous Detection of Water-Soluble and Fat-Soluble Vitamins

Time period (min)	Vitamins	Molecular weight (Da)	Precursor ion (m/z)	Collision energy (eV)	Fragment ion for quantification (m/z)	Retention time (min)	Other fragment ions (m/z)
0 - 4	B_1, Thiamine	300	265.0 $[M]^+$	20	122	2.36 ± 0.03	144, 156
	Thiamine-[$^{13}C_4$]	304	269.0 $[M]^+$	20	122	2.35 ± 0.00	251, 160, 148
	B_6, Pyridoxamine	168	169.1 $[M+H]^+$	18	152 $[M+H -H2O]^+$	2.31 ± 0.02	-
4 - 8.5	B_3, Nicotinamide	122	123.2 $[M+H]^+$	0	123	5.48 ± 0.15	105, 80
	Nicotinamide-[d_4]	126	127.2 $[M+H]^+$	0	127	5.38 ± 0.04	109, 83
8.5 - 16	B_2, Riboflavin	376	377.1 $[M+H]^+$	23	243	14.19 ± 0.00	359
	Riboflavin-[$^{13}C_4$,$^{15}N_2$]	382	383.2 $[M+H]^+$	23	249	14.21 ± 0.00	365
	B_5, Pantothenic acid	219	220.0 $[M+H]^+$	18	90	13.60 ± 0.08	202, 184
	Pantothenate-[$^{13}C_3$,^{15}N]	223	224.2 [M+H]+	18	94	13.57 ± 0.06	205, 188
	B_7, Biotin	244	245.0 $[M+H]^+$	16	227 $[M+H -H_2O]^+$	14.44 ± 0.07	-
	Biotin-[d_2]	246	247.1 $[M+H]^+$	16	229 $[M+H -H_2O]^+$	14.43 ± 0.06	-
	B_9, Folic acid	441	442.0 $[M+H]^+$	19	295	13.88 ± 0.00	424, 313
16 - 23	A, Retinol	286	269.3 $[M+H-H_2O]^+$	25	213	20.36 ± 0.00	157, 119, 93
	Retinol-[d_5]	291	274.2 $[M+H-H_2O]^+$	25	218	20.35 ± 0.00	162, 124, 93
	25(OH)-D_3	400	401.1 $[M+H]^+$	16	383 $[M+H -H_2O]^+$	20.36 ± 0.03	365
23 - 29.5	D_3, Choleocalciferol	384	385.4 $[M+H]^+$	22	367 $[M+H -H_2O]^+$	25.44 ± 0.04	259
	Choleocalciferol-[d_3]	387	388.3 $[M+H]^+$	22	370 $[M+H -H_2O]^+$	25.41 ± 0.09	259

	E, α-Tocopherol	430	429.4 $[M+H-H2]^+$	27	165	27.50 ± 0.10	205
	Tocopherol-[d_6]	436	435.6 $[M+H-H_2]^+$	27	171	27.48 ± 0.06	417, 211
29.5 - 34	K_1, Phylloquinone	450	451.6 $[M+H]^+$	25	187	32.17 ± 0.07	433, 225
	Phylloquinone-[d_7]	457	458.4 $[M+H]^+$	25	194	31.98 ± 0.08	440, 232

Table 2a. Calibration Data, Limits of Detection (LOD), Limits of Quantification (LOQ)

Vitamins	Calibration curves	R^2 Correlation	LOD (ng) in standard solution	LOQ (ng) in standard solution	LOD (ng) in serum	LOQ (ng) in serum	LOD (ng) in tear	LOQ (ng) in tear	Linear range (μM)
B_1, Thiamine	y = 0.3600x + 0.002	0.9985	0.043	0.14	0.094	0.31	0.088	0.29	0.01-50
B_2, Riboflavin	y = 0.2280x + 0.001	0.9993	0.055	0.18	0.087	0.29	0.066	0.22	0.009-100
B_3, Nicotinamide	y = 0.2539x + 0.033	0.998	0.60	2.0	0.37	1.2	0.24	0.79	0.1-100
B_5, Pantothenic acid	y = 0.2135x + 0.000	0.9974	0.60	2.0	0.45	1.5	0.36	1.2	0.08-200
B_6, Pyridoxamine	y = 0.0335x + 0.001	0.9984	0.082	0.27	0.16	0.54	0.32	1.1	0.04-200
B_7, Biotin	y = 0.1242x + 0.001	0.9988	0.30	1.0	-	-	-	-	0.05-10
B_9, Folic acid	y = 0.0221x + 0.000	0.9963	0.60	2.0	0.64	2.1	5.3	18	0.06-100
A, Retinol	y = 0.7444x + 0.186	0.9992	2.1	7.0	1.3	4.4	-	-	0.2-50
D_3, Cholecalciferol	y = 0.2915x + 0.005	0.9976	0.45	1.5	-	-	-	-	0.5-100
25(OH)-D_3	y = 0.1029x + 0.002	0.9987	2.5	8.2	-	-	-	-	0.2-100
E, α-Tocopherol	y = 0.2658x + 0.031	0.9954	1.4	4.6	1.1	3.6	0.28	0.92	0.1-100
K_1, Phylloquinone	y = 0.0546x + 0.001	0.998	0.74	2.5	-	-	0.12	0.41	0.07-100

Table 2b. Recovery (%), Intra-day and Inter-day Precision (RSD)

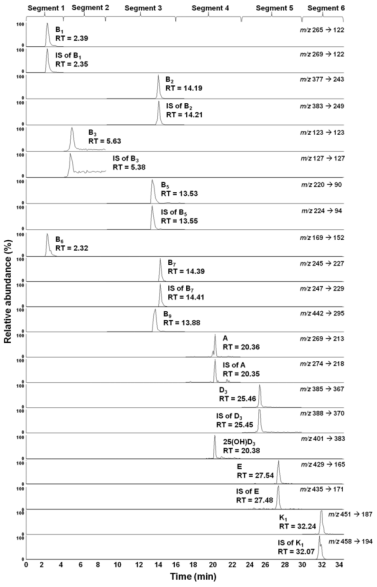
Vitamins	Intra-day precision in standard solution	Inter-day precision in standard solution	Intra-day precision in serum	Inter-day precision in serum	Intra-day precision in tear	Inter-day precision in tear	Recovery in serum	Recovery in tear
B_1, Thiamine	2.2	4.2	1.6	2.4	6.7	5.9	94.6	102
B_2, Riboflavin	3.2	4.4	4.1	2.4	6.0	7.4	100.7	98.4
B_3, Nicotinamide	3.4	7.2	2.2	3.9	7.2	7.6	98.5	91.4
B_5, Pantothenic acid	3.6	9.8	4.7	4.3	3.8	7.6	87.9	86.8
B_6, Pyridoxamine	3.0	4.8	4.7	6.4	8.5	8.1	93.7	92
B_7, Biotin	4.4	6.8	-	-	-	-	-	-
B_9, Folic acid	11	10	7.1	8.7	29	57	36.1	84.8
A, Retinol	8.1	10	9.8	12	-	-	89	-
D_3, Choleocalciferol	8.5	8.2	-	-	-	-	-	-
25(OH)-D_3	7.5	8.4	-	-	-	-	-	-

E, α-Tocopherol	3.2	4.9	4.6	2.5	6.0	7.7	94	91.7
K_1, Phylloquinone	5.0	9.4	-	-	6.4	7.9	-	87.2

Table 3. Vitamin Concentrations (μM) in Tears and Blood Serum of Five Tested Individuals

Vitamins	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
	Tear	Serum	Tear	Serum	Tear	Serum	Tear	Serum	Tear	Serum
B_1, Thiamine	0.022	0.054	0.038	0.14	N.D.	0.13	0.027	0.013	N.D.	0.053
B_2, Riboflavin	0.018	0.034	N.D.	0.036	0.060	0.034	0.032	N.D.	0.015	0.018
B_3, Nicotinamide	4.0	1.9	1.3	0.92	5.0	2.4	1.3	1.1	6.9	0.35
B_5, Pantothenic acid	0.49	0.44	0.12	0.12	0.78	0.46	0.23	0.13	0.21	0.16
B_6, Pyridoxamine	N.D.	0.62	0.083	0.71	0.12	1.3	N.D.	0.51	0.22	0.42
A, Retinol	-	1.1	-	2.9	-	2.7	-	1.4	-	1.0
E, α-Tocopherol	0.13	14	0.090	16	0.42	20	0.055	9.2	0.12	7.1

N.D. not detected.



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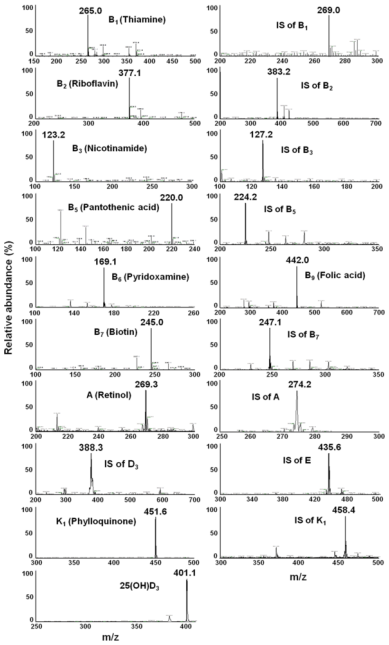

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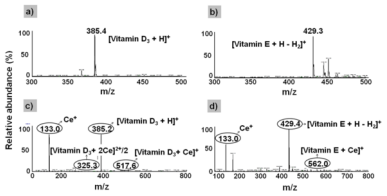

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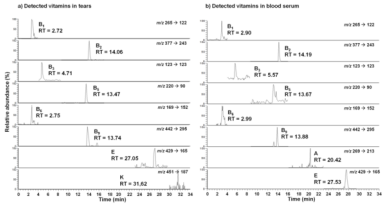

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Table 1. Chromatography and mass spectrometry results for simultaneous detection of water-soluble and fat-soluble vitamins

Time period (min)	Vitamins	Molecular weight (Da)	Precursor ion (m/z)	Collision energy (eV)	Fragment ion for quantification (m/z)	Retention time (min)	Other fragment ions (m/z)
0 - 4	B_1, Thiamine	300	265.0 $[M]^+$	20	122	2.36 ± 0.03	144, 156
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	Phylloquinone-[d_7]	457	458.4 $[M+H]^+$	25	194	31.98 ± 0.08	440, 232

Table 2a. Calibration data, limits of detection (LOD), limits of quantification (LOQ)

Vitamins	Calibration curves	R^2 Correlation	LOD (ng) in standard solution	LOQ (ng) in standard solution	LOD (ng) in serum	LOQ (ng) in serum	LOD (ng) in tear	LOQ (ng) in tear	Linear range (μM)
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B_2, Riboflavin	y = 0.2280x + 0.001	0.9993	0.055	0.18	0.087	0.29	0.066	0.22	0.009-100
B_3, Nicotinamide	y = 0.2539x + 0.033	0.998	0.60	2.0	0.37	1.2	0.24	0.79	0.1-100
B_5, Pantothenic acid	y = 0.2135x + 0.000	0.9974	0.60	2.0	0.45	1.5	0.36	1.2	0.08-200
B_6, Pyridoxamine	y = 0.0335x + 0.001	0.9984	0.082	0.27	0.16	0.54	0.32	1.1	0.04-200
B_7, Biotin	y = 0.1242x + 0.001	0.9988	0.30	1.0	-	-	-	-	0.05-10
B_9, Folic acid	y = 0.0221x + 0.000	0.9963	0.60	2.0	0.64	2.1	5.3	18	0.06-100
A, Retinol	y = 0.7444x + 0.186	0.9992	2.1	7.0	1.3	4.4	-	-	0.2-50
D_3, Cholecalciferol	y = 0.2915x + 0.005	0.9976	0.45	1.5	-	-	-	-	0.5-100
25(OH)-D_3	y = 0.1029x + 0.002	0.9987	2.5	8.2	-	-	-	-	0.2-100
E, α-Tocopherol	y = 0.2658x + 0.031	0.9954	1.4	4.6	1.1	3.6	0.28	0.92	0.1-100
K_1, Phylloquinone	y = 0.0546x + 0.001	0.998	0.74	2.5	-	-	0.12	0.41	0.07-100

Table 2b. Recovery (%), intra-day and inter-day precision (RSD)

Vitamins	Intra-day precision in standard solution	Inter-day precision in standard solution	Intra-day precision in serum	Inter-day precision in serum	Intra-day precision in tear	Inter-day precision in tear	Recovery in serum	Recovery in tear
B_1, Thiamine	2.2	4.2	1.6	2.4	6.7	5.9	94.6	102
B_2, Riboflavin	3.2	4.4	4.1	2.4	6.0	7.4	100.7	98.4
B_3, Nicotinamide	3.4	7.2	2.2	3.9	7.2	7.6	98.5	91.4
B_5, Pantothenic acid	3.6	9.8	4.7	4.3	3.8	7.6	87.9	86.8
B_6, Pyridoxamine	3.0	4.8	4.7	6.4	8.5	8.1	93.7	92
B_7, Biotin	4.4	6.8	-	-	-	-	-	-
B_9, Folic acid	11	10	7.1	8.7	29	57	36.1	84.8
A, Retinol	8.1	10	9.8	12	-	-	89	-
D_3, Choleocalciferol	8.5	8.2	-	-	-	-	-	-
25(OH)-D_3	7.5	8.4	-	-	-	-	-	-
E, α-Tocopherol	3.2	4.9	4.6	2.5	6.0	7.7	94	91.7
K_1, Phylloquinone	5.0	9.4	-	-	6.4	7.9	-	87.2

Table 3. Vitamin concentrations (μM) in tears and blood serum of five tested individuals

Vitamins	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
	Tear	Serum	Tear	Serum	Tear	Serum	Tear	Serum	Tear	Serum
B_1, Thiamine	0.022	0.054	0.038	0.14	N.D.	0.13	0.027	0.013	N.D.	0.053
B_2, Riboflavin	0.018	0.034	N.D.	0.036	0.060	0.034	0.032	N.D.	0.015	0.018
B_3, Nicotinamide	4.0	1.9	1.3	0.92	5.0	2.4	1.3	1.1	6.9	0.35
B_5, Pantothenic acid	0.49	0.44	0.12	0.12	0.78	0.46	0.23	0.13	0.21	0.16
B_6, Pyridoxamine	N.D.	0.62	0.083	0.71	0.12	1.3	N.D.	0.51	0.22	0.42
A, Retinol	-	1.1	-	2.9	-	2.7	-	1.4	-	1.0
E, α-Tocopherol	0.13	14	0.090	16	0.42	20	0.055	9.2	0.12	7.1

N.D. not detected.