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Simultaneous analysis of circulating 25-hydroxy-vitamin D₃, 25-hydroxy-vitamin D₂, retinol, tocopherols, carotenoids, and oxidized and reduced coenzyme Q10 by high performance liquid chromatography with photo diode-array detection using C18 and C30 columns alone or in combination

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Abstract

Circulating lipid-phase micronutrients (LPM) such as 25-hydroxylated D vitamers, retinol, tocopherols, carotenoids including their isomers, and coenzyme Q10 play important roles in health maintenance and disease prevention and can serve as useful biomarkers. We developed fast, affordable, and accurate HPLC assays that simultaneously measured all above LPM in a single run using UV/VIS detection at 265 nm, 295 nm, and 480 nm with (1) a C18 column alone; (2) a C30 column alone; or (3) each of these columns connected in series. The C18 column alone could separate all major LPM of interest in less than 17 min but insufficiently resolved the lycopene isomers, the 25-hydroxylated D vitamers, lutein from zeaxanthin and β - from γ -tocopherol. The C30 column alone separated all LPM of interest including many isomeric analytes but failed to resolve the Q10 compounds, which co-eluted with carotenoids. Connecting the C18 and C30 columns in series with a detector after the C30 column and a pressure resistant detector between the columns resulted in ideal resolution and accurate quantitation of all LPM of interest but required software capable of processing the acquired data from both detectors. Connecting the C18 and C30 columns in series with exclusively one detector after the C30 column resulted in carotenoid-Q10 interferences, however, this was remedied by heart-cutting 2D-LC with a 6-port valve between the columns, which resolved all analytes in 42 min. Faster run times led to some analytes not being resolved. Many variations of these methods are possible to meet the needs of individual requirements while minimizing sample material and turn-around-times.

Keywords

HPLC UVVIS detection; Circulating carotenoids; Retinoids; Tocopherols; Coenzyme Q10; Vitamin D metabolites

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This manuscript is dedicated to Laurence N. Kolonel, MD, PhD, in honor of his retirement from the University of Hawaii Cancer Center (formerly Cancer Research Center of Hawaii) which he built up to national and international recognition for prime research in epidemiology. His intelligence, foresight, mindful leadership, outstanding accomplishments and collegiality he shared with researchers and staff members alike during his stellar career was inspirational and will lead us to new frontiers.

1. Introduction

Modern studies in the life sciences require high-throughput biochemical analyses that provide high efficiency, robustness, accuracy, and affordability, while utilizing minimal sample volume to provide results for multiple biomarkers from a single blood specimen. Specific lipid-phase micronutrients (LPM) present in the human circulation, namely 25-hydroxy vitamin D₂ (25(OH)D₂) and 25-hydroxy vitamin D₃ (25(OH)D₃) (established markers of vitamin D status), vitamins A and E, carotenoids, and coenzyme Q10 (Q10) in its reduced (ubiquinol-10, UL10) and oxidized (ubiquinone-10, UN10) form, play important roles in nutrition, health maintenance, and disease prevention serving as helpful nutritional and health status biomarkers [1–4]. Consequently, there exists a high demand for reliable and affordable analytical methods capable of determining these LPM.

Numerous methods for analyzing these LPM have been reported in the past which applied predominantly LC in the high pressure format (HPLC), the ultra-high pressure format (UHPLC), or the two-dimensional LC format (LC × LC) using either normal-phase (NP) [5–8] or reverse-phase (RP) columns [9–24] with preference to the latter column type due to its robust and high throughput feature, affordability, and ability to quantify a high variety of compounds at low levels in biological and non-biological matrices. NP columns have been effective in separating all E vitamers including β- and γ-forms of tocopherols [7,25] and tocotrienols [8] as well as all of the major geometric isomers of carotenoids, especially when calcium hydroxide, alumina, or silica-based nitrile-bonded packing materials have been applied [26,27]. The use of NP columns, however, is limited by its commercial unavailability and difficult preparation techniques [28]. In addition, NP-HPLC is sensitive to small changes in mobile phase composition that result in long equilibrium times during gradient elution and, consequently, variable retention times [29]. In contrast, RP columns are readily available, maintain superior stability and robustness over NP columns [29], and are highly successful in separating relatively low and non-polar carotenoids while still providing sufficient resolution power to separate geometric isomers. Typical RP column type materials include C18 and, more recently, C30 phases. The former provides fast and good separations for a wide range of analytes, especially for those with relatively short chains and low molecular weights. However, for longer chain compounds, C18 columns have a lower degree of molecular shape recognition compared to C30 columns, which were specifically tailored for carotenoid analysis. For this reason, C30 columns are superior to C18 columns in separating geometric (*Z*- vs. *E*-) and other (e.g. α- vs. β-) isomers in addition to resolving α-, β-, γ- and δ- tocopherol despite its long (often 50 min plus) run times [28,30,31].

The LC based techniques reported so far have employed mostly photo diode-array detection (DAD) and less frequently electrochemical (ECD), fluorescence (FD), thermal lens spectrometry (TLSD), and mass spectrometry (MSD) detection [2]. These reports, however, restrict their analyses to individual analytes [32–42] or a limited combination thereof [10,11,13–20,23,43–50] as a result of problems that arise when numerous LPM are simultaneously analyzed in a single chromatographic run. Examples of these problems include analyte co-elution and the extensive range of analyte concentrations, both of which make quantitation impossible because of interference or the inability to reach detectable

levels. While MSD and, in part, TLSD can overcome such problems [2,9], these techniques often incur high cost of instrument acquisition and time-consuming maintenance and troubleshooting in addition to the requirement of operational technical skills, which are often not available or again, prohibitively expensive to obtain. Finally, TLSD is often not commercially available, particularly in the UV range.

For this study, we sought to establish several simple yet accurate, fast, and affordable HPLC–DAD based methods to simultaneously analyze all major aforementioned circulating LPM in a single chromatographic run using either a C30 or C18 column alone or variations of the C18–C30 columns connected in series from only 200 μ L plasma. In addition, each method utilizes internal standards (IS) that were either commercially available or custom synthesized to suit each of the 5 analyte groups (i.e. retinol, Q10, tocopherol, carotenoid, and vitamin D groups).

2. Materials and methods

2.1. Apparatus and chemicals

The HPLC system consisted of an autosampler with a quaternary pump (model Accela, ThermoFisher, San Jose, CA, USA) equipped with UV/VIS DAD (10 μ L volume, 50 mm cell path length, and certified for back pressures up to 70 bar or 1000 PSI) and ChromQuest chromatography software (all from Thermo Scientific, San Jose, CA, USA). A multiple wavelength UV/VIS detector model G1314A with a model G1314-60182 high pressure flow cell of 14 μ L volume, 10 mm cell path length, and certified for back pressures up to 400 bar or 5800 PSI (Agilent, Santa Clara, CA) was employed if used between analytical columns; the analogue signal was fed into the ChromQuest software via a SS420 box. Coulometric detection was performed with a Coulochem III detector and a 5011A analytical cell (ESA, Chelmsford, MA) and again, the analogue signal was fed into the ChromQuest software via a SS420 box.

HPLC-grade methanol (MeOH), dichloromethane (DCM), acetonitrile (MeCN), hexane, hydrochloric acid (HCl), methyl tert-butyl ether (MTBE), and potassium hydroxide (KOH) were purchased from Fisher Scientific (Fair Lawn, NJ). Ethanol (EtOH) (100%) was obtained from Pharmco (Brookfield, CT). Tetrahydrofuran (THF), butylated hydroxytoluene (BHT), ammonium acetate (NH₄OAc), 4-dimethylamino-pyridine, triethylamine, and bis-tris propane were acquired from Sigma Chemical Co (St. Louis, MO) as were retinol, dodecanoic anhydride, glacial acetic acid (AA), sodium borohydride, ubiquinone (UN10), and butanol (BuOH). Retinyl laurate, butylated or ethylated ubiquinone-10 (Bu-UN10, Et-UN10), and butylated or ethylated ubiquinol-10 (Bu-UL10, Et-UL10) were used as ISs for retinoids, ubiquinone-10 (UN10), and ubiquinol-10 (UL10), respectively, and were synthesized as described below. *Rac*-tocol, used as the IS for tocopherols, was purchased as a solution in hexane from Matreya, LLC (Pleasant Gap, PA, cat#1797). Echinenone (ECH), used as the IS for carotenoids, was a gift from Dr. Maria Stacewicz-Sapuntzakis/Dr. Phyllis Bowen (University of Illinois at Chicago) and was originally donated from Hoffman-LaRoche, Basel, Switzerland now DSM. 1 α (OH)D₃, used as the IS for 25-hydroxylated D vitamers, was obtained from Sigma–Aldrich (St. Louis, MO). IS stock standards were stored at 20 °C. Working IS solutions were prepared under dimmed or yellow lighting and diluted

from stock solutions in hexane (tocol, butylated or ethylated UN10 and UL10), EtOH (retinyl laurate), or THF (ECH) so that peak sizes were similar to the analytes in the plasma extracts. IS concentrations were checked spectrophotometrically using reported extinction coefficients (Table 1) [4,12,43,51] and were accepted if purity, as assessed by HPLC, was greater than 95%.

2.2. Chromatography

2.2.1. Wavelength selection—DAD was carried out routinely by scanning between 200 and 600 nm. Quantitation was performed at 265 nm for 25(OH)D₂, 25(OH)D₃, UL10 and UN10 (plus their respective ISs), at 295 nm for retinol and tocopherols (plus their respective ISs), and at 450 nm for carotenoids (luteins, zeaxanthins, cryptoxanthins, lycopenes, carotenes, ECH). 480 nm was chosen to maximize lycopene detection. Sufficient signal strength could be obtained at 295 nm for retinol.

2.2.2. Column and detector configurations—In all methods, one DAD was used after the last analytical column and/or after the 6-port valve and chromatographic separations were performed using: (a) a fused-core Kinetex C18 analytical column alone (100 mm × 3.0 mm ID; 2.6 μm; Phenomenex, Torrance, CA, USA) with a preceding filter cartridge (21 mm, 0.2 μm; Thermo Scientific, Bellefonte, PA) or a SecurityGuard C18 precolumn cartridge (4 mm × 3.0 mm ID; 10 μm ('C18'; Fig. 1a); (b) a 5SIL 200-3 C30 analytical column alone (150 mm × 2.0 mm; 3 μm) with a preceding ProntoSIL 200-3 C30 precolumn (10 mm × 2.0 mm ID; 5 μm; both columns from MAC-MOD Analytical Inc, Chadds Ford, PA) ('C30'; Fig. 1a); and (c) these C18 and C30 columns from above connected in series with the SecurityGuard C18 precolumn cartridge preceding the C18 column also noted above.

The C18–C30 serially connected columns were assembled in several different configurations as shown in Fig. 1b–d. In the configuration of Fig. 1b (referred to as the 'C18–C30' system), the columns were serially connected directly to each other with a DAD after the columns. The configuration shown in Fig. 1c (referred to as the 'C18-D-C30' system) was similar to that in Fig. 1b except that a second, pressure resistant detector ('D1') was inserted between the columns. In the configuration of Fig. 1d (the double valve switching method or the 'C18–C30dvs' system), a 6-port valve was inserted between the serially connected C18 and C30 columns and was switched twice during the run – from position II (which directed the flow from the C18 to the C30 column then to DAD) to position I (which directed the flow from the C18 column directly to the DAD) at 19.2 min, then back to position II at 26.0 min. To shorten the run times of the C18–C30dvs system, the method was modified such that the valve was switched only once during the run – from position II to position I after 10 min (referred to as single valve switching or the 'C18–C30svs' system) or by applying an isocratic elution and valve switching from position II to I at 4 min and back to II after 18 min (referred to as the 'C18–C30dvsI' system). All systems were kept at ambient laboratory temperature (ca. 23 °C) except for the autosampler, which kept injection solutions at 4 °C.

2.2.3. Mobile phases—The mobile phase components were identical for all configurations presented and consisted of A = MeOH/1.5% aq. NH₄OAc (90:10; v/v), B = MTBE/MeOH/1.5% aq. NH₄OAc (90:8:2; v/v/v), and C = MTBE/MeOH/1.0% aq. NH₄OAc (10:88:2; v/v/v). Gradient elution was performed at a flow rate of 0.4 mL/min for the resolution of all analytes for the C18 alone, C30 alone, C18-DC30, C18-C30, and C18-C30dvs systems as follows: 0–6.5 min at 100% A; 6.5–7.0 min linear gradient to 95%B/5%C, 7.0–13.0 min keep at 95%B/5%C; 13.0–19.0 min linear gradient to 73%B/27%C, 19.0–21.0 min linear gradient to 50%B/50%C, 21.0–22.0 min linear gradient to 20%B/80%C, 22.0–22.2 min linear gradient to 97%B/3%C, 22.2–26.0 min hold at 97%B/3%C, 26.0–37.0 min linear gradient to 10%B/90%C, 37.0–41.0 min linear gradient to 50%B/50%C, then switch in 0.2 min to initial 100% A and equilibrate for 5 min before subsequent injections.

The following elution was used for the C18–C30svs method: 0–10.0 min at 100% A; 10.0–12.0 min linear gradient to 95%B/5%C, 12.0–14.0 min keep at 95%B/5%C, 14.0–24.0 min linear gradient to 73%B/27%C; 24.0–25.0 min linear gradient to 50%B/50%C, 25.0–27.0 min linear gradient to 20%B/80%C, 27.0–27.1 min linear gradient to 97%B/3%C then hold 2 min at that ratio and equilibrate for 5 min at 100% A before subsequent injections. The C18–C30dvs method used 20% B and 80% C isocratically. Changing these mobile phases by including other solvents did not lead to any improvements.

2.3. Collection of plasma

Blood was collected anonymously from adult volunteers at the University of Hawai'i Cancer Center (both genders, mean age of 41 years) by venipuncture through licensed phlebotomists into green-top Li-heparin vacutainers. Blood collection was approved by the University of Hawai'i Committee on Human Subjects and all volunteers signed the approved consent form. The blood was immediately processed by centrifugation at 1050 × g for 20 min at 4 °C. The supernatant plasma was pooled then aliquoted into individual cryogenic vials and immediately stored at –80 °C.

2.3.1. Extraction and analysis of LPM from plasma—200 µL EtOH containing 0.25 mg/ml BHT and spiked with five ISs (tocol, retinyl laurate, ECH, 1α(OH)D₃ and Bu-UN10) was added to 200 µL freshly thawed plasma. This mixture was carefully mixed and incubated for 5 min (to avoid balling [52]) followed by extraction with 1.5 mL hexane. The hexane layer was transferred to and evaporated in HPLC amber vials at room temperature under nitrogen flow (N₂). The residue was reconstituted with 10 µL THF, sonicated for 10 s, then diluted with 40 µL MeOH/MTBE (9:1) followed by another 10 s sonication. The resulting extract was transferred to a 100 µl glass insert placed in an amber vial and centrifuged for 5 min at 1050 × g and 5 °C. Twenty micro-liters of the clear supernatant extract was injected into the HPLC system. All extraction and handling procedures were conducted under yellow light to avoid light-induced isomerization and analyte degradation. Concentrations were determined from external calibration curves after adjusting for recovery of the appropriate IS.

2.4. Internal standards

Carotenoids, retinoids, tocopherols, and 25-hydroxylated D vitamers were adjusted for recovery of the ISs ECH, retinyl laurate, tocol, and $1\alpha(\text{OH})\text{D}_3$, respectively. UN10 and UL10 were adjusted for recovery of Et-UN10 or Bu-UN10 and Et-UL10 or Bu-UL10, respectively.

2.4.1. Synthesis of retinyl laurate—Retinyl laurate synthesis from retinol was modified by the procedure of Lentz et al. [53]. Briefly, 0.025 g retinol, 600 μL triethylamine, 0.07 g dodecanoic anhydride, and 40 mM 4-dimethylamino-pyridine in DCM were combined in a boiling flask, which was then attached to a condenser where the mixture was heated in a 60 °C water bath. Aliquots were taken every 30–60 min to monitor completion of the conversion by HPLC.

The resulting mixture from above was pipetted onto a C18 SPE column conditioned with 1 mL MeOH and 1 mL H_2O . The column was washed with 1 mL MeOH and 1 mL 50% aq. MeOH then dried under vacuum. The column eluate was collected using 1 mL DCM then subjected to rotary evaporation to remove the solvents. Evaporation resulted in an oily substance that was subsequently reconstituted in 500 μL hexane. Using HPLC, the yield was approximately 98%.

2.4.2. Synthesis of ethylated and butylated coenzyme Q10 compounds—Et-UN10 and Bu-UN10 standards were synthesized analogous to a previous report [54]. Briefly, 0.1 g UN10 was dissolved in 1 mL hexane followed by addition of 4 mL EtOH or BuOH and 200 μL 1 M KOH. The mixture was stirred in a darkened container for 1 h at room temperature. An HPLC C18 system (Model Accela, ThermoFisher, San Jose, CA, USA) monitored the reaction progress, which was deemed complete when all starting material disappeared (UN10: RT = 6.1 min) and the new product peaks emerged (Et-UN10: RT = 8.3 min; Bu-UN10: RT = 13.1 min). The reaction was quenched by the addition of 15 μL AA and the resulting mixture was extracted twice with 5 mL hexane. The combined hexane fractions were washed once with 5 mL H_2O then dried under N_2 . Using HPLC, the yields were approximately 80%.

Et-UN10 and Bu-UN10 were subsequently reduced to Et-UL10 and Bu-UL10 for use as ISs for UL10. The oxidized starting material was dissolved in 1 mL hexane and 4 mL EtOH followed by addition of 0.08 g sodium borohydride. The mixture was stirred in the dark for 30 min at room temperature when the peaks of the starting materials disappeared with concomitant appearance of a new peak for the reduced product (Et-UL10: RT = 7.0 min, Bu-UL10: RT = 9.0 min). The resulting mixture was diluted with 10 mL hexane and washed twice with 8 mL H_2O . The pH of the combined H_2O layers was lowered to pH 5–6 using 1 M HCL then re-extracted with 2 mL hexane. The combined hexane layers were dried under N_2 . The resulting crude reduced products were purified by HPLC equipped with a semi-prep Spherex 5 C18 column (250 $\times$ 10 mm, Phenomenex, Torrance, CA, USA) and using a mobile phase containing 30% (v/v) DCM in MeOH at a flow rate of 1 mL/min and monitored by UV at 295 nm. After elution, the desired Et-UL10 and Bu-UL10 products were immediately dried under N_2 to give the final product as white powder.

The reduced Bu-UL10 and Et-UL10 forms are not stable and easily convert back to their oxidized forms when exposed to oxygen. However, our experiments have shown that stability is assured if kept dry at -80°C .

3. Results and discussion

Numerous analytical techniques have been introduced in the past for the analysis of biologically relevant LPM. Most of these techniques have been HPLC-based separations using a variety of detection techniques [10,11,13–20,23,32–50]. Since, to the best of our knowledge, none of the reported techniques included the entire array of the major circulating LPM (retinol, carotenoids, tocopherols, predominant vitamin D metabolites, and coenzyme Q10) in a single chromatographic run with a simple, fast, and affordable assay we developed such an assay and chose HPLC with UV/VIS detection due to its robustness, sensitivity, affordability, accuracy, and non-destructive nature [4,12,55–59].

3.1. Separation of LPM using Kinetex C18 column

The fused-core particle Kinetex C18 column (Fig. 2a) yielded excellent separation of all major analytes and particularly the Q10 compounds [4]; it was able to resolve retinol, α -, β + γ , and δ -tocopherol, the major carotenoids and most of their isomers (Table 1). This column also baseline separated ECH from all carotenoids and, most importantly, eluted UL10, UN10, and their ethylated or butylated ISs after all carotenoids in a very late and empty portion of the chromatogram. However, applying the C18 alone showed insufficient resolution of 25(OH)D₂, 25(OH)D₃, lycopene isomers, and the common eluting lutein/zeaxanthin pair. Moreover, 25-hydroxylated D vitamers were not separated from retinol.

3.2. Separation of LPM using ProntoSil C30 column

The C30 column alone (Fig. 2b) performed superiorly over the C18 column by baseline separating 25(OH)D₃ from retinol and resolving all tocopherol isomers (including β - from γ -tocopherol) and all carotenoid isomers including numerous *E*- from all-*Z*-lycopene isomers, the latter of which appeared in a very late and empty part of the chromatogram. However, the C30 column failed to resolve ECH from *cis*- β -cryptoxanthin even though ECH is often recommended to be used as an IS with C30 columns [60]. In addition, using the C30 column alone resulted in the co-elution of the Q10 compounds with many carotenoids making quantification of the Q10 compounds impossible. The coupling of the same type of columns in series as recommended by others [61] did not improve the resolution substantially and also increased run times significantly (data not shown).

3.3. Separation of LPM using serially coupled C18–C30 columns

When the C18 and C30 columns were serially connected with a DAD after the C30 column (C18–C30 system; Figs. 1b and 2c), retinol, 25(OH)D₂ and 25(OH)D₃ and all the tocopherols and carotenoids including their isomers were resolved. The Q10 compounds, however, co-eluted with carotenoids (similar to the C30 column alone) thus preventing quantitation of UL10 and UN10 (Table 1). This problem was solved with the C18–C30dvs system (Figs. 1d and 2d); integrating a 6-port valve between the columns allowed heart-cutting 2D-LC by forcing the mobile phase through both columns (valve position II) in the

first 19 min until zeaxanthin was detected. At this time the residual carotenoids had already eluted from the C18 column and onto the C30 column while the Q10 compounds were still being retained on the C18 column. Switching the valve to position I at 19.2 min directed the mobile phase from the C18 column directly to the DAD bypassing the C30 column. In this manner, elution of the residual carotenoids from the C30 column was interrupted, which allowed the Q10 compounds and their ISs to be measured after eluting from the C18 column and directly to the detector in the period of 20–23 min. The carotenoid chromatography was reinitiated by the subsequent valve switching to position II at 26 min and by the mobile phase gradient starting at a low elution power (i.e. low MTBE). Total run time of this C18–C30dvs system was 42 min. Despite the interruption of the carotenoid chromatography on the C30 column caused by valve switching, this method lead to excellent carotenoid chromatography that included the resolution of many lycopene isomers, as typical for C30 columns [48,60], without the presence of interfering Q10 compounds (Fig. 2d).

To shorten run times, this method was modified by applying a steeper mobile phase gradient and a single valve switching event (C18–C30svs system; data not shown). The chromatography of this C18–C30svs system was similar to the C18–C30dvs system and involved an initial isocratic elution with aq. MeOH for 10 min in valve position II, a time during which retinol was resolved from the 25-hydroxylated D vitamers similar to the C18–C30dvs system. By switching the flow from the C18 column directly to the DAD after 10 min (valve position I) and concurrently changing the mobile phase to an aq. MeOH/MTBE gradient, the remaining analytes retained on the C18 column were resolved in the same fashion as with the C18 column alone (Fig. 2a). Total run time of this C18–C30svs system was approximately 25 min, which is considerably shorter than the 42 min run of the C18–C30dvs system but at the expense of co-elution of lutein with zeaxanthin, β - with γ -tocopherol, and of all lycopene isomers.

An even shorter run time of approximately 20 min was achieved by using the C18–C30dvs system with an isocratic elution (20% B and 80% C) and a first valve switch from position II to I after 4 min followed by a second switch back to position II after 18 min (C18–C30dvs system; data not shown). This resulted in a chromatogram qualitatively similar to that obtained with the C18 column alone (Fig. 2a) except for the presence of a non-shifting baseline and the additional resolution of lutein from zeaxanthin and retinol at the very end of this run (retention time approx. 20 min) when they eluted from the C30 column. This could be further improved by including a second pump that allows the resolution on both columns simultaneously thereby shortening run times by another 8 min.

When the C18 and C30 columns were serially connected with a pressure resistant UV/VIS detector placed between and a DAD after the columns (C18-D-C30 system; Fig. 1c, HPLC traces were exactly identical to those shown in Fig. 2a and c), the signals could be recorded simultaneously with all compounds of interest being resolved. UL10, UN10, Bu-UN10, and BU-UL10 were separated on the C18 column and read from the pressure resistant detector “D1” (see 295 nm trace in Fig. 2a) while 25(OH)D₂, 25(OH)D₃, and all carotenoids plus their isomers were separated on the C30 column and read from detector “D” (see 265 nm and 450 nm trace, respectively, in Fig. 2c). Retinol (peak 1) and the tocopherols (peaks 6–9) could be accurately read from either detector. We considered this system superior to the

others, however, applying it required the use of a pressure resistant UV/VIS detector between the columns for the monitoring of the C18 eluates and another UV/VIS detector after the C30 column for the monitoring of compounds after chromatography on both columns in addition to the respective software to control the instruments and acquire and manipulate the recorded data. We achieved this using an Agilent model G1314 UV/VIS detector with a high pressure flow cell certified to withstand pressures up to 400 bar (which was sufficient since the C30 column never caused more back pressure than 150 bar in our system) and by using ChromQuest software which allowed the feeding of additional analogue signals to the existing DAD data from the second detector.

3.4. Method improvements

We tested several column types (Hypersil Gold, Gemini, Spherex, and others) and solvent combinations (MeCN, ethylacetate, DCM, and other modifiers) and found separations were best performed using a MeOH based gradient towards MTBE including an aqueous NH_4OAc buffer. BHT was included in the injection solutions to preserve analytes during storage and chromatography. Since BHT elutes as the first agent, it neutralizes any potential oxidizing components before the analytes elute thereafter.

In order to entirely redissolve all concentrated analytes without distorting peak shapes, we found THF/MeOH/MTBE (8:1.8:0.2, v/v/v) to be the best solvent combination. Similarly, we found the maximum injection volume to use without impairing peak shapes was 20 μL with the applied column sizes. If more sensitive UV detectors were available, an even smaller volume than the 200 μL of plasma used in this study would be sufficient since all analytes other than the vitamin D metabolites react at sufficiently high sensitivities by DAD or are abundantly concentrated to exceed detection limits. In all our systems peaks were identified and assigned by spiking experiments and by comparing UV/VIS scans obtained by DAD using authentic standards.

3.5. Optimization of UV/VIS monitoring

Using the C18–C30dvs system, we obtained sufficient signal strength to quantitate retinol, retinyl laurate, α -, β + γ , and δ -tocopherols, tocol, UL10, UN10, Bu-UL10 and Bu-UN10 by monitoring at 295 nm. Absorbance intensities of the carotenoids were optimized using 480 nm as the monitoring wavelength. At this wavelength, the very strong signals of most carotenoids (λ_{max} = ca. 450 nm) were reduced as to not exceed the dynamic range; at the same time the weaker signals of the lycopenes were maximized (λ_{max} = 473 nm). Similarly, retinol (λ_{max} = 325 nm) could be monitored at 295 nm or 350 nm, which resulted in a lower but still sufficiently sensitive and highly selective signal. Since DADs record all signals between 200 and 600 nm (or wider ranges depending on the instrument), chromatograms can be reanalyzed retrospectively and calibration data can be re-interrogated accordingly. Finally, the nondestructive nature of absorbance measurements allows the addition of other detection devices downstream for other sensitive and selective monitoring, e.g. FD or MSD [8].

3.6. Internal standard selection

In order to accurately adjust for losses and measurement inaccuracies of the wide array of LPM, ISs for each analyte groups are needed because each group behaves differently during sample handling, extraction, and analysis as a result of differential binding abilities to plasma proteins and other compounds, oxidation and degradation propensities, solubilities, and absorption at different wavelengths [12]. For the current study, an intelligent choice among the usual IS candidates was needed to avoid interferences with the analytes of interest while keeping the molecular structure similar to the referred analyte. In the past, we used custom designed synthetic esters of β -apo-carotenoate [12] as ISs for carotenoids. However, in the systems of our current study that resolved carotenoids on the C30 column, the various *Z*-isomers of the β -apo-carotenoates caused interferences with some carotenoid analytes. In contrast, ECH was not only available as a pure all *E*-isomer but also eluted during an empty portion of chromatograms in all systems without interference (except for borderline resolution in the C30 system) and, therefore, served as a suitable IS for carotenoids. Similarly, custom synthesized retinyl laurate (see Section 2) and commercially available $1\alpha(\text{OH})\text{D}_3$ and *rac*-tocol eluted in our systems without interferences and thus served as ideal ISs for retinol, 25-hydroxylated D vitamers and tocopherols, respectively. For UL10 and UN10, we custom synthesized ISs as both their diethyl and dibutyl analogues (see Section 2). These analogues are extremely similar structurally to UL10 and UN10 and displayed the same chemical behaviour as the analytes, particularly in regards to artificial oxidation (data not shown) [4]. This allows accurate back calculation of the original UL10/UN10 ratio since the reduced/oxidized ratio of the IS is known when added to the specimen (original ratio) and when measured (new ratio by artificial oxidation). For this reason and due to their elution without interferences, our custom synthesized Q10 analogues were deemed excellent choices as ISs.

3.7. Validation

Validation of the novel C18-D-C30 or C18-C30dvs systems were performed using the serum reference material 968 from the National Institute of Standards and Technologies (NIST, Gaithersburg, MD, USA) (Table 2a) or by participating in the Vitamin D External Quality Assessment Scheme (DEQAS, London, UK) and direct comparison to our traditional HPLC system [12], which is validated by participation in the Fat-Soluble Quality Assurance Programme from the NIST (Table 2b). At levels in healthy subjects, all values agreed with literature data and average coefficients of variation were low (mean 3.9%, median 1.4%).

3.8. Detection of vitamin D metabolites

A critical aspect of the presented HPLC systems is the low circulating levels of the $25(\text{OH})\text{D}_2$ and $25(\text{OH})\text{D}_3$ and their relatively low extinction coefficients that resulted in analyses near the detection limits at the lowest expected levels even when monitoring at the maximum absorbance (265 nm). For these reasons, plasma needed to be concentrated at least five times prior to HPLC analysis in order to obtain sufficient signal strength for accurate peak integration, which is similar to previous reports that concentrated extracts at least 7-fold [43,52]. In addition, we found that applying an initial elution of 85% aq. MeOH

for 9.5 min before starting the MTBE-containing gradient led to greater sensitivity with a limit of detection of 2.8 ng/mL (signal to noise ratio of 3) due to longer chromatography (elution at ca. 15 min). Detection limits of other analytes are presented in Table 1.

Our attempts to improve the detection limit of 25(OH)D₂ and 25(OH)D₃ by coulometric monitoring failed. Despite increasing the electrolyte content (4.5% NH₄OAc) of the mobile phase and lowering its pH to 4.4 as recommended for coulometric detection of carotenoids and tocopherols (ESA Inc. application note 10–1176), in addition to using high potentials (up to 820 mV), the sensitivity and detection limits compared to absorbance monitoring at 265 nm could not be improved. Moreover, increasing the sensitivity of coulometric detection by decreasing the pH of the mobile phase to 2 or lower [62] using corrosive perchloric acid would inadvertently increase the risk of degradation of other compounds as well as impose undue strain on the HPLC hardware. Furthermore, the presence of THF (needed to dissolve carotenoids efficiently for extraction) masks the coulometric 25(OH)D signals. Finally, from our previous experience, the use of high potentials often leads to frequent failures of the coulometric cell that consequently results in recurring and costly replacements. Use of Cookson reaction products of 25(OH)D were not a feasible option to improve signal strength either since pre-column derivatization of the vitamin D compounds would affect and alter all carotenoids while post-column derivatization was not possible due to the reagent being fluorescent [63,64]. TLSD using laser light at 476 nm was found to result in up to 100-fold lower detection limits for carotenoids vs. absorbance monitoring at 450 nm in conventional HPLC systems [65] but this was reduced to 2–10 fold better detection limits with gradient elution [66]. TLSD is currently very difficult to implement owing to its commercial unavailability. In addition, it is uncertain whether TLSD has advantages over UV/VIS for compounds other than carotenoids, particularly for those absorbing in the UV range.

4. Conclusion

Previously reported LC methods applying highly sophisticated UHPLC and LC × LC require long run times (up to 30 min and longer), do not include all analytes of interest, and are not feasible for routine operations that require fast turn-around times at an affordable cost. On the other hand, our HPLC systems presented are robust, simple, easy to implement, fast, accurate, inexpensive, and sufficiently sensitive to include all analytes of interest and can be custom designed and simplified to reduce run times and adjust to individual project needs. Most detailed analyses of all LPMs considered in this study are possible with only one (very affordable) HPLC–DAD system, serially connected C18 and C30 columns, and a 6-port valve that can be triggered at given times by the HPLC software to allow the focus on certain analytes in a heart-cutting 2D-LC approach (C18–C30dvs system). Alternatively, the same detailed analysis but with shorter run times is possible by applying the serially connected C18 and C30 columns with a pressure resistant UV/VIS detector between and a DAD after the columns (C18–D–C30 system), which requires adequate software to acquire and control all signals. Many alternative variations of the methods presented are possible including the use of isocratic mobile phases that can be custom designed to optimize resolution and turn-around times depending on the analytes of interest. Finally, the non-

destructive nature of UV/VIS detection allows the use of additional monitoring techniques (FD, ECD, MSD, TLSD etc.) and/or the conservation of all or parts of the injected sample.

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D = diode array or multiple wavelength UV/VIS detector; D1 = pressure resistant diode array or UV/VIS detector

Fig. 1.

HPLC configurations. The fused-core C18 and the C30 columns were used either individually alone (a) or connected in series so that the C18 column was followed; (b) directly by the C30 column then by the detector (C18–C30 system); (c) by one pressure resistant detector followed by the C30 column, then a second detector (C18–D–C30 system); (d) a 6-port valve which, in position I, directed the flow directly to the detector and, in position II, directed the flow to the C30 column and then to the detector (C18–C30dvs system).

Fig. 2. HPLC traces of a pooled plasma extract using as mobile phase a MeOH/MTBE gradient containing NH₄Ac buffer (see Section 2.2.3) and as stationary phase (a) Kintex-C18 column alone, (b) a C30 column alone, (c) a Kinetex-C18 with a C30 column in series (C18–C30 system, configuration as shown in Fig. 1b), and (d) a Kinetex-C18 with a C30 column in series with double valve switching (C18–C30dvs system, configuration as shown in Fig. 1d). Black, green and red traces were obtained by monitoring at 265 nm, 295 nm, and 480 nm, respectively. Peak assignments are shown in Table 1 as validated by co-chromatography.

Table 1

Analytes and internal standards included in the assay.

Peak #	Compound	a		b		c		d		λ^a	ϵ (A) ^c	LOD
		t_R [t-rel]	k	t_R [t-rel]	k	t_R [t-rel]	k	t_R [t-rel]	k			
Analytes												
1	Retinol	3.70, [1.00]	1.31	3.40, [1.00]	1.83	8.93, [1.00]	1.63	7.80, [1.00]	1.60	325 (295, 265)	52,487 (325) ^d	0.9
2	25(OH)D ₃	nd		4.90, [1.44]	3.08	10.01, [1.12]	1.94	8.70, [1.12]	1.90	265	18,200 (265) ^d	2.8
3	25(OH)D ₂	nd		5.60, [1.65]	3.67	10.01, [1.12]	1.94	nd		265	19,400 (265) ^d	2.8
4	<i>trans</i> Lutein	9.10, [2.46]	4.69	13.10, [3.85]	9.92	16.84, [1.89]	3.95	15.90, [2.04]	4.30	450 (480)	152,010 (453) ^e	0.5
5	<i>trans</i> Zeaxanthin	9.10, [2.46]	4.69	14.50, [4.26]	11.08	17.88, [2.00]	4.26	17.60, [2.26]	4.87	450 (480)		0.5
6	δ -tocopherol	9.90, [2.68]	5.19	9.90, [2.91]	7.25	15.12, [1.69]	3.45	13.30, [1.71]	3.43	295	3516 (298) ^d	21.4
7	γ -Tocopherol	10.60, [2.86]	5.63	10.30, [3.03]	7.58	15.67, [1.75]	3.61	14.10, [1.81]	3.70	295	3809 (298) ^d	18.3
8	β -Tocopherol	10.60, [2.86]	5.63	10.50, [3.09]	7.75	16.20, [1.81]	3.76	14.30, [1.83]	3.77	295	3725 (296) ^d	
9	α -Tocopherol	10.90, [2.95]	5.81	10.90, [3.21]	8.08	16.60, [1.86]	3.88	15.00, [1.92]	4.00	295 (265)	3265 (292) ^d	21.2
10	α -Cryptoxanthin	11.50, [3.11]	6.19	18.60, [5.47]	14.50	20.34, [2.28]	4.98	28.30, [3.63]	8.43	450 (480)		
11	<i>trans</i> β -Cryptoxanthin	11.60, [3.14]	6.25	19.00, [5.59]	14.83	21.04, [2.36]	5.19	31.50, [4.04]	9.50	450 (480)	136,013 (451) ^f	0.6
12	<i>cis</i> β -Cryptoxanthin	11.80, [3.19]	6.38	nd		nd		nd		450 (480)		
13	<i>cis</i> + <i>trans</i> Lycopenes	15.90, [4.30]	8.94	<i>b</i>		<i>b</i>		<i>b</i>		480 (450)		
14	Dihydro lycopene	16.70, [4.51]	9.44	<i>b</i>		<i>b</i>		<i>b</i>		480 (450)		
15	α -Carotene	17.10, [4.62]	9.69	21.50, [6.32]	16.92	23.31, [2.61]	5.86	34.50, [4.42]	10.50	450 (480)	137,124 (451) ^f	0.6
16	<i>trans</i> β -Carotene	17.40, [4.70]	9.88	22.50, [6.62]	17.75	23.97, [2.68]	6.05	35.20, [4.51]	10.73	450 (480)	139,057 (451) ^f	0.7
17	<i>cis</i> β -Carotene	18.00, [4.86]	10.25	nd		22.72 [2.54]	5.68	nd		450 (480)		
18	Ubiquinol-10	18.60, [5.03]	10.63	dwi		22.50, [2.52]	5.62	19.80, [2.54]	5.60	295	3510 (290) ^g	30
19	Ubiquinone-10	20.60, [5.57]	11.88	dwi		23.50, [2.63]	5.62	21.60, [2.77]	6.20	295	14,200 (275) ^g	50
20	ζ -Carotene/9- <i>cis</i> β -carotene	nd		20.00, [5.88]	15.67	24.34, [2.73]	6.16	nd		450 (480)		
21	15- <i>cis</i> Lycopene	nd		33.70, [9.91]	27.08	26.29, [2.94]	6.73	37.40, [4.79]	11.47	480 (450)	110,000 (468) ^h	
22	13- <i>cis</i> Lycopene	nd		34.20, [10.06]	27.50	27.31, [3.06]	7.03	38.20, [4.90]	11.73	480 (450)		
23	9- <i>cis</i> Lycopene	nd		36.70, [10.79]	29.58	28.88, [3.23]	7.49	39.40, [5.05]	12.13	480 (450)		

Peak #	Compound	a		b		c		d		λ^a	$\varepsilon (\lambda)^c$	LOD
		t_R [t-rel]	k	t_R [t-rel]	k	t_R [t-rel]	k	t_R [t-rel]	k			
24	trans lycopene	nd		38.40, [11.29]	31.00	30.43, [3.41]	7.95	40.70, [5.22]	12.57	480 (450)	186,304 (474) ^f	
25	5-cis Lycopene	nd		38.5 [11.32]	31.08	30.58, [3.42]	7.99	40.80, [5.23]	12.60	480 (450)	184,000 (470) ^h	
Internal standards												
A	1 α -25(OH)D ₃	7.40, [2.00]	3.63	9.10, [2.68]	6.58	nd		11.60, [1.49]	2.87	295 (265)		
B	Tocol	9.70, [2.62]	5.06	9.40, [2.76]	6.83	13.94, [1.56]	3.10	12.50, [1.60]	3.17	295		
C	Echinenone	12.20, [3.30]	6.63	19.80, [5.82]	15.50	21.42, [2.40]	5.30	32.10, [4.12]	9.70	450 (480)		
D	Retinyl laurate	12.70, [3.43]	6.94	12.60, [3.71]	9.50	19.09, [2.14]	4.61	18.50, [2.37]	5.17	325 (295, 265)		
E	Ethylated ubiquinone-10	21.50, [5.81]	12.44	dwi		nd		dwi		295		
F	Butylated ubiquinone-10	22.70, [6.14]	13.19	dwi		24.90, [2.79]	6.32	dwi		295		

a, Kinetex C18 column alone; b, ProntoSIL C30 column alone; c, C18–C30 columns in sequence (C18–C30; no valve included); d, C18–C30 columns in sequence with double valve switching (C18–C30dvs); all systems used with the same mobile phase gradient (see Section 2.2.3)
nd, not detectable; dwi, detectable with interference (not quantifiable); LOD, limit of detection (ng/mL) at a signal to noise ratio of 3, if no value are shown then data were not available.

t_R , retention time of the analyte in minutes; t-rel = $t_R/\text{Retinol}$; $k = (t_R - t_0)/t_0$, where t_0 is the dead volume time of unretained compounds in minutes; t_0 (minutes) for $a = 1.6$, $b = 1.2$, $c = 2.2$, $d = 3.0$; ε = molar extinction coefficient;

λ = wavelength in nm used for detection for best peak size (in parantheses alternative wavelength used with lower signal strength but acceptable peak size and higher selectivity if wavelength is higher).

^aWavelength in nm used for detection (in parantheses alternative wavelength with lower signal strength but acceptable peak size and higher selectivity when higher wavelength is selected).

^bResolved as individual isomers as shown in peaks# 21, 22, 23, 25.

^cData from [12,67].

^dIn ethanol.

^eIn dioxane.

^fIn hexane.

^gIn isopropanol.

^hIn hexane/2% dichloromethane.

Table 2

a

Validation of the C18-D-C30 system^a using serum reference material 968.^b

Compound	NIST assigned value [ng/mL] ^b	C18-D-C30 system determined values [ng/mL (CV)] ^c	CV between these methods (%)
Retinol	341	317.0 (5%)	5
25(OH)D ₃	7	5 (18%)	24
25(OH)D ₃	24.1 ^d	26.8 (8%)	8
25(OH)D ₃	28.3 ^d	28.7(7%)	1
<i>trans</i> -Lutein	67	73.3 (5%)	6
<i>trans</i> -Zeaxanthin	31	36.3 (8%)	11
δ-Tocopherol	9	8.4 (10%)	5
β + γ-Tocopherol	1860	1922.8 (4%)	2
α-Tocopherol	6530	7053.6 (7%)	5
α-Cryptoxanthin	na	16.0 (12%)	
β-Cryptoxanthin	41	38.4 (9%)	5
<i>trans</i> -Lycopene	135	143.9 (7%)	5
α-Carotene	11	9.0 (7%)	12
<i>trans</i> -β-Carotene	88	99.1 (7%)	8
Ubiquinol-10 + ubiquinone-10	900	997.6 (3%)	7

b

Validation of the C18–C30dvs system^a using a pooled plasma sample.

Compound	Spherex-C18 ^b (CV)	Kinetex C18–C30dvs ^b (CV)	CV between these methods (%)
Retinol	978 (0%)	897 (2%)	6.1
25(OH)D ₃	nd	40.0 (10%)	13.1 ^c
25(OH)D ₂	nd	bdl	
<i>trans</i> -Lutein	232 (2%)	232 (6%)	0.0
<i>trans</i> -Zeaxanthin	nd	63 (9%)	
δ-tocopherol	nd	110 (12%)	
β + γ-Tocopherol	940 (1%)	923 (2%)	1.3
α-Tocopherol	11,242 (1%)	11,597 (2%)	2.2
α-Cryptoxanthin	32 (2%)	32 (10%)	0.0
β-Cryptoxanthin	148 (0%)	165 (0%)	7.7
<i>cis</i> -β-Cryptoxanthin	50 (4%)	ni	
<i>cis</i> + <i>trans</i> -	333 (2%)	361 (9%)	5.7
Lycopene			
dihydro lycopene	78 (8%)	79 (4%)	0.9

b**Validation of the C18–C30dvs system^a using a pooled plasma sample.**

Compound	Spherex-C18 ^b (CV)	Kinetex C18–C30dvs ^b (CV)	CV between these methods (%)
α -Carotene	90 (1%)	109 (5%)	13.5
<i>trans</i> - β -Carotene	349 (0%)	342 (4%)	1.4
<i>cis</i> - β -Carotene	31 (1%)	ni	
Ubiquinol-10	nd	1183 (3%)	
Ubiquinone-10	ni	403 (8%)	
15- <i>cis</i> -Lycopene	nd	26 (10%)	
13- <i>cis</i> -Lycopene	nd	52 (4%)	
9- <i>cis</i> -Lycopene	nd	35 (10%)	

^aUsing Kinetex-C18 + Prontosil-C30 columns in series without valve switching but with two detectors (C18-D-C30 system) as described in Section 2 and Fig. 2c.

^bObtained from the National Institute of Standards and Technology (NIST), Gaithersburg, MD.

^cMean and coefficients of variation (CV) of duplicate analysis.

^dObtained from the Vitamin D External Quality Assessment Scheme (DEQAS, London, UK).

CV, coefficient of variation; bdl, below detection limits; nd, not detectable; ni, not identified; if no value are shown then data were not available.

^aUsing Kinetex-C18 + Prontosil-C30 columns in series with dual valve switching (C18–C30dvs system) as described in Section 2 and Fig. 2d; mean of triplicate analysis.

^bUsing our traditional NIST validated method with a Spherex-C18 column [12]; mean of duplicate runs.

^cValidated with samples #391–393 from DEQAS (6.3–29.6 ng/mL).