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Comparison of high-performance liquid chromatography/tandem mass spectrometry and high-performance liquid chromatography/photo-diode array detection for the quantitation of carotenoids, retinyl esters, α -tocopherol and phyloquinone in chylomicron-rich fractions of human plasma

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Abstract

Rationale—Bioavailability of essential lipophilic micronutrients and carotenoids is of utmost interest for human health, as the consumption of these compounds may help alleviate major nutritional deficiencies, cardiovascular disease, and cancer. High-performance liquid chromatography/photo-diode array detection (HPLC-PDA) and high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) were compared for the quantitative analysis of α - and β -carotene, β -cryptoxanthin, lutein, lycopene, α -tocopherol, phyloquinone, and several retinyl esters from chylomicron-containing triglyceride rich lipoprotein (TRL) fractions of human plasma obtained from two clinical trials.

Methods—After selecting an efficient extraction method for the analytes, both the HPLC/PDA and the HPLC/MS/MS methods were developed and several parameters validated using an HP 1200 series HPLC system interfaced with a HP 1200 series diode-array detector (Agilent Technologies, Santa Clara, CA, USA) and a QTRAP 5500 (AB Sciex, Foster City, CA, USA) via an atmospheric pressure chemical ionization (APCI) probe operated in positive ion mode.

Results—For lycopene, α - and β -carotene, HPLC/MS/MS was up to 37 times more sensitive than HPLC-PDA. PDA detection was shown to be up to 8 times more sensitive for lutein. MS/MS signals were enhanced by matrix components for lutein and β -cryptoxanthin, as determined by referencing to the matrix-independent PDA signal. In contrast, matrix suppression was observed for retinyl palmitate, α -carotene, and β -carotene. Both detectors showed similar suitability for α -tocopherol, lycopene and retinyl palmitate (representing ~73% of total retinyl esters). MS/MS exclusively allowed the quantitation of minor retinyl esters, phyloquinone, and (Z)-lycopene isomers.

Conclusions—HPLC/MS/MS was more sensitive than HPLC-PDA for six of the eight analytes and represents a powerful tool for the analysis of chylomicron samples and potentially other biological samples of limited sample size. When internal standards are available for the target

carotenoid, employing MS/MS detection may reduce the necessary blood sample volume, which is particularly advantageous for minimizing risk and discomfort to human subjects during clinical studies.

Fat-soluble vitamins, including vitamin A, vitamin E, and vitamin K, are essential nutrients provided in the human diet. Some carotenoids, including α -carotene, β -carotene, and γ -cryptoxanthin, are vitamin A precursors, while carotenoids such as lutein and lycopene have been associated with other health benefits.^{1–3} Understanding the absorption of these compounds from the human diet is essential to study their bioavailability and to provide sound dietary recommendations.

After the consumption of a meal containing fat-soluble nutrients, these compounds are absorbed by intestinal cells, packaged into chylomicrons, and ultimately released into the blood circulation.⁴ The compounds of interest can be isolated from the chylomicron-containing triglyceride rich lipoprotein (TRL) fractions of the blood plasma by ultracentrifugation and extraction with lipophilic solvents. Subsequent instrumental analysis allows for the estimation of the bioavailability of a compound.

Carotenoid bioavailability has been studied widely by monitoring the levels of these compounds in chylomicron-rich fractions of human plasma by high-performance liquid chromatography (HPLC) coupled to a photodiode array (PDA) detector and/or an electrochemical (EC) detector.^{5–7} For the simultaneous quantitation of carotenoid and tocopherol isomers, a rapid HPLC-PDA method has been developed for vitamin-supplemented (α -tocopherol and β -carotene, i.e. ATBC) drinks.⁸ However, more sensitive identification and quantitation methods for these compounds are necessary, particularly for samples from human studies, since the analysis of minor carotenoids and retinyl esters at low biological levels would require a large sample volume. Many strides have been made in recent years, particularly in mass spectrometry, offering distinct advantages over the other methods of detection.⁹ Higher sensitivity might facilitate the quantitation of minor components such as retinyl esters or carotenoid isomers. Moreover, the risk for patients during a clinical trial might be minimized due to the reduction of the necessary blood sample volume or biological sample size. Thus, we developed and partially validated an HPLC/MS/MS method to analyze fat-soluble vitamins and carotenoids in one HPLC run. Our method involves an optimized extraction procedure and avoids saponification, which enables distinct measurements of esterified forms of retinol. The main focus of this study was the comparison of analytical parameters of the HPLC/MS/MS and the HPLC-PDA methods, investigating extracts of chylomicron-rich fractions from two clinical trials, which contained challenging lipid matrix components.

Experimental

Reagents

HPLC grade methyl *tert*-butyl ether (MTBE), toluene, acetone, and ethanol, and Optima grade hexane, water, and methanol, and ACS grade sodium chloride were purchased from Fisher Scientific (Pittsburg, PA, USA). High-purity formic acid (>95%) and polyvinyl alcohol (PVA) with average molecular weight from 70 000–100 000 Da was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate was obtained from JT. Baker (Phillipsburg, NJ, USA). Lutein, zeaxanthin, and β -carotene were acquired from Chromadex (Irvine, CA, USA). Retinol, unlabelled β -carotene, retinyl palmitate, γ -cryptoxanthin, phyloquinone, and α -tocopherol were purchased from Sigma-Aldrich. Lycopene was purified and crystallized as described previously.¹⁰ d_8 - β -Carotene was purchased from Cambridge Isotopes (Andover, MA, USA).

Clinical trials

Two human clinical trials were performed, the details of which will be published elsewhere. Both studies were approved by the Institutional Review Board of the Ohio State University. In trial 1, fasted healthy adult subjects were fed raw carrots containing approximately 28 mg β -carotene, 18 mg α -carotene, 0.5 mg lutein, 47 μ g phyloquinone, and 3 mg α -tocopherol with a breakfast meal containing 23 g lipid from avocado. Blood samples were taken at 0, 2, 3, 4, 5, 6, 8, 10, and 12 h. During trial 2, fasted healthy adult subjects received 400-506 g fresh papaya (*Carica papaya* L. cv. 'Pococí') slices containing 1.3 mg β -carotene, 2.1 mg α -cryptoxanthin, and 13.0 mg lycopene as determined by HPLC-PDA.¹¹ Additionally, subjects were fed 150 g of a low-fat yogurt enriched with soy oil to a lipid content of 10% (w/w). Blood samples were drawn at 0, 2, 3, 4, 5, 6, 8, and 9.5 h after test meal consumption.

Chylomicron isolation

The following procedure and the subsequent carotenoid extractions were carried out under subdued light. Since the isolation of chylomicron-rich fractions was conducted in two different laboratories for trial 1 and trial 2, isolation protocols differed slightly. For trial 1, venous blood was drawn via needle and syringe from the lower forearm into 7 mL glass EDTA vacutainer tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Plasma was immediately separated from red blood cells by centrifuging at 1000 *g* (3000 rpm) at 4°C for 10 min. Blood plasma was then placed into cryovials and stored at 4°C before chylomicron isolation. Polyallomer tubes (13 mm $\times$ 51 mm) were washed with a 5% PVA solution (w/v) overnight, rinsed three times, and allowed to dry. Plasma (2.5 mL) was transferred into a polyallomer tube and overlaid with 1 mL of NaCl solution having a density of 1.006 kg L⁻¹.¹² Samples were subjected to ultracentrifugation for 33 min at 40 700 rpm in a SW 50.1 swinging bucket rotor of a Beckman L8-M ultracentrifuge to isolate the TRL fraction containing the chylomicrons. The lower plasma fraction was drained from the tube and the TRL-containing supernatant (top 0.5 mL) was removed and set aside. The tube was rinsed twice with 250 μ L of saline solution which was combined with the TRL fraction. The TRL fraction was frozen at -80°C until extraction. All TRL fractions were isolated from fresh (i.e. previously unfrozen) plasma.

For trial 2, the blood samples were centrifuged at 1700 *g* (4000 rpm) at 4°C for 10 min. Separation of TRL fractions from plasma was performed as described above, except that 0.8 g potassium bromide were added to the plasma samples prior to overlaying with NaCl solution and ultracentrifugation (WX Ultra 80, Thermo Fisher Scientific, Asheville, NC, USA) following a modified method of Terpstra.¹³ After removing the TRL fraction, the polyallomer tube was rinsed once with 250 μ L of saline solution, and the fractions were stored at -80°C. Prior to carotenoid extraction, the TRL samples were made up to 1.0 mL with saline solution.

Extraction

First, a 0.5 mL aliquot of the TRL fraction was mixed with 0.5 mL ethanol. After vortexing, 2 mL of the extraction solvent were added. The sample was probe-sonicated for 8 s thrice and then centrifuged for 5 min at 300 *g*. The upper non-polar layer was removed and the remaining aqueous plasma mixture was re-extracted as described above, unless otherwise specified. The two non-polar extracts were combined in a glass vial and dried under nitrogen gas at <25°C. The dried extract was stored at -80°C until HPLC-PDA/MS/MS analysis. Preliminary testing revealed that these extracts were stable at -80°C for at least 5 days. Various extraction solvent combinations, previously used for extraction of carotenoids and fat-soluble compounds from biological samples, were tested, including hexane alone,¹⁴ hexane/acetone (1:1, v/v),¹⁵ and hexane/ethanol/acetone/toluene (10:6:7:7, v/v/v/v).¹⁶

Recovery experiments were performed by adding 100 μL of a standard mixture containing all analytes of interest at levels expected to be observed at 5 h into 5 h TRL samples.

HPLC-PDA/MS/MS analysis

TRL extracts and standards were re-dissolved in 100 μL MTBE containing the d8- β -carotene internal standard (0.042 nmol). Then, the samples were sonicated in a water bath, followed by the addition of 100 μL of MeOH and centrifugation (15 000 rpm, 2 min, model 5424, Eppendorf, Hamburg, Germany) to pellet any solid matrix remaining in the sample. The compounds were separated using reversed-phase chromatography with a YMC C30 column (4.6 mm $\times$ 150 mm, 5 μm particle size; Waters, Milford, MA, USA). Samples were separated on an HP 1200 series HPLC system with an HP 1200 series diode-array detector (Agilent Technologies, Santa Clara, CA, USA). The column was operated at 40°C and the injection volume was 20 μL . Total run time was 19 min at a flow rate of 1.8 mL min⁻¹. The following gradient was employed to separate the samples: 0% B with a linear gradient to 100% B over 16 min, holding at 100% B for minute, and then returning to 0% B and holding for min. Solvent A = 80:20 MeOH/water (v/v) and solvent B=78:20:2 MTBE/MeOH/water (v/v/v). Besides the described solvent composition without further additives, two additional solvent modifications were tested. Ammonium acetate was added at 0.4 g L⁻¹ solvent. Furthermore, the water proportion of solvent A and B was replaced by 0.1% aqueous formic acid. After several optimization experiments, the solvent combination containing ammonium acetate proved to most effectively enhance chromatographic separation and MS signal intensity of precursor ions for all carotenoids tested, but particularly for the carotenes β -carotene, γ -carotene, and lycopene (Fig. 1). The LC solvents were therefore enriched with 0.4 g ammonium acetate per liter of solvent throughout the method development.

The eluent from the HPLC-PDA was serially interfaced with a QTRAP 5500 mass spectrometer (AB Sciex, Foster City, CA, USA) via an atmospheric pressure chemical ionization (APCI) probe operated in positive ion mode. After various optimization experiments, the following parameters were chosen to analyze the final samples: curtain gas, 30 psi; source temperature, 450°C; ion source gas 1 (GS1), 45 psi; ion source gas 2 (GS2), 0 psi; declustering potential (DP), 100 V; entrance potential (EP), 10 V; collision cell exit potential (CXP), 11 V. Various precursor-product ion combinations for each analyte were identified in preliminary experiments. Only those precursor-product ion combinations which were selective for the compounds of interest and for which background interference was minimized or eliminated were chosen for the final method (Tables 1 and 2). Final data acquisition was carried out using multiple reaction monitoring (MRM) windows with dwell times of 50 ms for each channel, and 5 ms pauses between channels. All data were acquired and peak areas integrated using Analyst 1.5.1 software (AB Sciex, Foster City, CA, USA).

Quantitation

Retinol and zeaxanthin were identified in the chromatogram using authentic standards (labeled in Fig. 2), but were not further investigated in this study. For the analysis of β -carotene, the ratio of native β -carotene to d8- β -carotene internal standard (IS) was used to generate a calibration curve. The remaining carotenoids and other micronutrients were quantitated using calibration curves generated with authentic standards at five different dilution levels (0.24, 2.4, 6.0, 12, 36 pmol on-column for β -carotene, γ -carotene, δ -cryptoxanthin, lutein, lycopene, and retinyl palmitate; 0.0012, 0.012, 0.03, 0.06, 0.18 pmol on-column for phyloquinone and α -tocopherol). For PDA quantitation, the peak area at the UV/Vis absorption maximum of each analyte was used. For MS/MS quantitation, the specific precursor-product fragment denoted in Table 1 was chosen for each compound. However, all precursor-product fragments listed in Table 1 were monitored during the

experiment as sufficient duty cycle was available for each precursor-product pair, providing additional confirmation of compound identity.

Validation

Limit of detection (LOD) and limit of quantitation (LOQ) were determined by using the least-squares regression method of the residuals of the standards at each dilution level. According to Vial and Jardy,¹⁷ the y-intercept was used to calculate LOD (x3) and LOQ (x10). To determine precision and accuracy of the HPLC-PDA/MS/MS method, quality control (QC) standards were run on multiple days. The standard mixture was created in solvent, dried under nitrogen gas, and stored dry at -80°C until use. Each QC injection contained 6 pmol of α -carotene, β -carotene, β -cryptoxanthin, lutein, lycopene, and retinyl palmitate and 0.03 pmol of α -tocopherol and phyloquinone.

Results and Discussion

Extraction recovery

A variety of extraction solvents and solvent combinations have been described in the literature for removing lipophilic analytes like carotenoids from TRL fractions.^{12,18–20} As a first step in plasma sample preparation for HPLC, most methods use an organic solvent such as methanol, ethanol, or acetonitrile to precipitate proteins.²¹ Adding ethanol and vigorously vortexing the TRL fraction before the addition of the extraction solvent was vital for an acceptable extraction recovery of the analytes. Multiple extraction solvents were tested during a series of standard addition recovery experiments, maintaining the ratio of 1:4 (v/v) TRL fraction to extraction solvent (see Experimental section). Since all extraction solvents provided comparable recovery results, a repeated extraction with hexane was chosen for simplicity and the results are summarized in Table 2.

Validation results

Precursor-product transitions unique to each compound were chosen for each analyte. The instrument speed (minimum dwell of 2 ms, delay of 3 ms) allowed us to monitor multiple precursor-product transitions without sacrificing sensitivity. Thus, several transitions for each compound were monitored to increase confidence in identification, and the relative ratio of multiple precursor-product transitions for each compound was monitored to ensure that we were indeed detecting the analyte of interest. In our samples, we observed that generally higher product mass ions were more selective to a particular analyte, but less sensitive. Thus, for most analytes, the lower mass product ions were chosen for quantitation (Table 1). For lutein and retinyl esters, the corresponding *in-source* fragment $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ and $[\text{M}+\text{H}-\text{fatty acid}]^+$ showed a much higher intensity as compared to the molecular ion $[\text{M}+\text{H}]^+$ and, therefore, was used as precursor ion for MS/MS analysis (Table 1). The LOD and LOQ for all analytes investigated are provided as pmol injected onto the HPLC column in Table 2. LC/MS/MS was more sensitive for most analytes except for lutein, where PDA was more sensitive than LC/MS/MS by 7-fold, respectively. No clear sensitivity advantage was observed for α -tocopherol between the two detection methods. In contrast, LC/MS/MS was 3.6-fold more sensitive for β -carotene, 3.9-fold more sensitive for α -carotene, and a remarkable 36-fold more sensitive for lycopene as compared to PDA. For phyloquinone, which is a comparatively poor absorber of light in the UV-visible range, LC/MS/MS was the only viable method of detection, particularly when UV-absorbing background compounds from the TRL fractions were present.

Our LOQ values for the LC/MS/MS analysis of lutein, β -cryptoxanthin and β -carotene are comparable to those values reported by Hao *et al.*,²² and our LOQ values for lycopene by LC/MS/MS are similar to those reported by Fang *et al.*²³

In contrast, our LC/MS/MS LOD value for α -tocopherol (4.2 fmol) is 121-fold lower than that reported by Hao *et al.*²² and 5.7-fold lower than that reported by Andreoli *et al.*²⁴ Although the MS/MS detection limit was quite low, precision and accuracy values were not within an acceptable range, and thus PDA is the favorable method for quantitation of α -tocopherol using our method.

Sensitivity parameters have also been reported for selected ion monitoring (SIM, LC/MS) by various groups. Our LOQ values (LC/MS/MS) for retinyl palmitate (1.91 pmol) and β -carotene (0.44 pmol) were very similar to those reported by van Breemen *et al.*²⁵ and Matsumoto *et al.*,²⁶ respectively, for the LC/MS analysis of selected ions (SIM). Likewise, we observed higher sensitivity for phyloquinone (LOQ=8.8 fmol) than Martinis *et al.*,²⁷ who reported an LOQ value of 0.11 pmol for the precursor ion as compared to 0.009 pmol in our method. Loss in analyte signal during fragmentation was expected with our tandem mass spectrometry as compared to the SIM methods of van Breemen *et al.*,²⁵ Matsumoto *et al.*²⁶ and Martinis *et al.*²⁷ However, this was not observed for the above-mentioned analytes.

Some studies have employed electrochemical (EC) detection to quantitate carotenoids at low levels typically observed in biological samples. In our study, our LC/MS/MS LOD for β -carotene was 304 fmol, as compared to 10 fmol reported earlier by EC detection.⁵ In contrast, we report a LOD of 24 fmol for lycopene by MS/MS, while 50 fmol has been reported by EC for lycopene. Likewise, the LOD for α -tocopherol by LC/MS/MS was 4 fmol, while EC LOD has been reported as 150 fmol.²⁸ While EC appears to be more sensitive for some carotenoids, MS/MS analysis provides confirmation of the compound via precursor and product ions consistent with standards. EC provides less selectivity as the measured dominant oxidation potentials are not specifically unique to individual compounds and unknown interfering compounds may be present.

As shown in Table 2, detection accuracy was >90% for all analytes except α -tocopherol by MS/MS analysis. Except for β -carotene, the precision for all analytes was better for the PDA measurement as compared to MS/MS measurement, demonstrating that the PDA response is more stable than that of MS/MS. The higher precision for β -carotene by MS/MS analysis was likely due to the use of the stable isotope internal standard, which can account for instrumental variability. The precision for α -tocopherol was comparatively high regardless of whether the 205 product ion (Table 2) or the 165 precursor ion (data not shown) was chosen. Overall, precision for most analytes was acceptable.

The developed method using HPLC-PDA/MS/MS for the determination of carotenoids, retinyl esters, α -tocopherol, and phyloquinone was applied to TRL fractions acquired during two clinical trials in 2011 (trials 1 and 2). Figure 2 illustrates the chromatographic separation, as monitored by PDA and corresponding MS/MS signals of a representative TRL sample (t=6 h, trial 2). Figure 3 displays the absorption curves for some of the investigated compounds normalized to the compound level measured at t=0 h. It should be noted that Fig. 3 is meant to illustrate MS/MS vs. PDA response differences for different analytes, and standard error of the mean (SEM) reflects inter-individual subject differences and not differences in response. Table 3 provides a quantitative estimate of the effect of matrix on the area under the concentration-time curve (AUC) values derived from MS/MS detection as compared to PDA detection. The effects of matrix on MS/MS response are further described below.

Figures 3(A) and 3(B) show β -carotene and α -carotene absorption curves for the average of 12 subjects from trial 1. As expected, both time plots showed a concentration maximum at 5–6 h after meal consumption (Figs. 3(A), 3(B)). Clearly, for both β - and α -carotene, the raw MS/MS signal (without internal standard correction) delivered lower values as

compared to the PDA signal. This difference was substantially higher at carotenoid maximum concentration levels (i.e. Fig. 3(A), $t = 5$ h; Fig. 3(B), $t = 6$ h) as compared to other time points. As noted in Table 3, matrix suppression reduced $AUC_{MS/MS}$ values for β -carotene by 53% and for α -carotene by 25%, as compared to the respective AUC_{PDA} values. Since other lipophilic compounds show similar absorption behavior after the consumption of a meal, the signal suppression observed may be related to the co-elution of such lipids present in the sample matrix.¹² Mechanisms of ion suppression are discussed elsewhere^{29,30}.

The use of a stable isotope internal standard (d8- β -carotene) allowed the MS/MS signal of native β -carotene to be corrected for matrix suppression (Fig. 3(A)). This adjustment was successful as the MS/MS signal normalized to IS directly overlaid the PDA signal. Although co-elution could be an issue for PDA detectors in some cases, the concurrence of the PDA and the normalized MS/MS signals allowed mutual cross-validation. Table 3 further highlights how effectively the IS corrects for matrix suppression, with an average of 6% difference between $AUC_{MS/MS}$ and AUC_{PDA} values. This is in contrast to the matrix suppression (53%) observed when values were calculated without the stable isotope IS. Hence, the use of an internal standard represents a prerequisite for accurate measurements when using MS/MS for the determination of β -carotene in TRL samples with our instrument; β -carotene elutes just 0.2 min prior to α -carotene in the method. Due to their close proximity and the assumed similarity in matrix suppression, we attempted to use the α -carotene IS to compensate for β -carotene matrix suppression. Unfortunately, the α -carotene IS over-compensated for the matrix suppression of β -carotene and, thus, was not useful (data not shown).

Various retinyl esters (compounds 7, 8, 9, 10) as well as free retinol (compound 1) in small amounts were observed in the TRL samples (Fig. 2). Particularly at earlier time points (0, 2, 3, 4 h), retinyl palmitate was the only retinyl ester quantifiable by PDA, whereas the MS/MS signal also delivered acceptable signal-to-noise (S/N) ratios for other, minor retinyl esters. Therefore, the MS/MS signal for total retinyl esters delivered on average a 4% higher absorption curve as compared to PDA in the samples studied (Fig. 3(C), Table 3). The PDA signal in Fig. 3(C) represents retinyl palmitate only, which was the most abundant retinyl ester (~73% of total retinyl esters determined by MS/MS). AUC_{PDA} for retinyl palmitate only was approximately 15% higher than $AUC_{MS/MS}$ for retinyl palmitate only (Table 3). Thus, the application of an internal standard would be preferable for retinyl palmitate due to a minor amount of ion suppression.

The free retinol (compound 1) detected in the TRL samples might represent a baseline remnant, which was not derived from the test meal. After feeding isotopically labeled d8- β -carotene, our group, in collaboration with others, showed that the TRL fraction only contained labeled d4-retinyl esters, whereas labeled free d4-retinol was not detected.²⁰ At the same time, unlabeled free retinol was found in the TRL fractions, although no unlabeled β -carotene was fed. Hence, the presence of unlabeled free retinol was related to native circulating species²⁰.

By analogy to retinyl esters, MS/MS analysis of lycopene allowed the quantitation of further (Z)-lycopene isomers (compounds 13, 14, 15). At some time points, these isomers were not quantifiable by PDA (S/N ratio <10). Thus, as shown in Fig. 3(D), the AUC_{PDA} for total lycopene was on average 20% lower than the respective $AUC_{MS/MS}$ for total lycopene. For estimating the matrix effect, only (all-E)-lycopene and (5Z)-lycopene isomer (both eluting in peak 16) were considered, since the determination of both compounds was feasible by MS/MS and PDA (Fig. 3(D)). The $AUC_{MS/MS}$ signal was 8% lower than the AUC_{PDA} , indicating an apparently small matrix effect on average; β -cryptoxanthin and lutein absorption curves are shown in Figs. 3(E) and 3(F). Using the PDA signal as reference, the

MS/MS signal delivered artificially high results for the β -cryptoxanthin and lutein ion pairs. Regarding β -cryptoxanthin, the inconsistent signal enhancement for different ion pairs indicated that different co-eluting matrix compounds may be responsible for the signal enhancement of each of the two transitions at m/z 553 $\rightarrow$ 473 and 553 $\rightarrow$ 429. The apparent signal enhancement was more consistent for lutein (Fig. 3(F)). Furthermore, a matrix-related cause for the observed ion enhancement of both analytes is probable, since the differences between PDA and MS/MS were larger at data points with higher expected levels of matrix (3–6 h). Regarding lutein, a more than 2.2-fold matrix enhancement was revealed when comparing the $AUC_{MS/MS}$ of the ion pairs at m/z 551 $\rightarrow$ 345 (120 nmol*h/L) and m/z 551 $\rightarrow$ 429 (115 nmol*h/L) with the reference AUC_{PDA} (52 nmol*h/L). As shown in Fig. 3(E), for β -cryptoxanthin, the MS/MS ion pair at m/z 553 $\rightarrow$ 473 showed a slightly stronger enhancement as compared to the ion pair at m/z 553 $\rightarrow$ 429 of β -cryptoxanthin. The use of an internal standard would be ideal in order to compensate for the observed matrix enhancement.

The level of α -tocopherol fed was quite low, and the variable MS/MS response (as highlighted in Table 2 and earlier in the text), made comparisons between PDA and MS/MS difficult. Unlike the other analytes, the PDA signal was unavailable for phyloquinone in the real TRL samples. However, in preliminary experiments, TRL test samples were spiked with phyloquinone at high enough levels that PDA response could be compared to MS/MS response, and no matrix effect was observed (data not shown). However, it should be noted that the MS/MS product ions studied for phyloquinone showed very consistent behavior in all samples.

Conclusions

In conclusion, a stable isotope labeled internal standard (IS) was found to be necessary for the proper quantitation of β -carotene in TRL samples by MS/MS. In addition, a stable isotope labeled IS, when available, is recommended for MS/MS quantitation of lutein, β -cryptoxanthin, and β -carotene in lipid-rich TRL fractions, due to matrix enhancement and suppression. In contrast, deviations of PDA and MS/MS signals were less pronounced for retinyl palmitate and lycopene. Furthermore, MS/MS detection was necessary for the quantitation of retinyl esters and lycopene (Z)-isomers present at low levels and for phyloquinone at all levels. While the inherent absence of ion suppression/enhancement made HPLC-PDA a better choice for quantitation of some carotenoids and α -tocopherol, in-line MS/MS detection clearly provides compound identification confirmation, which could not be obtained by PDA or electrochemical detection at these low levels.

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References

1. Sesso HD, Liu S, Gaziano JM, Buring JE. Dietary lycopene, tomato-based food products and cardiovascular disease in women. *J Nutr.* 2003; 133:2336. [PubMed: 12840203]
2. Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC. A prospective study of tomato products, lycopene, and prostate cancer risk. *J Natl Cancer Inst.* 2002; 94:391. [PubMed: 11880478]
3. Johnson EJ, Vishwanathan R, Schalch W, Poon LW, Wittwer J, Johnson MA, Hausman D, Davey A, Green RC, Gearing M. Brain levels of lutein (L) and zeaxanthin (Z) are related to cognitive function in centenarians. *FASEB J.* 2011; 25 975.21.
4. During, A.; Harrison, E. *Physiology of the Gastrointestinal Tract.* Academic Press; New York: 2006. Digestion and intestinal absorption of dietary carotenoids and vitamin A; p. 1735-1752.

5. Ferruzzi MG, Sander LC, Rock CL, Schwartz SJ. Carotenoid determination in biological microsamples using liquid chromatography with a coulometric electrochemical array detector. *Anal Biochem.* 1998; 256:74. [PubMed: 9466800]
6. Brown MJ, Ferruzzi MG, Nguyen ML, Cooper DA, Eldridge AL, Schwartz SJ, White WS. Carotenoid bioavailability is higher from salads ingested with full-fat than with fat-reduced salad dressings as measured with electrochemical detection. *Am J Clin Nutr.* 2004; 80:396. [PubMed: 15277161]
7. Li S, Nugroho A, Rocheford T, White WS. Vitamin A equivalence of the β -carotene in β -carotene-biofortified maize porridge consumed by women. *Am J Clin Nutr.* 2010; 92:1105. [PubMed: 20810977]
8. Schieber A, Marx M, Carle R. Simultaneous determination of carotenes and tocopherols in ATBC drinks by high-performance liquid chromatography. *Food Chem.* 2002; 76:357.
9. Kopec, RE.; Cooperstone, JL.; Cichon, MJ.; Schwartz, SJ. Analysis methods of carotenoids. In: Xu, Z.; Howard, LR., editors. *Analysis of Antioxidant-Rich Phytochemicals*. Wiley-Blackwell; Oxford: 2012. p. 105-148.
10. Kopec RE, Riedl KM, Harrison EH, Curley RW Jr, Hruszkewycz DP, Clinton SK, Schwartz SJ. Identification and quantification of apo-lycopenals in fruits, vegetables, and human plasma. *J Agric Food Chem.* 2010; 58:3290. [PubMed: 20178389]
11. Schweiggert RM, Steingass CB, Esquivel P, Carle R. Chemical and morphological characterization of costa rican papaya (*Carica papaya* L.) hybrids and lines with particular focus on their genuine carotenoid profiles. *J Agric Food Chem.* 2012; 60:2577. [PubMed: 22243655]
12. van Vliet T, Schreurs WH, van den Berg H. Intestinal β -carotene absorption and cleavage in men: response of β -carotene and retinyl esters in the triglyceride-rich lipoprotein fraction after a single oral dose of β -carotene. *Am J Clin Nutr.* 1995; 62:110. [PubMed: 7598052]
13. Terpstra AHM. Isolation of serum chylomicrons prior to density gradient ultracentrifugation of other serum lipoprotein classes. *Anal Biochem.* 1985; 150:221. [PubMed: 4083481]
14. Nomura AM, Stemmermann GN, Lee J, Craft NE. Serum micronutrients and prostate cancer in Japanese Americans in Hawaii. *Cancer Epidemiol Biomarkers Prev.* 1997; 6:487. [PubMed: 9232334]
15. Ferruzzi MG, Nguyen ML, Sander LC, Rock CL, Schwartz SJ. Analysis of lycopene geometrical isomers in biological microsamples by liquid chromatography with coulometric array detection. *J Chromatogr Biomed.* 2001; 760:289.
16. Craft NE, Wise SA, Soares JH. Individual carotenoid content of SRM 1548 total diet and influence of storage temperature, lyophilization, and irradiation on dietary carotenoids. *J Agric Food Chem.* 1993; 41:208.
17. Vial J, Jardy A. Experimental comparison of the different approaches to estimate LOD and LOQ of an HPLC method. *Anal Chem.* 1999; 71:2672.
18. Bohn T, Blackwood M, Francis D, Tian Q, Schwartz SJ, Clinton SK. Bioavailability of phytochemical constituents from a novel soy fortified lycopene rich tomato juice developed for targeted cancer prevention trials. *Nutr Cancer.* 2011; 10.1080/01635581.2011.630156
19. Unlu NZ, Bohn T, Clinton SK, Schwartz SJ. Carotenoid absorption from salad and salsa by humans is enhanced by the addition of avocado or avocado oil. *J Nutr.* 2005; 135:431. [PubMed: 15735074]
20. Fleshman MK, Riedl KM, Novotny JA, Schwartz SJ, Harrison EH. An LC/MS method for d8- β -carotene and d4-retinyl esters: β -carotene absorption and its conversion to vitamin A in humans. *J Lipid Res.* 2012; 53:820. [PubMed: 22308509]
21. Bruce SJ, Tavazzi I, Parisod V, Rezzi S, Kochhar S, Guy PA. Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/mass spectrometry. *Anal Chem.* 2009; 81:3285. [PubMed: 19323527]
22. Hao Z, Parker B, Knapp M, Yu L. Simultaneous quantification of α -tocopherol and four major carotenoids in botanical materials by normal phase liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry. *J Chromatogr A.* 2005; 1094:83. [PubMed: 16257293]

23. Fang L, Pajkovic N, Wang Y, Gu C, van Breemen RB. Quantitative analysis of lycopene isomers in human plasma using high-performance liquid chromatography-tandem mass spectrometry. *Anal Chem.* 2003; 75:812. [PubMed: 12622371]
24. Andreoli R, Manini P, Poli D, Bergamaschi E, Mutti A, Niessen WMA. Development of a simplified method for the simultaneous determination of retinol, -tocopherol, and -carotene in serum by liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization. *Anal Bioanal Chem.* 2004; 378:987. [PubMed: 14598012]
25. van Breemen RB, Nikolic D, Xu X, Xiong Y, van Lieshout M, West CE, Schilling AB. Development of a method for quantitation of retinol and retinyl palmitate in human serum using high-performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry. *J Chromatogr A.* 1998; 794:245. [PubMed: 9491562]
26. Matsumoto H, Ikoma Y, Kato M, Kuniga T, Nakajima N, Yoshida T. Quantification of carotenoids in citrus fruit by LC-MS and comparison of patterns of seasonal changes for carotenoids among citrus varieties. *J Agric Food Chem.* 2007; 55:2356. [PubMed: 17300198]
27. Martinis J, Kessler F, Glauser G. A novel method for prenylquinone profiling in plant tissues by ultra-high pressure liquid chromatography-mass spectrometry. *Plant Methods.* 2011; 7:23. [PubMed: 21777468]
28. Puspitasari-Nienaber NL, Ferruzzi MG, Schwartz SJ. Simultaneous detection of tocopherols, carotenoids, and chlorophylls in vegetable oils by direct injection C30 RP-HPLC with coulometric electrochemical array detection. *J Am Oil Chem Soc.* 2002; 79:633.
29. Gosetti F, Mazzucco E, Zampieri D, Gennaro MC. Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *J Chromatogr A.* 2010; 1217:3929. [PubMed: 20004403]
30. Remane D, Meyer MR, Wissenbach DK, Maurer HH. Ion suppression and enhancement effects of co-eluting analytes in multi-analyte approaches: Systematic investigation using ultra-high-performance liquid chromatography/mass spectrometry with atmospheric pressure chemical ionization or electrospray ionization. *Rapid Commun Mass Spectrom.* 2010; 24:3103. [PubMed: 20941756]

Figure 1.

Influence of MS source temperature and LC solvent additives on MS signal intensity for precursor ions using SIM in APCI positive mode. AA: ammonium acetate (solid line), FA: formic acid (dashed line), H₂O: no additive (dotted line). AA at 450°C provided optimal signal for all analytes tested. For more details, see Experimental section.

Figure 2.

HPLC separation of lipophilic micronutrients from a human TRL sample at $t=6$ h after meal consumption, showing maximum levels of analytes of interest monitored at (A) 295 nm, (B) 450 nm, and (C) 325 nm. Dotted lines assign UV-Vis peaks to the corresponding MS/MS traces. For peak assignments, see Table 1.

Figure 3.

Baseline-corrected plasma TRL concentrations of (A) β -carotene, (B) α -carotene, (C) retinyl esters, (D) lycopene, (E) β -cryptoxanthin, and (F) lutein as measured by HPLC-PDA (solid line) and HPLC/MS/MS (dotted lines) during the 12 h (trial 1) and 9.5 h (trial 2) after consumption of the respective test meals. Normalization of the MS/MS signal by the internal standard (d8- β -carotene) was demonstrated for β -carotene only (dashed line, A). Analysis by MS/MS for total retinyl esters (C) and total lycopene (D) delivered higher results as compared to PDA, since quantitation of minor retinyl esters and lycopene isomers was only feasible by MS/MS (C, D). Values are means $\pm$ SEM from 12 (trial 1) and 16 (trial 2) subjects, except for lutein, where only data from 2 subjects (trial 1) demonstrated a response visible with both detection systems.

Table 1
UV-Vis spectra and MS data of the investigated analytes

No.	Retention time (min)	Compound identity	HPLC-PDA spectrum max (nm)	HPLC/APCI(+)-MS/MS experiment <i>m/z</i> precursor ion > <i>m/z</i> product ion	Collision energy for MS/MS experiment [#]
1	4.4	retinol	325	n.a.	n.a.
2	8.7	(<i>all</i> /E)-lutein	430/446/472	551.5 [*] > 533.0, 551.5 [*] > 429.5, 551.5 [*] > 345.3 [§]	20, 20, 20
3	8.9	(<i>all</i> /E)-zeaxanthin	430/452/478	551.5 [*] > 533.0, 551.5 [*] > 429.5, 551.5 [*] > 345.3	20, 20, 20
4	8.9	-tocopherol	294	431.4 > 205.1 [§] , 431.4 > 165.1	30, 30
5	9.8	phyllanthone	n.d.	451.4 > 227.2, 451.4 > 197.0, 451.4 > 187.2 [§]	40, 35
6	11.1	(<i>all</i> /E)- cryptoxanthin	430/454/478	553.3 > 473.5, 553.3 > 429.5 [§]	20, 25
7	11.1	retinyl ester 1	n.d.	269.2 [*] > 239.2, 269.2 [*] > 213.2 [§]	20, 35
8	11.6	retinyl ester 2	n.d.	269.2 [*] > 239.2, 269.2 [*] > 213.2 [§]	20, 35
9	11.8	retinyl ester 3	n.d.	269.2 [*] > 239.2, 269.2 [*] > 213.2 [§]	20, 35
10	12.0	retinyl palmitate	330	269.2 [*] > 239.2, 269.2 [*] > 213.2 [§]	20, 35
11	12.9	(<i>all</i> /E)- carotene	430/446/472	537.4 > 415.4, 537.4 > 413.4 [§]	30, 30
12a	13.1	(<i>all</i> /E)- carotene	430/454/478	537.4 > 415.4, 537.4 > 413.4 [§]	30, 30
12b	13.1	(<i>all</i> /E)-d8- -carotene [‡]	430/454/478	545.4 > 423.4 [‡] , 545.4 > 421.4 [§]	30, 30
13	14.3	(Z)-lycopene isomer 1	n.d.	537.4 > 455.6, 537.4 > 427.4, 537.4 > 413.4 [§]	25, 20, 25
14	14.5	(Z)-lycopene isomer 2	n.d.	537.4 > 455.6, 537.4 > 427.4, 537.4 > 413.4 [§]	25, 20, 25
15	15.2	(Z)-lycopene isomer 3	n.d.	537.4 > 455.6, 537.4 > 427.4, 537.4 > 413.4 [§]	25, 20, 25
16	16.0	(<i>all</i> /E)-lycopene and (5Z)-lycopene	446/472/502	537.4 > 455.6, 537.4 > 427.4, 537.4 > 413.4 [§]	25, 20, 25

^{*} *in source* fragment as precursor ion

[#] Collision energy used for the respective MS/MS is displayed in the same order as the precursor ion/product ions (MRM)

[§] Precursor-product ion pair selected for quantitation

[‡] Internal standard

n.a.: not applicable

n.d.: not determined

Table 2
Analytical parameters for HPLC-PDA and HPLC/MS/MS analysis of standards

Compound name	Detector type	Wavelength [nm] and MS/MS transition	Extraction recovery [#] [%](n = 5)	Calibration linearity (n = 4)	LOD [pmol on-column]	LOQ [pmol on-column]	Accuracy (n = 7)	Inter-day precision (n = 7)
(<i>all-E</i>)-lutein	PDA	446	99.3 ± 5.8	0.9976	0.042	0.139	98%	6.0%
	MS/MS	551.5 345.3	–	0.9983	0.323	1.077	94%	12%
(<i>all-E</i>)-cryptoxanthin	PDA	452	101.0 ± 3.3	0.9984	0.381	1.270	97%	5.6%
	MS/MS	553.3 429.5	–	0.9983	0.148	0.493	97%	15%
(<i>all-E</i>)- β -carotene	PDA	446	100.9 ± 5.3	0.9987	0.473	1.575	100%	4.7%
	MS/MS	537.4 413.4	–	0.9984	0.133	0.443	93%	14%
(<i>all-E</i>)- β -carotene	PDA	452	99.5 ± 0.7	0.9963	1.184	3.945	97%	6.2%
	MS/MS	537.4 413.4	–	0.9997	0.304	1.014	102%	4.5%
(<i>all-E</i>)-lycopene	PDA	472	100.2 ± 2.5	0.9989	0.899	2.995	93%	8.3%
	MS/MS	537.4 413.4	–	0.9997	0.025	0.082	96%	11%
retinyl palmitate	DAP	330	95.9 ± 9.7	0.9959	0.613	2.042	99%	3.7%
	MS/MS	269.2 213.2	–	0.9999	0.574	1.912	107%	7.2%
phyloquinone	PDA	248	100.5 ± 4.3	N/A	N/A	N/A	N/A	N/A
	MS/MS	451.4 187.2	–	0.9981	0.003	0.009	97%	13%
-tocopherol	PDA	295	100.0 ± 4.3	0.9965	0.003	0.011	100%	10%
	MS/MS	431.4 205.1	–	1.0000	0.004	0.014	79%	41%

N/A: not available

[#]Values are reported as mean ± standard deviation

Table 3
Estimated matrix effect as determined by comparing AUC_(MS/MS) and AUC_(PDA)

Analyte	Average matrix effect [*]
-cryptoxanthin	33±20% [#]
lutein	134±16% [#]
retinyl palmitate	-15±11%
-carotene	-25±14%
-carotene with IS [‡]	6±8%
-carotene without IS	-53±15%
(<i>all-E</i>)-lycopene and (<i>5Z</i>)-lycopene	-8±23%

^{*} Number of subjects as indicated in Fig. 3. For each individual subject, $[-(AUC(ms/ms)-AUC(pda))/AUC(pda) \times 100\%]$ was calculated. Averages and standard deviations were then calculated. Positive and negative values represent enhanced and suppressed AUC signals, respectively

[#] Signal enhancement

[‡] Internal standard