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Simultaneous quantification of α-tocopherol and four major carotenoids in botanical materials by normal phase liquid chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry

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Abstract

A rapid and sensitive method for the simultaneous quantification of α-tocopherol, β-carotene, β-cryptoxanthin, lutein and zeaxanthin in botanical materials has been developed using normal phase liquid chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry (NP-LC–APCI-MS/MS). A systematic extraction procedure for different botanical materials was described and the extraction solvents were matched with normal-phase LC requirement. Quantification was performed by using the external standards and standard calibration curves were linear between 35 and 8505 ng/mL for α-tocopherol, 64–15,552 ng/mL for β-carotene, 40–9720 ng/mL for β-cryptoxanthin, 70–17,010 ng/mL for lutein and 64–15,552 ng/mL for zeaxanthin with regression coefficient $r^2 > 0.9996$. The limits of quantification (LOQ) were 35, 64, 40, 70 and 66 ng/mL for α-tocopherol, β-carotene, β-cryptoxanthin, lutein and zeaxanthin, respectively.

Keywords: Tocopherol; Carotenoids; Botanical materials; NP-LC–APCI-MS/MS

1. Introduction

A number of edible botanical materials with nutraceutical functions such as grain, vegetable, vegetable oils and fruits contain a wide spectrum of lipophilic antioxidants including tocopherols and carotenoids [1–4]. Epidemiologic and clinic studies indicated that an increased intake of grain, vegetable and fruit might result in an higher plasma concentration of those antioxidants, which has been associated with a reduced risk of chronic degenerative diseases, such as cancer [5], cardiovascular disease [6], oral leukoplakia [7,8], skin damage [9], and aging-related eye disease [10,11]. Reliable and sensitive methods are in high demand for rapid determination of tocopherol and carotenoids in food, botanical, nutraceutical, and clinic materials to better quantify these beneficial components.

Generally, a good and practical analytical method should have an ideal combination of sample extraction, LC separation and detector functionality. The compatibilities within those three aspects are very critical for a method development. Based on their chemical properties, tocopherols and carotenoids can be extracted by hydrophobic solvents like hexane or chloroform, separated by using either normal or reverse phase LC column and quantified by UV, fluorescence, MS and NMR detectors. Previous efforts were mainly focusing on developing the novel LC protocols to improve the separation of α-tocopherol and carotenoids, which included using normal phase nitrile-bonded silica [12], adding CH_2Cl_2 in the mobile phase of C_{18} column separation [13,14], lowering the column temperature [1,15] or applying special C_{30} reverse phase column [16]. Within those efforts, the C_{30} column, which was first introduced by Sander at National Institute of Standards and Technology (NIST) [16], really provided an excellent separation for carotenoid stereo-isomers. However, its extended running time up to 80 min without equilibration

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time limited its application in practice [17]. Additionally, injection repeatability could be easily failed if hydrophobic extraction solvent like hexane, which was often used in the extraction protocol for tocopherols and carotenoids, was injected into C_{30} column. To avoid such interference between extraction solvent and mobile phase in C_{30} LC system, a special on-line SPE needs to be setup [18]. Until now, we have still not seen a method publication to address the compatibility among extraction solvent, LC mobile phase and LC–MS interfaces for α-tocopherol and carotenoid separation.

The present study was conducted to develop a simple, rapid and sensitive LC–MS method for the simultaneous quantification of α-tocopherol and four common carotenoids from different botanical materials at ambient temperature. This study not only focuses on LC separation of α-tocopherol and four carotenoids but also takes the special cautions of the combination of sample extraction with LC separation and LC separation with MS detection. For tocopherol and carotenoid extraction, this study describes a systematic procedure for different botanical materials. Dry hexane is the solvent used at the end of the extraction procedure, which has least interference with the mobile phase for LC separation. Meanwhile, the developed mobile phase system with high ratio of organic solvents can enhance the analyte evaporation in the APCI vaporizer for better detection sensitivity. This procedure also can be used for other field studies because the botanical matrix is generally more complex than clinic samples such as human plasma for quantification of lipophilic antioxidants [1,19].

2. Experimental

2.1. Chemicals and materials

α-, δ-, and γ-Tocopherols were purchase from Sigma (St. Louis, MO, USA). Lycopene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, neoxanthin and violxanthin were obtained from Indofine Chemical Company (Hillsborough, NJ, USA). Acetonitrile, methanol, tetrahydrofuran and water were HPLC grade from JT Baker (Phillipsburg, NJ, USA). Formic acid was from Mallinckrodt, (Irvine, CA, USA). Olive oil was purchased from local supermarket in Maryland, wheat bran was obtained from Dr. Scott Haley in the Department of Soil and Crop Science, Colorado State University, vegetables were obtained from Farmers Market in Colorado and freeze-dried immediately, and carrot and tomato juices were purchased from local markets in New Jersey. Syringeless filter device (0.2 μm PVDF membrane) was purchased from Whatman Inc. (Clifton, NJ, USA).

2.2. Preparation of standards and quality control samples

Since fat-soluble α-tocopherol and carotenoids are light sensitive, cautions were taken to minimize exposure of the standard solution by using either amber glassware or ordinary glassware protected with aluminum foil. Into a 10 mL volumetric flask, 2–5 mg of α-tocopherol, β-carotene, β-cryptoxanthin, lutein, zeaxanthin was weighed, respectively, and hexane was added to final volume as stock solution for each standard compound. The stock solution was then diluted with hexane to the required concentrations. Final concentration range in calibration standard were 35/64/40/70/64, 105/192/120/210/192, 315/576/360/630/576, 945/1728/1080/1890/1728, 2835/5184/3240/5670/5184, and 8505/15552/9720/17010/15552 ng/mL (ppb) for α-tocopherol/β-carotene/β-cryptoxanthin/lutein/zeaxanthin, respectively. Three levels of QC samples, 300/500/300/500/500, 1000/2000/1000/2000/2000, and 2500/5000/2500/5000/5000 ng/mL (ppb) for α-tocopherol/β-carotene/β-cryptoxanthin/lutein/zeaxanthin, respectively, were prepared, aliquoted, stored frozen at −20 °C. Calibration standard and QC sample were freshly prepared just before running each sample set. No significant component degradation was found during the calibration standard and QC sample preparation.

2.3. Preparation of samples

2.3.1. Vegetable oils and dry powder materials

Into 25 mL volumetric flask, 200–1000 μL of vegetable oil sample was weighed and hexane was added to make the final volume. Clear solution was obtained without any additional steps. With 8 mL of hexane, 200–1000 mg of dry flour powder materials was directly extracted at ambient temperature for 15 min using sonication. The supernatant was collected after centrifugation at 600 rpm for 5 min at ambient temperature and the solid residue was re-extracted two more times following the same condition. The three supernatants were combined and the final volume was adjusted to 25 mL using a volumetric flask. The extraction solution was filtered through a 0.20 μm PVDF membrane filter before LC–MS analysis.

2.3.2. Fresh fruit juices and aqueous liquid

Aqueous liquids (1–10 mL) such as fruit juices were weighted and partitioned with 8 mL hexane, and the hexane layer was collected. The resulted aqueous layer was re-extracted two more times with hexane under the same experimental conditions. The three hexane extracts were combined and dehydrated using anhydrous sodium sulfate. The final volume of the dried extract was adjusted to 25 mL with volumetric flask and the solution was filtrated through a 0.20 μm PVDF membrane filter before LC–MS analysis.

2.3.3. Wet hard botanical tissues

With 10 mL of methanol/tetrahydrofuran (1:1, v/v), 200–1000 mg of wheat bran was extracted at ambient temperature overnight and then sonicated for another 15 min. The extraction was repeated two more times following the same procedure. The resulting extraction was combined and centrifuged at 600 rpm for 5 min at ambient temperature. After centrifugation, the supernatants were collected. The

combined supernatant was dried under gentle stream of nitrogen gas and the solid residue was further extracted two times with 10 mL of hexane with 10 min sonication each time. The final volume of combined hexane extract was adjusted to 25 mL with a volumetric flask and the solution was filtered through a 0.20 μm PVDF membrane filter before LC–MS analysis. All the sample solutions were stored at −20 °C until LC–MS analysis.

2.4. Liquid chromatography and mass spectrometry

The standard and sample solutions were analyzed using LC–APCI-MS/MS (liquid chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry) or LC–ESI-MS/MS (liquid chromatography–electrospray ionization-tandem mass spectrometry) methods. Briefly, LC analysis was performed using a TSQ Quantum tandem mass spectrometry (Thermo-Electron Company, San Jose, CA, USA) equipped with an APCI or ESI interface and Agilent 1100 LC system (Agilent Technologies, Palo Alto, CA, USA). The LC separation was achieved by using a Zorbax RX-SIL column (Agilent Technologies, Palo Alto, CA, USA), 2.1 mm i.d. × 150 mm, 5 μm particle size, at room temperature. α-Tocopherol and four carotenoids were eluted using a mobile phase of hexane as solvent A and 1% isopropanol in EtOAc (v/v) as solvent B. The gradient procedure was as follows: (1) the gradient was linear from 1% to 10% of solvent B in the first 5 min, and (2) 10% of solvent B was increased to 50% from 5 to 20 min. The LC column was re-equilibrated for another 10 min with 1% of solvent B, prior to injection of the next sample. The flow rate was 0.5 mL/min and the injected volume was 5 μL. The TSQ Quantum was operated in the positive-ion mode under the following conditions: nitrogen (>99.7%) was used for sheath gas and auxiliary gas at pressure of 35 psi and 5 units, respectively. The APCI vaporizer temperature was maintained at 550 °C and the corona discharge needle current was set at 4.0 μA in APCI interface. ESI spray voltage was set at 4.5 kV in ESI interface. The temperature of the heated capillary was maintained at 300 °C. A collision induced dissociation (CID) was achieved using argon as the collision gas at the pressure adjusted to more than 0.8 mTorr above the normal, and the applied collision offset energy was set from −35 to −45 eV for individual analyte. The best energy level was adjusted against analyte standards each time before running the sample. Identification of α-tocopherol and the four carotenoids was accomplished by comparing the LC retention time and selected reactant monitoring (SRM) analysis of the sample peaks with that of the certified pure commercial α-tocopherol and four carotenoid standards. The quantitative *m*/*z*: from 431.4 (molecular ion) to 165.2 (major fragment) was set for α-tocopherol, 537.5 (molecular ion) to 119.2 (major fragment) was set for β-carotene, and *m*/*z*: 553.5 → 119.2, 551.5 → 119.2 and 569.5 → 119.2 were set for β-cryptoxanthin, lutein and zeaxanthin, respectively. Data was acquired with Xcalibur software system (Thermo-Electron Company, San Jose, CA, USA). The quantification for α-tocopherol and each carotenoid compound was conducted using the total ion counts of the responding external standard.

3. Results and discussion

3.1. Sampling preparation

3.1.1. Edible oils and dry fine powder

The edible oil samples can be directly dissolved into hexane and be analyzed without any further sample treatment because hexane is a good solvent of oils containing α-tocopherol and carotenoids. The fine dry materials like grain flour powder can also be extracted directly with hexane because hexane can be easily penetrated into those dried materials and dissolve α-tocopherol and carotenoids. The contents of individual tocopherol and carotenoids in vegetable oils depend fundamentally on the species, cultivars, state of ripeness and agronomic conditions, and in general, undergo a considerable variation during storage and preparation as edible oils [20–22]. A typical chromatogram of olive oil is shown in Fig. 1(A).

3.1.2. Aqueous liquid samples

α-Tocopherol and carotenoids in aqueous liquid can be partitioned with hexane because of their solubility. However, it is critical to completely remove the moisture from the hexane extract after extraction and prior to injection into LC. The moisture could be removed by anhydrous sodium sulfate. The reason to remove the moisture is that trace amount of water may change the performance of the silica column and fail the injection repeatability in method validation. The carotenoid profiles in tomato and orange juices have been reviewed [23]. Lycopene and β-carotene were predominant carotenoids present in tomato and carrot juices, respectively. They were very easily distinguished by retention time (1.02 min is for β-carotene and 1.19 min is for lycopene) in current chromatographic condition. A typical chromatogram of carrot juice is shown in Fig. 1(B).

3.1.3. Wet hard botanical tissues

The wet hard tissues such as wheat bran are difficult to be directly extracted by hexane because their surface moisture and tissue membrane structures. Methanol/tetrahydrofuran (1:1, v/v) was used as extraction solvent for those kinds of materials because methanol is not only considered as the most powerful penetrating solvent for common botanical materials but also mixable with moisture on the surface of wet hard tissues. The tetrahydrofuran used here is to enhance the solubility of α-tocopherol and carotenoids during the extraction. It was also noted that the methanol has to be completely removed before the sample solution was injected into the silica column to retain the reproducibility of the solid phase performance. Our previous study indicated that contents of

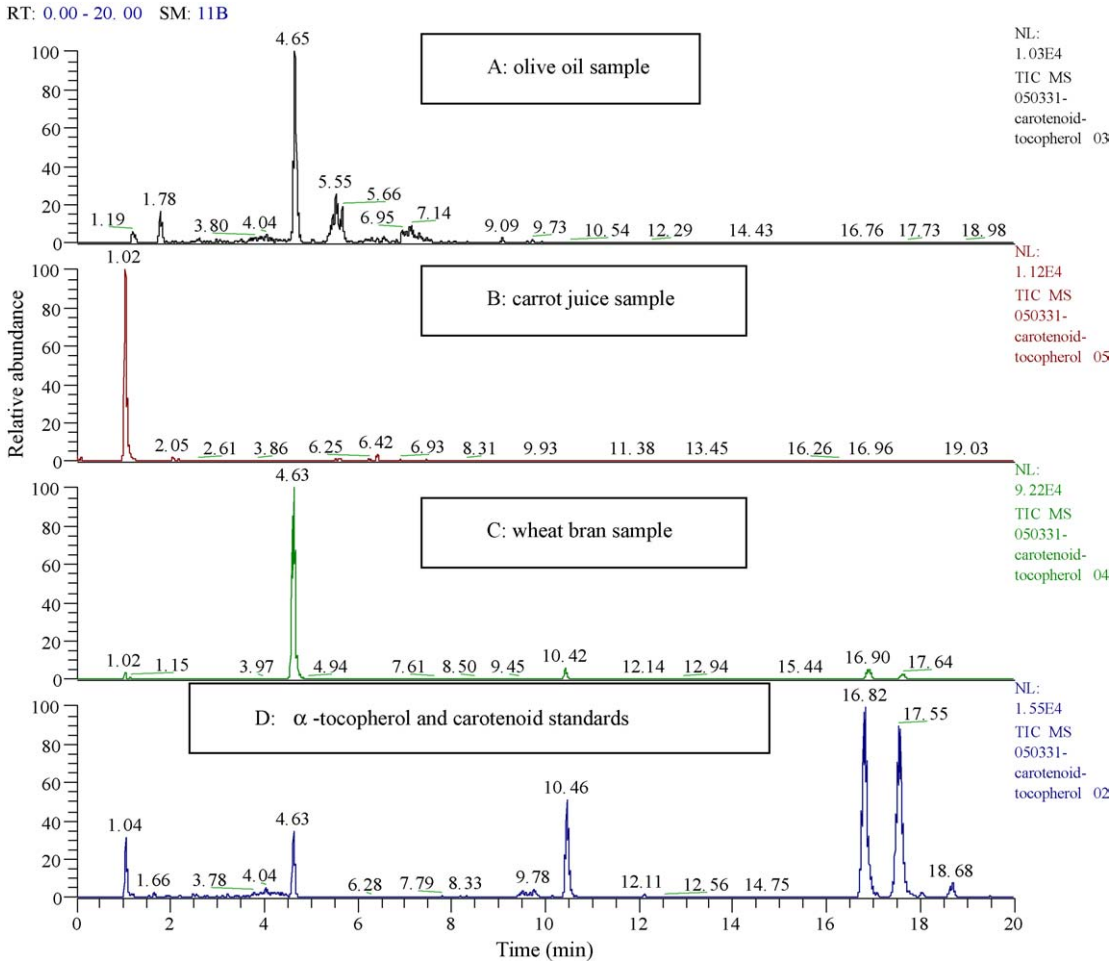

Fig. 1. LC–APCI-MS chromatograms of (A) olive oil sample (peak at 1.19 min is lycopene), (B) carrot juice sample, (C) wheat bran sample and α-tocopherol and (D) carotenoid standards. (1) β-Carotene (RT = 1.04 min), (2) α-tocopherol (RT = 4.61 min), (3) β-cryptoxanthin (RT = 10.46 min), (4) lutein (RT = 16.81 min), and (5) zeaxanthin (RT = 17.57 min).

α-tocopherol and four carotenoids in wheat brain were highly related to the source of materials [2]. A typical chromatogram of wheat bran is shown in Fig. 1(C).

The influence of polar solvents from sample extraction on normal phase LC performance was investigated in the present study. Methanol, tetrahydrofuran or water from the sample extraction can dramatically alter the retention time of the interested compounds in bare-silica column. If methanol and tetrahydrofuran are needed for sample extraction, they have to be removed under nitrogen gas prior to sample injection into LC system. Moisture also significantly alter the retention performance of the silica column and has to be removed using anhydrous Na_2SO_4 before sample injection. This may prevent the extended re-equilibration time required to condition the column after running each sample. This might partially explain why a normal phase LC often requires longer re-equilibration time than a reversed phase LC. In fact, the effects from polar solvent like water or methanol in silica column is just like injecting hexane or ethyl acetate solvents into C_{18} or C_{30} reverse-phase column [17]. Generally, re-equilibration in the reverse phase LC does not take long because non-polar organic solvents are not commonly used in the sample extraction for reverse phase chromatography. Therefore, the present LC procedure has reproducible retention time and requires less re-conditioning since the extra precaution was taken during sample preparation.

3.2. LC separation of α-tocopherol and the four carotenoids

Lutein and zeaxanthin of the four common carotenoids have a same molecular weight and very similar conjugated system in the structures, and therefore, their resolution on the common reverse phase column is poor [24]. Furthermore, UV and even tandem MS cannot provide the identity information during the quantification without a good separation. Low temperature, CH_2Cl_2 application in mobile phase of C_{18} column, nitrile-bonded silica normal phase LC system and special C_{30} column have been developed for better separation of α-tocopherol and carotenoids, especially for lutein and zeaxanthin, but those attempts have increased the time and financial cost and precaution for analysts during the

analysis [1,12–17]. The LC separation obtained with bare-silica column using APCI-MS detection in our current LC system provided a rapid and complete separation of the α-tocopherol and four carotenoids, especially for separating lutein and zeaxanthin (see Fig. 1(D)). The total running time including re-equilibration time was 30 min. Normally, the APCI interface needs a higher flow rate than ESI for better detection sensitivity. The medium size normal phase column (2.1 mm × 150 mm) used in this protocol might give a better sensitivity than the conventional 4.6 mm i.d. column while providing enough mobile phase (0.5 mL/min) for APCI interface that may not be obtained using a 1.0 mm i.d. column.

3.3. Mass spectrometry of α-tocopherol and the four carotenoids in ESI and APCI modes

Mass detectors measure mass to charge ratios of ionized molecules and the ionized molecules can be formed under the varieties of ionization techniques, which include EI, CI, FAB and API. Both ESI and APCI techniques in API category involve ionization of sample molecules at atmospheric pressure and so they are often called as soft-ionization techniques because proton transfer between donor and acceptor need much less energy than electron-capture or electron-loss processes in the normal EI processes, even the extended polyene chain in carotenoids can stabilize either a negative or positive charge in their molecular ions [$M^{\bullet+}$ or $M^{\bullet-}$] [25]. When samples are acquired using the electrospray ionization (ESI) interface, the ions are pre-formed in liquid phase before the analyte ever reaches the source probe. Most commonly, this can be accomplished by adding a proton donor such as formic acid to analytes. However, in our situation, fat-soluble α-tocopherol and four carotenoids lack protonation sites in liquid phase and their proton transfers become hard to occur. Competing with these proton transfer processes, the molecular ion $[M]^{\bullet+}$ (electron-loss) instead of $[M+1]^{\bullet+}$ formation becomes predominant via ESI interface [26]. Of course, the sensitivity obtained under this unusual phenomenon by ESI is sacrificed to a certain degree. To improve the sensitivity for our analytes, fat-soluble α-tocopherol and four carotenoids, the MS detection via APCI interface after LC separation was applied. When samples were acquired using the APCI interface, α-tocopherol and four carotenoids become the gas-phase before they reach the corona discharge needle. All the mass spectra of α-tocopherol and the four carotenoids were returned to common [M + 1] phenomenon. These protonated $[M+1]^{\bullet+}$ molecular ions from gas-phase ion-molecular reactions inside APCI interface might be considered as either a straightforward CI process or a result that the heating energy from APCI vaporizer promotes α-tocopherol and four carotenoids to various excited states that exhibit unusually high proton affinity compared with the ground-state molecules [25]. The mass spectra of α-tocopherol and a typical carotenoid, β-carotene via ESI and APCI are displayed in Fig. 2. The detection using APCI mode is 100 times more sensitive than that with an ESI mode.

3.4. Post-column conversion of lutein to dehydro-lutein

The thermal stability is a major concern for APCI approach. The influence of APCI vaporizer temperature, which converts the analytes to gas phase, on method sensitivity was investigated. Increasing temperature up to 550 °C improved the sensitivities for all tested compounds. However,

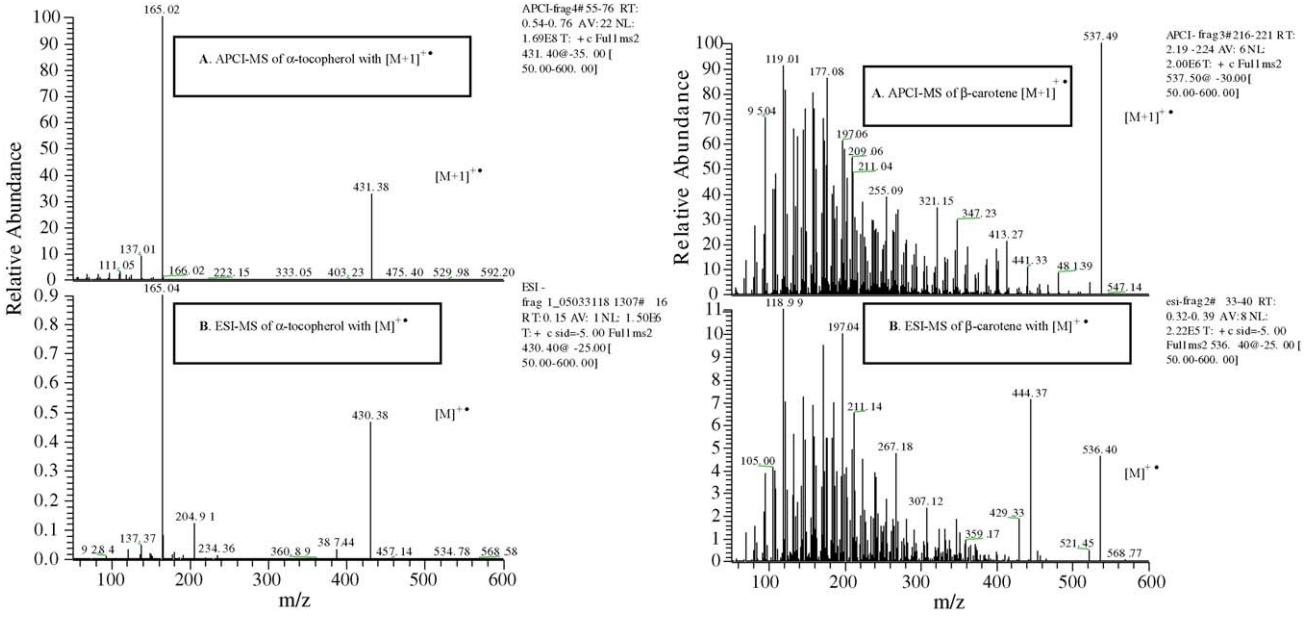

Fig. 2. Mass spectra of α-tocopherol (left) and β-carotene (right) via ESI (lower ones) and APCI (upper ones) interfaces.

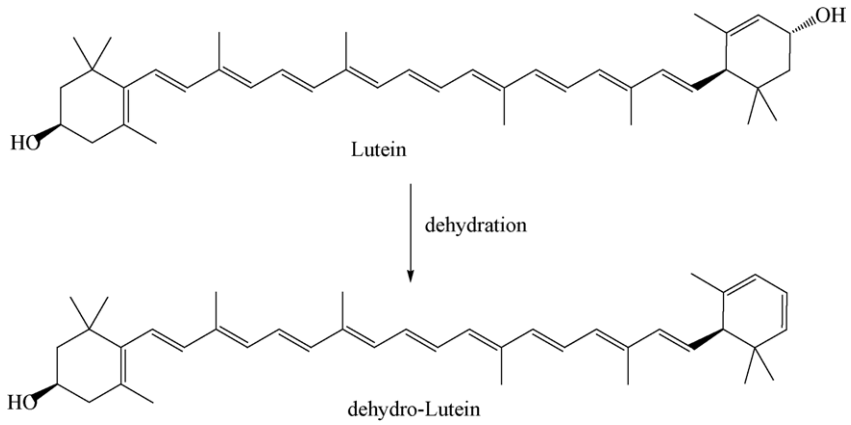

Fig. 3. Dehydro-lutein formation from lutein under high temperature in APCI vaporizer.

the thermal dehydration of lutein becomes significant when temperature was greater than 450 °C. The formation of dehydro-lutein is shown in the following (Fig. 3).

This dehydration happened post-column and pre-ionization at corona discharge needle because lutein still displayed *m*/*z* at 569 [M + 1] under lower APCI vaporizer temperature and dehydration ion is 551 [568 − H_2O + 1] instead of 550 [568 − H_2O]. The proton unit was added on dehydration molecule [568 − H_2O] before the soft-ionization. The SRM from 551.4 to 119.2 instead of from 569.5 to 119.2 was used for lutein quantitation because this post-column dehydration.

Zeaxanthin can also be dehydrated under high temperature but the ratio is much lower than lutein because it lacks the allyl alcohol skeleton (CH=CHCHOH) in the structure, which displays in the following (Fig. 4).

Therefore, the SRM from 569.5 to 119.2 was used to quantify zeaxanthin in the samples.

3.5. Selectivity and sensitivity

The blank botanical control samples, without any antioxidant activities, were tested for matrix interference. They did not show interfering peaks at retention time of compounds of interest. Method selectivity was also tested against other structurally similar compounds. δ- and γ-Tocopherols did not interfere with α-tocopherol, and furthermore, neoxanthin/violxanthin, the two other carotenoid compounds, did not interfere with the tested four carotenoids since their *m*/*z* and retention time were different. Lycopene, which has the same *m*/*z* as β-carotene and might be observed at the β-carotene SRM channel (*m*/*z* from 537.5 to 119.2), was separated from β-carotene peak. The retention times for β-carotene and lycopene were 1.04 and 1.19 min, respectively, under the present chromatographic condition.

When blank control samples were spiked with the analytes at their limits of quantification (LOQ) at a signal to noise ratio of 10.0, which were 35, 64, 40, 70 and 66 ppb for α-tocopherol, β-carotene, β-cryptoxanthin, lutein and zeaxanthin, the observed concentrations were within 10% of the theoretical concentration values (%R.E.). The relative errors (%R.E.) are presented in Table 1. The relative standard deviations (RSD) were 9.05, 9.62, 9.33, 9.52 and 9.52 for α-tocopherol, β-carotene, β-cryptoxanthin, lutein, and zeaxanthin of the spiked LOQ samples, respectively. The estimated limit of detection (LOD) at a signal to noise ratio of 3.3 was about 11, 21, 13, 23 and 21 ppb for α-tocopherol, β-carotene, β-cryptoxanthin, lutein and zeaxanthin, respectively, suggesting that the new method has excellent detecting sensitivity.

3.6. Precision, accuracy and linearity

Table 1 shows the validation data on accuracy and precision of each standard concentration. The regression coefficients of the five validation curves were all greater than 0.99 with a *P*-value smaller than 0.05. For the quadratic regressions, all intercept and concentration square (curvature) terms are not statistically significant at 95% confidence level ($P > 0.05$). However, concentration term is significant for both linear and quadratic regressions ($P < 0.05$) in all cases. Quantitation of α-tocopherol and the four carotenoids

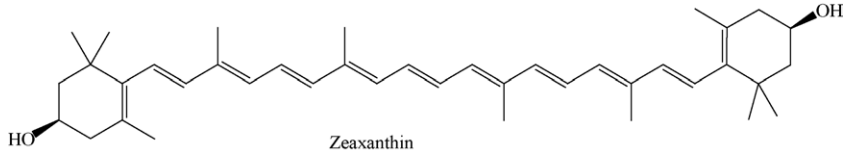

Fig. 4. Zeaxanthin structure.

Table 1
Linearity of calibration standards from five validation curves

α-Tocopherol	Concentration of standard (ng/mL)						Slope	r^2
	35	105	315	945	2835	8505		
Mean	37.7	114.3	305.5	1028.7	2719.4	8534.4	30.01	0.9996
%RSD	9.05	4.41	2.09	2.75	2.00	4.00		
%R.E.	7.70	8.83	−3.01	8.85	−4.08	0.35		

β-Carotene	Concentration of standard (ng/mL)						Slope	r^2
	64	192	576	1728	5184	15552		
Mean	65.7	200.1	532.1	1862.4	5055.2	15584.6	23.32	0.9998
%RSD	9.62	4.21	2.21	2.38	3.80	5.38		
%R.E.	2.61	4.24	−7.62	7.78	−2.48	0.21		

β-Cryptoxanthin	Concentration of standard (ng/mL)						Slope	r^2
	40.0	120.0	360.0	1080.0	3240.0	9720		
Mean	42.0	119.1	361.1	1011.8	3327.5	9698.3	19.55	0.9998
%RSD	9.33	3.89	4.37	5.89	4.18	3.52		
%R.E.	5.08	−0.74	0.31	−6.31	2.70	−0.22		

Lutein	Concentration of standard (ng/mL)						Slope	r^2
	70.0	210.0	630.0	1890.0	5670.0	17010.0		
Mean	72.7	220.2	628.7	1806.3	5764.5	16988.3	15.43	0.9999
%RSD	9.52	2.58	3.36	2.73	2.89	2.82		
%R.E.	3.82	4.87	−0.20	−4.43	1.67	−0.13		

Zeaxanthin	Concentration of standard (ng/mL)						Slope	r^2
	64	192	576	1728	5184	15552		
Mean	66.4	201.3	574.8	1651.4	5270.2	15531.6	21.04	0.9999
%RSD	9.52	2.11	3.95	2.24	2.19	1.85		
%R.E.	3.82	4.86	−0.20	−4.43	1.66	−0.13		

were performed using external standards. The five-point calibration curves with the standard solution of α-tocopherol and four carotenoids can cover the concentration ranges in different botanical materials.

4. Conclusion

The present study developed a practical method for the simultaneous determination of α-tocopherol, β-carotene, β-cryptoxanthin, lutein and zeaxanthin in botanical materials. This method not only gave a rapid and complete separation condition for those target analytes but also provided an excellent insight of the extraction solvent and LC mobile phase. Additionally, high ratio of organic mobile phase (hexane and ethyl acetate) could enhance the analyte evaporation inside APCI vaporizer and improve the detection sensitivity.

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