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Determination of retinol and alpha-tocopherol in avocado nanoemulsion with monolithic column

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Abstract

A high performance liquid chromatographic procedure was developed for separation of retinol and α-tocopherol from avocado oil. The extraction methods were studied to extract of oil from avocado sample. This method was developed and validated for various chromatographic conditions for simultaneous determination of retinol and α-tocopherol in avocado oil samples. The oil sample was analyzed on Chromolith C-18 using methanol and water as mobile phase with gradient elution system. The analyzes were detected at 292 and 325 nm, respectively by spectrophotometric detection. The flow rate was 2 mL min^{-1} to complete separation of both analytes and was achieved in 6 minutes. Under the optimum conditions, retinol and α-tocopherol could be determined within a concentration range of 1-40 µg mL^{-1} which can be expressed by the regression equation y = 32222x + 5107 and y = 5742x + 3400 respectively. The limit of detection and quantitation were found to be 0.044µg mL^{-1} and 0.120 µg mL^{-1} for retinol and 0.134 µg mL^{-1} and 0.364 µg mL^{-1} for α-tocopherol respectively. The proposed method was applied to the determination of retinol and α-tocopherol contents in avocado oil samples and found to be 0.002-0.036 mg g^{-1} and 0.033-0.142 mg g^{-1} respectively. The development method is simple, rapid and suitable for analysis of retinol and α-tocopherol in avocado nanoemulsion lotion and avocado oil samples.

Key Words: HPLC, Chromolith, Retinol, α-tocopherol, Avocado oil

Introduction

Avocado (*Persea Americana* Miller), colloquially known as alligator pear, aguacate or butter pear, is a vital commercial tropical fruit. It is now being recognized as one of the finest fruits, which is very rich in dietary fiber, vitamins (A, B, C, E, folacin, niacin etc.) as well as minerals (iron, magnesium, potassium etc.).

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Avocado fruit is also high in mono-unsaturated fatty acids like oleic and palmitoleic acids as well as omega-6 poly-unsaturated fatty acid and calories. Moreover, it is packed with several health profiting plant nutrients [1 2]. Avocado can be botanically classified into four strains namely, West Indian, Guatemalan, West Indian-Guatemalan hybrid and Mexican [3-4]. This plant is originated from South America and Mexico. It was introduced into Thailand by missionaries about a hundred years ago. Due to its high nutrition and good taste, avocado fruit is becoming more popular in Thailand [5]. The prior study on avocado's constituents revealed that it contained potentially anti carcinogenic lipophilic compounds i.e. carotenoids [6]. The lipophilic extract of avocado inhibited prostate cancer cell growth, inducted apoptosis in human breast cancer cell and suppressed liver injury [7-9].

Table 1 Fruit characteristics of some avocado cultivars in Thailand

Cultivars	Flower type	Shape	Weight (g)	Skin Surface	% Fat	Pulp Colour	Taste
Buccanaer	B	pear	250-300	rough	12-18	yellow	excellent
Booth 7	B	round	300-400	green, nearly smooth	12	dark yellow	good
Booth 8	B	pear	300-400	green, nearly smooth	6-12	bright yellow	good
Fuerte	B	pear	150-250	green, smooth	18	yellow	excellent
Hass	A	oval	150-250	rough, black	18-25	yellow	excellent
Peterson	A	round	200-350	smooth	12	dark yellow	good
Ruehle	B	pear	200-350	smooth	10	yellow	good

In addition, the cholesterol-lowering effects of avocado fruit pulp in hypercholesterolemic and hyperlipidemic animal models have been studied [1]. Avocado is a high metabolic rate fruit, completing its ripeness within 5 to 7 days at 25 °C, after harvesting [10]. The ripening process of avocado fruit is of great importance to generate data regarding the changes of individual fatty acids and antioxidant capacity of lipophilic and hydrophilic avocado extracts [11]. Avocados harvested from four different locations were investigated to characterize their contents of carotenoids, fatty acids, and tocopherol using HPLC, gas chromatography, and mass spectrometry [12]. However, no such data regarding the monolithic HPLC analysis combined of retinol and α-tocophenol is available for avocado oil so far. Hence, the thin layer chromatography [13] and the high performance liquid chromatography [14-15] were applied in this study.

Recently, highly porous monolithic silica rod columns have been introduced, which have a bimodal pore structure with a large surface area [16-18]. Due to this fact, higher flow-rates can be applied while the column back-pressure remains low. These new columns not only enable flow-rates up to 10 mL min^{-1} with good column performance, resulting in very short run times, but also a very rapid column equilibration allowing a fast method change-over.

In this study, the HPLC-monolithic silica rod column was developed for simple, rapid and reliable HPLC method with spectrophotometric detection for the quantitation of retinol and α-tocopherol in avocado oils nanoemulsion lotion. This method has been applied to the development of the HPLC method using monolithic silica rod column technology and their applications to the quantitation of retinol and α-tocopherol in avocado nanoemulsion lotions and avocado oils (from Hass, Booth 7 and Buccanaer) collected from Phetchaboon and Tak provinces in northern Thailand.

Materials and Methods

Chemical and solutions. All chemicals used were of analytical reagent grade. Deionised distilled water was used throughout the experiment (Milli-Q water purification system, Millipour Co., USA.). The standard of retinol and α-tocopherol were purchased from Sigma (Saint Louis, USA). Tween 80 and Span 80 were purchased from Fluka (Buchs, Switzerland). Methanol, ethanol, acetonitrile, n-hexane and petroleum ether were obtained from Carlo-Erba (Italy). The stock standard solutions of retinol and α-tocopherol were prepared in methanol to provide a concentration of 1 mg mL^{-1}. These stock solutions were freshly prepared each time and stored below 4 ^{o}C and protected from light. The solutions were diluted with methanol to the desired concentration levels just before performing the analysis.

Materials. Table 1 shows the fruit characteristics of some avocado cultivars in Thailand [6]. Fresh avocado fruit was purchased from commercial sources in Phetchabun and Tak provinces, Thailand. The avocado samples were washed. The pulps were collected while the fruit was fresh and a proportion was dried in a hot air oven at 50 ^{o}C for 36 h. The dried material was ground to a fine powder, passed through a 60-mesh sieve and kept in an air-tight container at 4 ^{o}C until further use. The sample of fresh pulp that was collected was kept in an air-tight container at 4 ^{o}C until further use.

Instruments. HPLC analyses were carried out with Shimadzu Model SCL-10A liquid chromatograph, thermostatic column compartment, online degasser and an UV-visible detector model SPD-10A. The analytical column used was a 25 × 4.6 mm Chromolith® Flash RP-18e column (Merck KGaA 64271 Darmstadt, Germany). Mobile phase was a mixture containing varying ratios of methanol and water with gradient elution system and vacuum-filtered through 0.45 μm nylon membrane (Whatman® GD/X syringe filters, Germany) before use.

Table 2 Result and some physical properties of different varieties of avocado

Varieties of avocado	Sample type	Oil yield (%)		
		Cold extraction	Hot extraction	
		DI water:n-hexane (50:50)	n-hexane	petroleum ether
Hass	Fresh	1.52	0.42	0.32
	Dried	10.98	13.50	-
Booth 7	Fresh	4.19	0.13	0.17
	Dried	11.67	13.81	-
Buccanaer	Fresh	2.82	0.15	0.21
	Dried	13.05	14.01	-

The following instruments were also used; simultaneous spectrophotometer (UV mini-1240, Shimadzu, Japan) was used to scan the spectra of retinol and α-tocopherol, pH-meter (model pH 900, Precisa, Switzerland), water bath and shaker (model SB-200-10, Thailand), ultrasonicator (model 889, Cole Parmer, USA), centrifuge (Thermo Scientific, Japan), high pressure homoginizer (emulsiFlex®, model C3, Canada), particle sizes analyzer (Zetasizer ZS, Malvern instrument, United Kingdom), polytron (PT-MR 3000, Kinematica AG, Switzerland) and a rotary evaporator (EYELA N-N series, Buchi, Switzerland).

Sample preparation. Fresh avocado (*Persea americana* Miller) fruit was purchased from commercial sources in Phetchabun (Hass and Booth 7) and Tak provinces (Buccanaer).

For fresh pulp avocado, 1 kg of fresh pulp avocado was chopped into small pieces. The fresh pulp avocado was cold extracted with water and n-hexane (50:50, v/v) and sonicated for 3 h followed by centrifugation for 30 min at 76000 rpm. The avocado oil was separated using reparatory funnel and kept in dark at 4 ^{o}C [19-20].

For dried pulp avocado, 1 kg of fresh pulp avocado was chopped into small pieces and dried in a hot air oven at 50 ^{o}C for 36 h. Then the dried pulp avocado was powdered and extracted with n-hexane and petroleum ether by Soxhlet extraction process for 3 h. The organic solution was evaporated to dryness at 60 ^{o}C by mean of a rotary evaporator. The 0.5 g of avocado oil from both was transferred into a 5 mL volumetric flask and made up to volume with methanol. An aliquot (0.1g/mL) of this solution was filtered through a 0.45 µm nylon membrane. Then 20 µL of this solution was injected into HPLC system.

Nanoemulsion preparation. Aqueous (Tween 80 (15 g) and water (68 g)) and oil (Span 80 (15 g) and avocado oil (2 g)) phases were prepared separately. The water phase was poured into the oil phase. The nanoemulsion was continuously stirred for 1 hour and pre-homogenized with a polytron at 8000 rpm for 20 min. Then the nanoemulsion was more dispersed using a high pressure homogenizer at 1000 bars for 8 cycles. The final concentration of avocado oil, surfactants and water in the formula were mixed of 2, 30 and 68 (% g) respectively. The 0.5 g of avocado nanoemulsion lotion (0.01 g oil) was transferred into a 5 mL volumetric flask and made up to volume with methanol. An aliquot (0.002 g oil/mL) of this solution was filtered through a 0.45 µm nylon membrane. Then 20 µL of this solution was injected into HPLC system.

HPLC separation procedure. All analyses were performed at room temperature. The chromatographic conditions were carried out in the gradient elution mode using a mixture of methanol and water as mobile phase (Table 3). The flow rate was set at 2.0 mL min^{-1}. The analytical column was a 25 × 4.6 mm Chromolith® Flash RP-18e column. Each sample and standard aliquots of 20 µL was injected into the analytical column. The effluent from the analytical column was monitored by UV detection at 292 and 325 nm. The quantitation was achieved based on peak area of retinol and α-tocopherol. Calibration graphs of analytes were constructed by plotting peak areas versus various concentrations of retinol and α-tocopherol.

Results and Discussion

Avocado oil. The color of the avocado oil in each sample ranged from yellow to brown at room temperature. The oil was soluble in n-hexane and petroleum ether, this solubility was useful in the extraction and isolation of the avocado oil. The oil yield in dry pulp avocado sample was significantly higher than fresh pulp avocado sample (Table 2). The highest value was 14.01 % and 4.19 % in dry and fresh samples, respectively.

Nanoemulsion characterization

Droplet size: The mean droplet size and the size distribution were determined by photon correlation spectroscopy with a particle size analyzer at 25 ^{o}C by diluting 10 µL of the nanoemulsion with 10 mL of DI

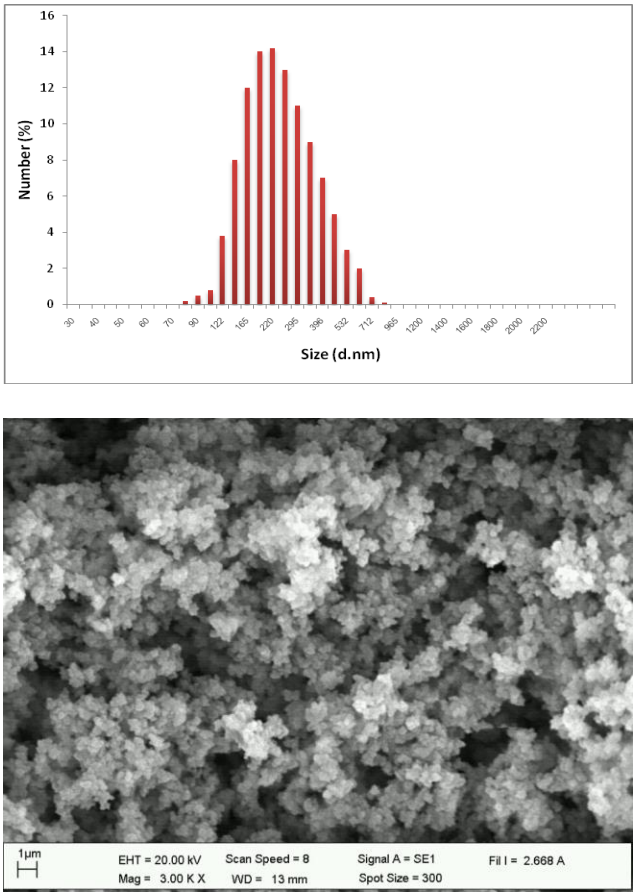

Figure 1 Average droplet sizes and SEM of the avocado nanoemulsion formulation

water. The size distribution was showed by polydispersity index (PI) values. The effect of number of homogenization cycle on droplet size of avocado oil nanoemulsions, upon increasing the number of cycles from 2 to 8, a decreased in droplet size and PI value was investigated. From these results showed that, avocado oil formulations which passed through the homogenizer for 8 cycles showed the lowest droplet size and PI values. The average droplet sizes and the PI values of the formulation were found to be 220 ± 0.6 nm and 0.095, respectively (Figure 1). The PI values lower than 0.25 indicated a close size distribution providing good stability of nanoemulsion.

Surface charge: The surface charge was determined using a particle size analyzer . The nomoemulsion sample was done by diluting 10 µL of the nanoemulsion with 10 mL of DI water and measuring the zeta potential (ZP). The ZP values of avocado nanoemulsion were below -32 mV. The ZP characterizes of particles and thus it gives information about repulsive forces between particles and droplets, to obtain stable nanoemulsion by preventing flocculation and coalescence of the nanodroplets (ZP value above ± 30 mV).

Method optimization. A preliminary experiment was carried to investigate the spectral characteristics of the retinol and α-tocopherol. The absorption spectrum was studied by batch wise spectrophotometric method, while the absorption spectrum was obtained by scanning the wavelength over the range of 200-400 nm. The UV spectrum of retinol and α-tocopherol standard solutions showed the absorption maxima at 325 and 292 nm, respectively. Secondly, the optimization work mainly addressed the program of the chromatographic elution in order to maximize both resolution and sensitivity. An aqueous solution of retinol and α-tocopherol, methanol (A), and water (B) was still adopted as components of the mobile phase, whereas the optimized elution program for avocado oil sample is reported in Table 3.

The best sensitivity compromise for all analytes was reached using two different operative wavelengths (325 nm for retinol and 292 nm for α-tocopherol). The chromatographic separation was performed at a flow rate of 2.0 mL min^{-1}. The method proposed is rapid: all analytes were completely eluted within 5 min and the whole chromatographic run was completed in 6 min. Figure 2 shows the chromatograms obtained by analyzing a mixture of standards (Figure 2(A)), and an avocado oil sample (Figure 2(B)).

Method validation

Range and linearity: Under the selected chromatographic conditions, the linear ranges of the signal response for retinol and α-tocopherol were studied over the concentration range of 1.0-100.0 µg mL^{-1}. The linearity of each calibration graph was determined using the optimal experimental parameters. Eight standard solutions ranging from 1.0-40.0 µg mL^{-1} in concentration, in five replicates each, were injected into the HPLC system. The calibration graph was obtained by plotting the absorbance of thesolutions against the standard concentrations. Linear calibration graphs over the concentration range 1.0-40.0 µg mL^{-1} of retinol and α-tocopherol were obtained with the regression equations: $y = 32222x + 5107$ ($r^2 = 0.9990$) and $y = 5742x + 3400$ ($r^2 = 0.9990$), respectively.

Detection limit and quantification limit: Limits of detection (LOD) of retinol and α-tocopherol were each estimated from their respective calibration curves using the expression 3.3 SD/S where SD is standard deviation of the blank (or the intercept of the calibration curve) and S is the slope of calibration curve and limit of quantitation (LOQ) = 10 SD/S [21]. The limit of detection (LOD) and limit of quantitation (LOQ) values were found to be 0.044 µg mL and 0.120 µg mL^{-1} for retinol and 0.134 µg mL^{-1} and 0.364 µg mL^{-1} for α-tocopherol respectively.

Precision: The precision of the method was determined by measuring the repeatability (intraday precision) and the intermediate precision (inter day precision), both expressed as relative standard deviation (R.S.D). The precision was evaluated by assaying six

replicate injections of 5, 10, and 20 µg mL^{-1} of retinol and α-tocopherol standard solutions respectively. The repeatability was evaluated for each sample on the same day under the same experimental conditions. The intermediate precision was evaluated by assaying each sample on three different days. The results of repeatability and intermediate precisions are shown in Table 4.

Accuracy: Accuracy of the method was assessed with recovery using the addition of four known concentration levels (5, 10, 20 and 30 µg mL^{-1}). All samples were injected in three replicates for each concentration. The concentration found was calculated against the concentration added. The recoveries of analyte were in the ranges of 104.11-114.66 % and 97.27-106.16 % for retinol and α-tocopherol respectively (Table 5). Additives and excipients did not interfere in the determination of those active ingredients since the samples used to evaluate recovery were prepared with those additives and excipients present.

Interferences: Effect of some possible excipients in formulation (Tween 80 and Span 80) was investigated. The synthetic sample solution containing 20 µg mL^{-1} of retinol and α-tocopherol and different concentration of interferences were tested, and the peak areas obtained were recorded. Interestingly, Tween 80 and Span 80 had no significant effect on the determination of retinol and α-tocopherol.

Comparing Analytical Columns: The comparison of the analytical columns was investigated using the proposed optimized conditions. The retinol and α-tocopherol standard solutions (5 µg mL^{-1}) were injected into the HPLC system using three analytical columns. The analytical columns were 25 × 4.6 mm Chromolith® Flash RP-18e column, 125 × 4.6 mm ODS® C18 column and 100 × 4.6 mm RESTEK® C18 column respectively. Retinol and α-tocopherol separated on the Chromolith® Flash RP-18e column gave the best sensitivity peak and short analysis time. Whereas when tested with the same conditions, the ODS® C18 analytical column gave low sensitivity peak, and the RESTEK® C18 column gave lowest sensitivity, broad peak and long analysis time (Figure 3).

Application of HPLC in separation of retinol and α-tocopherol in avocado nanoemulsion lotions. The proposed HPLC method was applied to the determination of retinol and α-tocopherol in avocado nanoemulsion lotions. The sample was prepared according to sample preparation (see Experimental) and the content of retinol and α-tocopherol in each sample solution was determined using the optimum conditions. The samples gave well-defined peaks. There is no interference peak present in either sample. The average contents of retinol and α-tocopherol of avocado nanoemulsion lotion from avocado samples purchased

Table 3 Gradient elution program for the chromatographic separation of retinol and α-tocopherol in avocado oil samples

Step	Time (min)	Mobile phase composition (%)		Flow rate (mL min^{-1})	Wavelength (nm)
		methanol (A)	water (B)		
1	0.00	85	15	2	325
2	1.50	85	15	2	325
3	1.51	95	5	2	292
4	6.00	95	5	2	292

Table 4 Repeatability and intermediate precision for the studied retinol and α-tocopherol standard solutions (n=7)

Compounds	concentration (µg mL^{-1})	Intra-day precision (mean ± S.D.)		Inter-day precision (mean ± S.D.)	
		Concentration found (µg mL^{-1})	% R.S.D	Concentration found (µg mL^{-1})	% R.S.D
Retinol	5	4.99 ± 0.02	1.61	5.01 ± 0.02	1.02
	10	10.09 ± 0.07	1.90	10.06 ± 0.11	1.00
	20	19.88 ± 0.28	1.04	19.58 ± 0.48	1.01
α-tocopherol	5	5.12 ± 0.02	1.07	5.05 ± 0.02	1.05
	10	10.21 ± 0.07	1.47	10.10 ± 0.07	1.21
	20	20.08 ± 0.28	1.80	19.98 ± 0.28	1.33

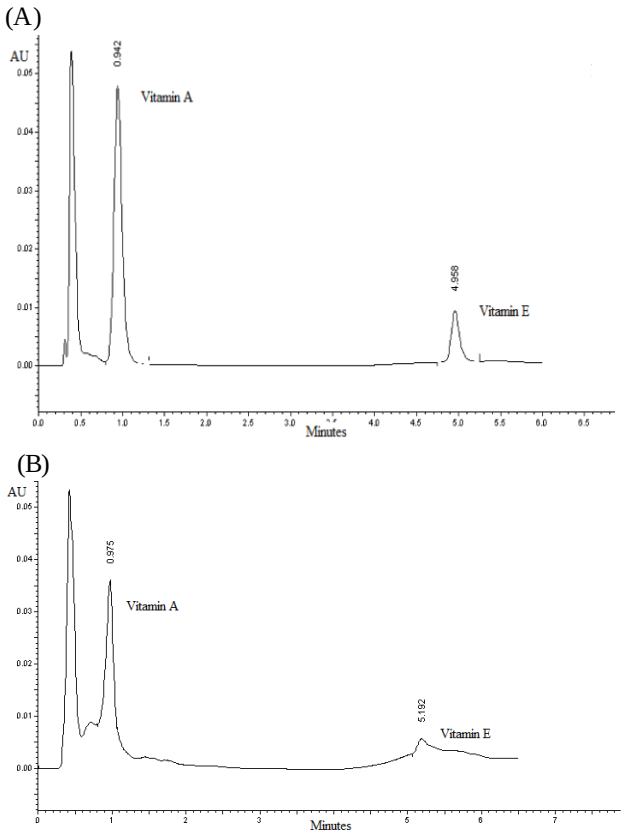

Figure 2 (A) Chromatogram of a standard mixture of vitamin A (retinol) and vitamin E (α-tocopherol), each at a concentration of 10 mg L^{-1}; (B) chromatogram of an avocado nanoemulsion lotion sample (Booth 7)

from Phetchabun province (Hass and Booth 7) were found to be 0.002, 0.036 and 0.033, 0.102 mg g^{-1} respectively and avocado sample purchased from Tak province (Buccanaer) was found to be 0.024 and 0.142 mg g^{-1} respectively.

The results of development HPLC method was compared with the publication method [15]. The results were determined by the calculated t-values at the 95 % confidential limit and t-values less than the Table list t-value. Therefore, these two methods are not significantly different in any of the samples (Table 6).

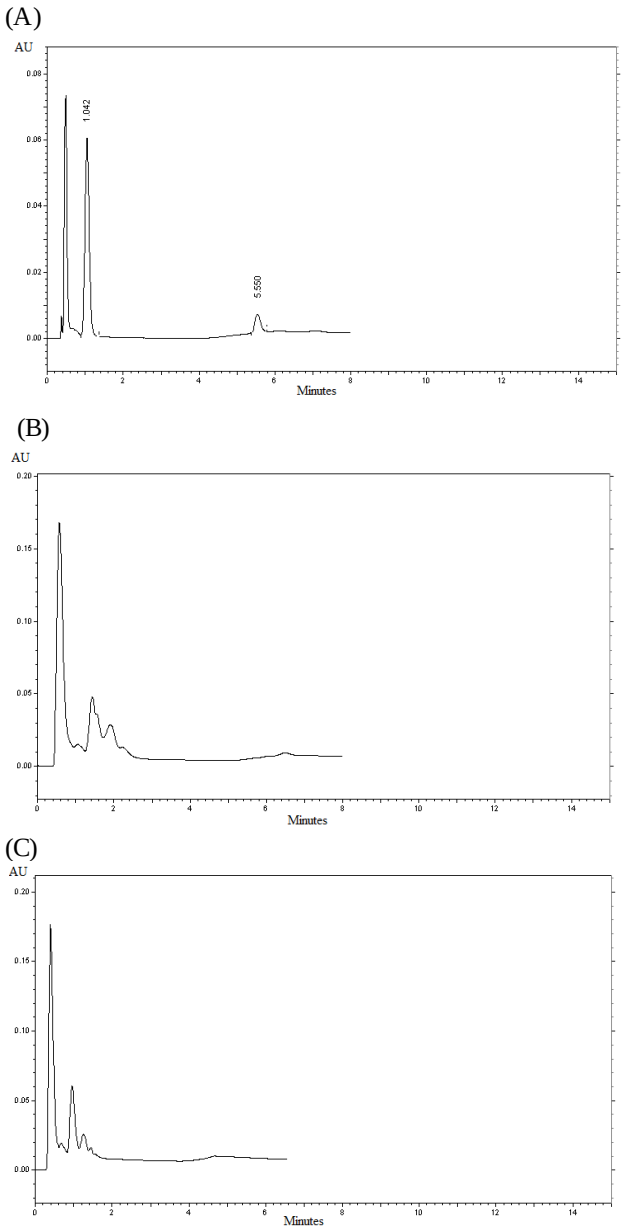

Figure 3 Comparison of the analytical columns; (A) 25 × 4.6 mm Chromolith® Flash RP-18e column, (B) 125 × 4.6 mm ODS® C18 column and (C) 100 × 4.6 mm RESTEK® C18 column

Conclusion

In conclusion, the proposed HPLC procedure can be used for the determination of retinol and α-tocopherol in avocado oil and avocado nanoemulsion lotion samples. The detection limit of this method is reasonable. Sample pretreatment is not necessary. This method is simple, fast, relatively inexpensive, precise, accurate and sensitive. Therefore, the speed of analysis and the precision make this method suitable for quality control of retinol and α-tocopherol in avocado samples. It is therefore suitable for quality control in cosmetic and pharmaceutical formulations containing avocado oil.

Table 5 Accuracy of the proposed HPLC method (n=5)

Retinol	Concentration (mg L^{-1})		% Recovery (average ± S.D)	α-tocopherol	Concentration (mg L^{-1})		% Recovery (average ± S.D)
	Added	Found			Added	Found	
	5	5.50 ± 0.13	109.97 ± 2.52		5	5.31 ± 0.13	106.16 ± 2.52
	10	10.38 ± 0.35	103.84 ± 3.47		10	10.25 ± 0.35	102.51 ± 3.47
	20	22.93 ± 0.26	114.66 ± 1.73		20	21.21 ± 0.26	106.07 ± 1.73
	30	31.23 ± 0.23	104.11 ± 1.15		30	29.18 ± 0.23	97.27 ± 1.15

Table 6 Comparison between the proposed HPLC and the published methods for determination of retinol and α-tocopherol

Nanoemulsion lotion sample	Proposed HPLC method		Published method [Lozano et al., 1993]	
	Amount found			
	retinol (mg g^{-1})	α-tocopherol (mg g^{-1})	retinol (mg g^{-1})	α-tocopherol (mg g^{-1})
Hass	0.002	0.033	0.002	0.035
Booth 7	0.036	0.102	0.033	0.100
Buccaneer	0.024	0.142	0.022	0.143

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