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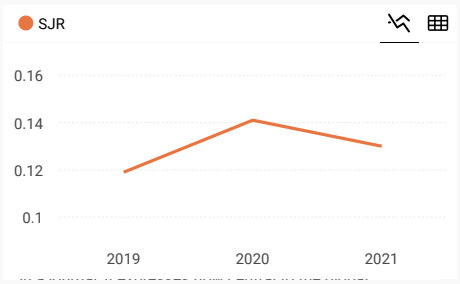

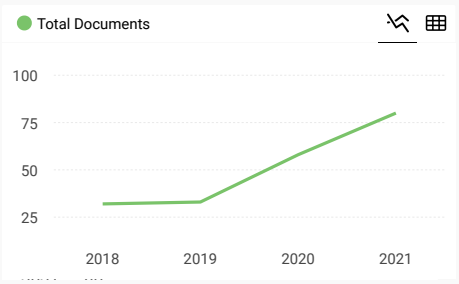

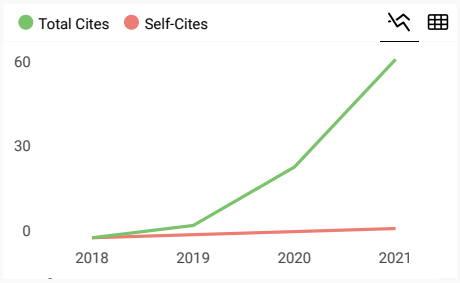

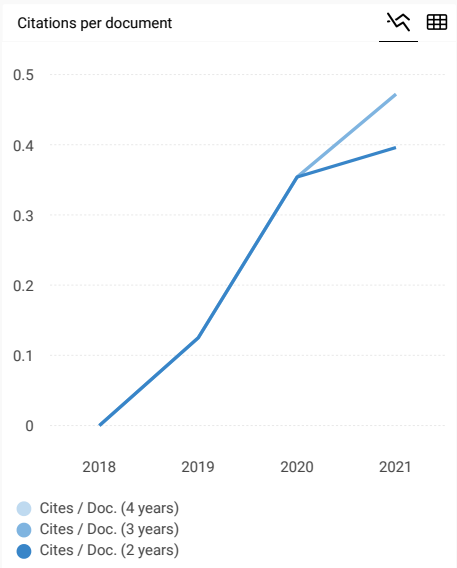

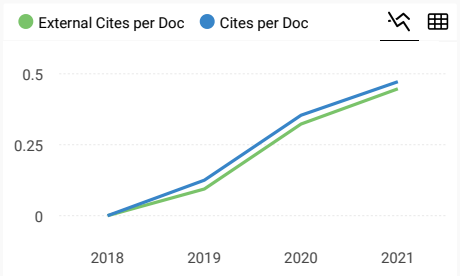

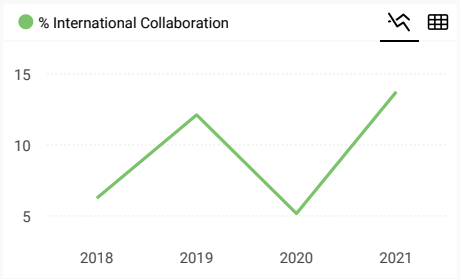

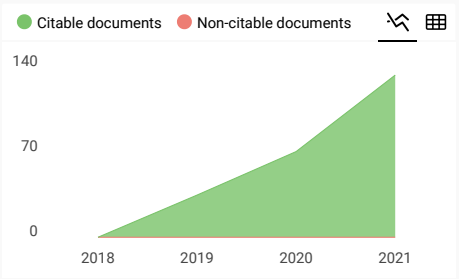

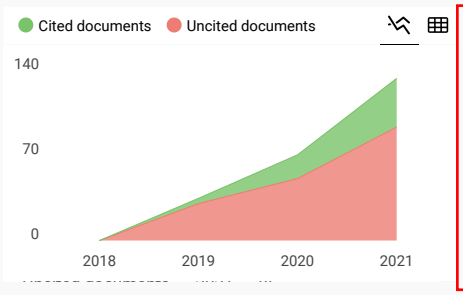

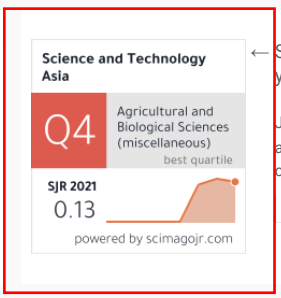

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Science & Technology Asia

11 July 2022

Dear Asst.Prof.Dr. Saowapa Chumanee

Referring to your submission of the manuscript* entitled **"Comparison of Tocopherol Contents in 13 Cold-Pressed Vegetable Oils Extraction from the Mini Screw Press Model T3 by RP-HPLC/FLD"** by Sasikarn Panpraneecharoen, Saowapa Chumanee for publication in Science & Technology Asia, we are pleased to inform you that your manuscript has been accepted for publication in Science & Technology Asia Vol.27, No.3, July-September, 2022.

Thank you for your contribution. We look forward to receiving your submission again soon.

Best wishes,

(Assoc. Prof. Dr. Wutiphol Sintunavarat)
Editor

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Original research article

Comparison of Tocopherol Contents in 13 Cold-Pressed Vegetable Oils Extracted from the Mini Screw Press Model T3 by RP-HPLC/FLD

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ABSTRACT

Optimization and validation of reversed-phase high-performance liquid chromatography with fluorescence detection (RP-HPLC/FLD) for the determination of tocopherols (δ, β+γ, and α) were studied. In a simple sample preparation, oils were diluted in ethanol without saponification and injected directly onto Zorbax Eclipse plus C18. Acetonitrile and methanol (70:30) mixture was used as a mobile phase with a flow rate of 1 mL/min. Fluorescence detection was operated at 296 nm of excitation wavelength and 330 nm of emission wavelength. Tocopherols were separated at 30 °C in less than 15 min after injection. The method showed to be good linear ($r^2 > 0.999$). Mean recoveries were 97.6-100.7%, with intra- and inter-day RSD less than 3.79 and 4.11%, respectively. The detection limits and quantification limits of the method were 0.08-0.21 and 0.23-0.71 µg/mL. The technique had been applied to analyze tocopherols in thirteen cold-pressed vegetable oils extracted using a mini screw press, which were virgin oils. The results showed statistically significant differences ($P < 0.5$) between the kinds of oil sample. The total tocopherols content ranges from 3.62 mg/kg in Japanese pumpkin oil to 1890.71 mg/kg in sacha inchi oil.

Keywords: Cold-pressed oil; HPLC; Screw press; Tocopherols; Vegetable oil

1. Introduction

Cold-pressed vegetable oils that are not heat-treated or chemically extracted are 100% natural products (virgin oils) with characteristics of intense taste, color, and unique aroma [1, 2]. There are two types of cold oil extraction machines: hydraulic press and screw press. Compared to a hydraulic press, the advantages of a screw press are its slight yield, and its continuous

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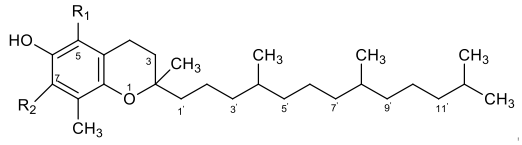
mode of operation [3]. The cold press oil production technique is a simple and environmentally friendly method without using chemicals, resulting in nutritious and safe products for human health [4]. Vegetable oils produced this way are good sources of tocopherols, carotenoids and chlorophylls, polyphenols, and other antioxidants [5-7]. Tocopherols (Ts) are vitamin E, a fat-soluble compound composed of four isomers (*α*-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol). The structure of tocopherols has a chromanol ring and a saturated phytyl tail, and different isoforms at 5- or 7- position of the chromanol ring with either an H or a CH_3 group as shown in Fig. 1. Tocopherols are potent antioxidants [8, 9], prevent rancidity [10], have the capability to reduce the risk of cancer [11], and reduce inflammatory angiogenesis [12]. Vitamin E has also led to the development of numerous new formulations in cosmetics and skin care products [13]. The principal dietary sources of tocopherols are vegetable oils such as corn, soybean, sesame, and cottonseed. γ-T is the most abundant form of tocopherol in the US diet, being three to five times more abundant than *α*-T and δ-T, whereas β-T is present only in minute amounts [14]. Tocopherol isomer has the most antioxidant activity, which is *α*-T [15-17].

High-performance liquid chromate-gramphy (HPLC) techniques commonly have been used for the determination of tocopherols, which can employ either normal-phase (NP) or reversed-phase (RP) column, with fluorescent and ultraviolet detector [18-21]. The fluorescent detector is preferred over the ultraviolet detector in analyzing tocopherols in samples because of its specificity and sensitivity [22]. NP-HPLC provides separation of all tocopherols, while RP-HPLC cannot separate the β-T and γ-T, which are co-eluted. Nevertheless, the RP-HPLC has advantages over NP-HPLC, because of fast equilibration, reproducibility of retention time, low volatility of solvents, good selectivity, and reduction of the use of hazardous solvents [23-25]. Several methods for sample preparation have been studied for the determination of tocopherol content in samples, such as direct solvent extraction [26-28], pressurized liquid extracton [29, 30], QuEChERS extraction [31], ultrasound-assisted saponification [32], and matrix solid-phase dispersion [33]. There were certain advantages and disadvantages to each of the extraction methods. So, the choice of an extraction method for the release of tocopherols depends on the physical and chemical characteristics of the sample and also on the available resources and instruments [34].



α-T: R_1= CH_3 , R_2= CH_3
β-T: R_1= CH_3,R_2= H
γ-T: R_1= H , R_2= CH_3
δ-T: R_1= H, R_2= H

Fig. 1. The structure of tocopherols.

The aim of this work was the comparison of tocopherol contents in thirteen cold-pressed vegetable oils extracted from the mini screw press model T3. Moreover, we optimized a quick and simple method for routine analysis of δ-T, (β+ γ)-T, and *α*-T in vegetable oils by RP-HPLC with fluorescence detection, avoiding a saponification step and using an environmentally friendly solvent. The cold-pressed vegetable oil samples were diluted in ethanol and sonicated. An aliquot of the samples was injected directly into a C18 column.

2. Materials and Methods

2.1 Reagents and standard preparation

The analytical standards of tocopherols (*α*-T, β-T, γ-T, and δ-T) were purchased from Sigma-Aldrich (St.Louis,

MO, USA) and stored at -20 ºC until use. Acetonitrile was purchased from J.T. Baker (Deventer, The Netherlands). Methanol and ethanol were obtained from Carlo Erba (Milan, Italy), all of HPLC grade. Purified water was produced from the arium® pro ultrapure water system (18.2 MΩcm, Sartorius, Germany).

The standard stock solutions of α-T, β-T, δ- T (1 mg/mL), and γ-T (0.1 mg/mL) were prepared in ethanol and stored at -20 ºC protected from light. The working standard solutions were made by dissolving the stock solutions in ethanol at appropriate concentration before use and all solutions stored at 4 ºC.

2.2 Cold-pressed vegetable oils and sample preparation

Thirteen samples of cold-pressed vegetable oils in this study obtained from Nature health and innovation Co., Ltd. (Saraburi, Thailand) include sacha inchi (*Plukenetia volubilis*), chaulmoogra (*Hydnocarpus anthelminthicus*), chilli pepper (*Capsicum annuum*), hemp (*Cannabis sativa*), almond (*Prunus dulcis*), avocado (*Persea americana*), neem (*Azadirachta indica*), moringa (*Moringa oleifera*), flaxseed (*Linum usitatissimum*), sesame (*Sesamum indicum*), black cumin (*Nigella sativa*), Japanese pumpkin (*Cucurbita moschata*), and coconut (*Cocos nucifera*). All samples belong to customers were took seeds for cold-pressed oil extraction by Mini Screw Press Model T3 that was produced by Thai people for small and medium enterprises (SME). The mini screw press consists of a press system and oil pan which were manufactured from stainless steel (food grade) and has a dimension (L×W×H) of 650×280×410 mm, the motor of 200 w, the ratio of 1:50, power of 220 V, and weight of 17 kg (Fig. 2). The screw rotated at a constant speed of 30 rpm. Sample seeds, about 0.5-2 kg, were poured into the hopper and the screw pressed the grain in the pressing chest, then extracted oil dripped on the pan into the container. The extracted crude was stored in a container to settle for 24 hr. The crude oil was filtered through oil filter paper to separate other fine particles in the oil and then kept at room temperature.

Fig. 2. The mini screw press (Model T3) for cold-pressed vegetable oils.

The sample preparation for determining tocopherol content in cold-pressed vegetable oils was performed according to Tahoun and Sheata [21] with slight modifications. A 0.1 g oil sample was dissolved in 10 mL of ethanol in a brown flask with a screw cap to avoid excessive exposure to light and air. The sample solution was sonicated in an ultrasonic bath for 5 min, filtered through a 0.22 μm PTFE filter membrane, and then the filtrate injected into the HPLC system.

2.3 Instrument

Chromatography was performed using Agilent 1260 Infinity HPLC system (Agilent Technology, USA) consisting of quaternary pump VL (G1311C), degasser (G1322A), autosampler (G1329B), thermostatted column compartment (G1316A), fluorescence detector (G1321C) and software OpenLab CDS ChemStation Edition C.01.07 for system control and data collection.

2.4 Chromatographic condition

The chromatographic separation of tocopherols was achieved by RP-HPLC using a ZORBAX Eclipse Plus C18 (4.6×150 mm, 3.5 µm; Agilent Technology) with guard column ZORBAX Eclipse Plus C18 (4.6×5 mm, 1.8 µm). The mobile phase was a mixture of acetonitrile and methanol (70:30, v/v) under isocratic elution at a flow rate of 1 mL/min with a total runtime of 17 minutes. The injection volume was 10 µL and the column temperature was regulated at 30 °C. The mobile phase was filtered through a 0.45 PTFE membrane filter before use. The fluorescence detector was set at excitation wavelength 296 nm and emission wavelength 330 nm.

2.5 System suitability tests

System suitability testing (SST) ensures that the complete testing system (including instrument, reagents, columns, and analysts) is suitable for the intended application [35]. SST was carried out by injecting standard working solutions of tocopherols, each at concentrations of 1 µg/mL in six replicates. Parameters evaluated include retention time, peak area, number of theoretical plates, retention factor, resolution factor, and tailing factor

2.6 Method validation

The HPLC-FLD method for the determination of tocopherols was validated according to the AOAC (2012) guideline [36]. The following validation characteristics were evaluated: linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, and precision.

2.6.1 Linearity and range

The linearity was constructed for each tocopherol in the range of 0.1-10 µg/mL. The concentration of each standard solution was prepared at level of 0.1, 0.5, 1.0, 2.0, 3.0, 5.0, 7.0, and 10.0 µg/mL. The calibration curves were plotted as the concentration of α-T, (β+γ)-T, and δ-T versus the peak area at each level for three days. The coefficient of determination (r^2), slope, and intercept value were determined and the statistic was evaluated.

2.6.2 Limits of detection and limit of quantification

The limits of detection (LOD) and limits of quantification (LOQ) of *α*-T, (β+γ)-T, and δ-T were calculated by the following equation according to an AOAC (2012) guideline [36]: LOD = 3.3 σ/S and LOQ = 10 σ/S, respectively, where, σ is the standard deviation of the response and S is the slope of the linear regression equations.

2.6.3 Precision and accuracy

The intra- and inter-day precision and accuracy were assessed by recovery and relative standard deviation (RSD) values, respectively. The precision and accuracy were studied by spiking of *α*-T, (β+γ)-T, and δ-T with 3 levels concentrations into avocado oil sample at 0.5, 1.0 and 2.0 µg/mL.

2.7 Tocopherols quantification

The *α*-T, (β+γ)-T, and δ-T contents in cold-pressed vegetable oils were identified using the retention time parameter. The tocopherols concentration in theses samples (each sample injected in three times injection volume 10 µL) were estimated by measuring average peak area and were quantified by the use of linear regression from the calibration curve. Results were given in milligrams of each tocopherol per kilogram of oil (mg/kg), calculation as follows:

$$\text{concentration} = \frac{C \times V}{W}$$

where C = tocopherol concentration in sample, determined for calibration curve, mg/L,

V = volume of sample solution, L,

W = weight of oil sample, kg.

2.8 Statistical analysis

All samples were analyzed in triplicate and results expressed as mean ± standard deviation (SD). Data sets were evaluated using one-way analysis of variance (ANOVA). A Duncan's multiple range test was used to determine significant difference. The level of significance was set at $p<0.05$. Statistical analysis was performed by using a SPSS package (SPSS 22.0 for windows, IBM Singapore Pte Ltd).

3. Results and Discussion

3.1 Optimization of tocopherol separation

The chromatographic conditions for tocopherols in this work was evaluated using RP-column. An isocratic elution program was used to achieve the separation. The mobile phase consisted of acetonitrile and methanol (70: 30, v/v). Typically, the RP-HPLC columns were unable to separate the β-T and γ-T isomers, so they appeared the same peak. Other authors found similar results with different protocols for sample preparation and HPLC separation of tocopherols [32, 33, 37, 38].Moreover, RP-HPLC was preferred over normal-phase systems due to the reproducibility of retention time, fast equilibration and robustness of reversed-phase columns over other stationary phases [19].!Irakli et al., 2012 found that the column PerfectSil Target ODS 3, 3 μm, 250×4.6 mm could be partially separated but the separation required a long retention time [25]. The chromatographic separation of tocopherols in standard solution is shown in Fig. 3(A). The total run time was less than 16 min. The retention times of δ-T, (β+γ)-T, and α-T were about 10. 44, 12. 45, and 14. 56 min, respectively. Table 1 shows the values of system suitability parameters of tocopherols at a concentration of 1 mg/ mL. Retention time (R_t) and peak area were very reproducible, with the RSD (%) being below 1%. In the case of theoretical plates (N) , they were above 2000 for all tocopherols, which leads to the conclusion that the chromatographic column was efficient enough. Retention factor (k), selectivity factor (α) , and resolution factor (R_s) were above 2, indicating good separation of tocopherols. In the case of peak asymmetry, the good Gaussian shape of the peaks could be macroscopically shown from the chromatogram. Additionally, the tailing factor was below 2 for all retained peaks ranging from 1.16 for α-T to 1.23 for δ-T, indicating good peak symmetry. The results were acceptance criteria according to AOAC (2012) guideline [36].

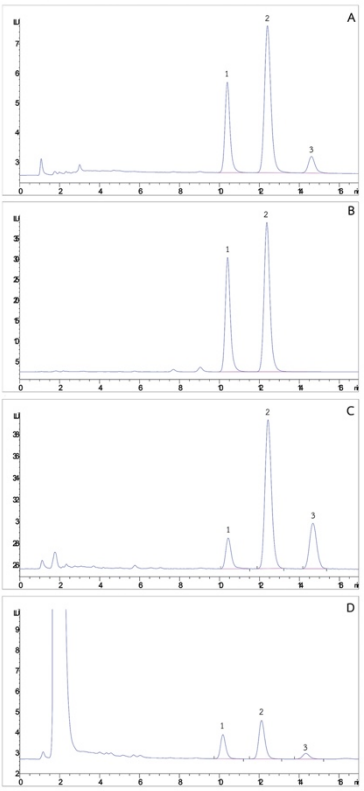


Fig. 3. Chromatogram of the (A) tocopherols standard solution at 1 μg/mL, (B) sacha inchi oil, (C) moringa oil, and (D) chili pepper oil; 1: δ-T (10.44 min.); 2: (β+γ)-T (12.45 min.); 3: α-T (14.56 min.).

Table 1. Term of system suitability test was carried out by injection solution of each tocopherol at concentration of 1 μg/mL.

Tocopherol	R_t (±SD)	%RSD	Peak area (±SD)	%RSD	Theoretical plates (N)	Retention factor (k)	Selectivity Factor (α)	Resolution factor (R_s)	Tailing factor
δ-T	10.44±0.07	0.68	56.30±0.96	0.73	23016	8.40			1.23
(β+γ)-T	12.45±0.10	0.77	119.61±0.95	0.79	16272	10.22	1.22	6.06	1.18
α-T	14.56±0.07	0.48	14.32±0.96	0.82	19851	12.12	1.19	2.62	1.16

3.2 Method validation

The linear correlation was found between the peak areas and the concentration of δ-T, (β+γ)-T, and α-T. The results for linear regression equations and coefficient of determination (r^2) are summarized in Table 2. The coefficient of determination values (r^2>0.9999) shows that regression analysis has had a good linearity. The limits of detection (LOD) and limits of quantification (LOQ) values were found to be 0.08 and 0.23 μg/mL for δ-T, 0.09 and 0.28 μg/mL for (β+γ)-T, and 0.21 and 0.71 μg/mL for α-T, respectively. The data in Table 2 shows that the method was sensitive enough to detect concentrations for the analyzed compound in cold-pressed vegetable oil samples.

Table 2. Analytical characteristics of the method.

standards	Regression equations	r^2	LOD (μg/mL)	LOQ (μg/mL)
σ-T	y =53.55x + 2.16	1.0000	0.08	0.23
(β+γ)-T	y=56.28x + 6.71	0.9999	0.09	0.28
α-T	y=11.94x + 1.88	0.9996	0.21	0.71

The intra- and inter-day precision and accuracy results spiked standard into avocado oil sample were evaluated at three different concentrations at 0.5, 1.0 and 2.0 μg/mL for α-T, (β+γ)-T, and δ-T with three replicate injections per day and dose three days. The results are presented in Table 3. Excellent repeatability and satisfactory inter-day precision were noticed with RSD values lower than 3.79 and 4.11%, respectively, while mean recoveries were between 97.7 and 100.7% for the selected levels.

Table 3. The precision and accuracy data in spiked avocado oil sample (n=3).

Tocopherol	Sample blank (μg/mL)	Added (μg/mL)	Found (μg/mL)	Recovery (%)	Mean Recovery (%)	RSD(%)	
						Intraday	Interday
δ-T	0.1377 ±0.0028	0.50	0.6276±0.0107	98.0	97.7	0.82	2.19
		1.00	1.1105 ±0.0300	97.3		3.79	3.09
		2.00	2.0961 ±0.0379	97.9		0.91	1.94!
(β+γ)-T	0.0990 ±0.0026	0.50	0.5871±0.0205	97.6	97.6	3.09	4.11
		1.00	1.0861±0.0160	98.7		1.01	1.62
		2.00	2.0299±0.0270	96.5		1.40	1.40
α-T	nd!	0.50	0.5009±0.0182	100.2	100.7	3.55	3.63
		1.00	1.0460±0.0280	104.6		3.65	2.77
		2.00	1.9438±0.0265	97.2		1.02	1.36

Note: nd: not detected

3.3 Quantification of tocopherols in cold-pressed vegetable oils

The optimized method was applied to the determination of tocopherols in thirteen cold-pressed vegetable oils extracted from the mini screw press model T3. There were no interferences for the separation of tocopherol in the sample. Peak identification was performed by comparing the retention times with those of the standards. Representative chromatograms were obtained from the sample were illustrated in Fig. 4 (B-D). Sacha inchi oil, moringa oil and chili pepper oil chromatograms were selected as an example. Table 4 shows that the content of individual and total

tocopherols in thirteen cold-pressed vegetable oils are significant (p<0.05). The total tocopherols content ranges from 3.62 mg/kg in Japanese pumpkin oil to 1890.71 mg/kg in sacha inchi oil. No tocopherols were detected in coconut oil. According to the previous publication, the coconut oils' α-T and δ-T were not detected, and (β+γ)-T was valued below at 0.04 mg/kg [39].

Table 4. Tocopherol content for thirteen cold-pressed vegetable oils (mg/kg).

Oils	δ-T	(β+γ)-T	α-T	Total tocopherol
Sacha inchi (*Plukenetia volubilis*)	781.50±2.35^{a}	1109.21±5.92^{a}	nd	1890.71±7.93^{a}
Chaulmoogra (*Hydnocarpus anthelminthicus*)	100.41±2.02^{b}	48.53±2.00^{f}	nd	148.94±3.91^{e}
Chilli Pepper (*Capsicum annuum*)	33.97±1.41^{c}	54.06±1.91^{e}	46.77±4.95^{c}	134.80±7.35^{f}
Hemp (*Cannabis sativa*)	25.21±2.01^{d}	487.50±2.83^{b}	nd	512.71±4.78^{b}
Almond (*Prunus dulcis*)	24.59±0.01^{d}	39.17±0.05^{g}	197.74±8.08^{a}	261.49±7.98^{d}
Avocado (*Persea americana*)	12.60±0.26^{e}	12.63±0.67^{i}	nd	25.23±0.79^{j}
Neem (*Azadirachta indica*)	6.76±0.65^{f}	27.73±1.78^{h}	29.75±2.62^{d}	64.25±5.03^{h}
Moringa (*Moringa oleifera*)	5.42±0.21^{fg}	39.45±0.83^{g}	67.15±1.58^{b}	112.03±2.47^{g}
Flaxseed (*Linum usitatissimum*)	4.14±0.34^{g}	243.79±0.87^{d}	21.46±0.74^{e}	269.38±1.22^{d}
Sesame (*Sesamum indicum*)	nd	456.81±8.47^{c}	nd	456.81±8.47^{c}
Black cumin (*Nigella sativa*)	nd	40.78±0.61^{g}	nd	40.78±0.61^{i}
Japanese Pumpkin (*Cucurbita moschata*)	nd	3.62±0.09^{j}	nd	3.62±0.09^{k}
Coconut (*Cocos nucifera*)	nd	nd	nd	nd

Note: Value are express as means ± SD (n=3) in mg/Kg. Value in the same column with the same superscript letter are not significantly different (Duncan, p> 0.05). ND: not detected.

The mean δ-T of cold-pressed vegetable oils in descending order was sacha inchi > chaulmoogra > chilli pepper > hemp ≥ almond > avocado > neem ≥ moringa ≥ flaxseed and was not detected in sesame, black cumin, Japanese pumpkin, and coconut. The mean (β+γ)-T in descending order was sacha inchi > hemp > sesame > flaxseed > chilli pepper > chaulmoogra > black cumin ≥ moringa ≥ almond > neem > avocado > Japanese pumpkin. The results of mean α-T were found in 5 cold-pressed vegetable oils and the amounts in descending order were almond > chilli pepper > moringa > neem > flaxseed. In this study, the highest δ-T and (β+γ)-T levels were found in sacha inchi as 781.50 and 1109.21 mg/kg, respectively. In contrast, α-T was not detected in sacha inchi similarly in previous literature [40]. It was found that the amount of α-T in almond oil was the highest, equal to 197.74 mg/kg. However, comparing the quantity tocopherols in each vegetable oil with previous research differences would depend on the raw materials' countries of origin, different cultivars, and geographical and growing conditions [41].

4. Conclusion

Tocopherols are important phytochemical compounds with antioxidant activity and potential benefits for human health. This study presents a simple, fast, accurate, and precise method for the determination of tocopherols in thirteen cold-pressed vegetable oil extracted with mini screw press model T3 using RP-HPLC with fluorescence detection. The method proposed can be useful for the routine

analysis of δ-T, (β+γ)-T, and α-T in cold-pressed vegetable oils, and the results show that the lowest and highest of the total tocopherols were Japanese pumpkin oil and sacha inchi oil, respectively.

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