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Simultaneous high-performance liquid chromatography determination of three active compounds in snake fruit peel extracts

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ABSTRACT

A high-performance liquid chromatography method was developed to simultaneously quantitatively study neochlorogenic acid (Neo), chlorogenic acid (CGA), and procyanidin B4 (B4), which are the bioactive components of snake fruit (*Salacca zalacca* [Gaertn.] Voss). Snake fruit is an edible plant in the Arecaceae family with oval fruits resembling snake scales. They are frequently grown in Southeast Asia. To achieve separation, the gradient elution mobile phases used acetonitrile and 0.1% trifluoroacetic acid in water on a reversed-phase (C18) analytical column. The detecting wavelength was changed to 279 nm. Accuracy, precision, linearity, and limits of quantification and detection (LOQs and LODs) were all tested following the International Conference on Harmonization (ICH) requirements. Neo, CGA, and B4 were eluted in the proper order under ideal circumstances, and were subjected to chromatogram analysis for 20 min. All bioactive components had linear calibration curves with 0.59–300 µg/mL concentrations. LODs were 0.02–0.13 µg/mL, while LOQs were 0.10–0.44 µg/mL. These three examined chemicals had 83.06%–106.38% recoveries. The precision was <2%, which was within acceptable range. Furthermore, the validated method was performed simply, quickly, and effectively to monitor mixtures of these compounds from 30 extraction processes.

Keywords: chlorogenic acid; procyanidin; *salacca*; salak; validation

1. INTRODUCTION

Salacca zalacca (Gaertn.) Voss is a member of the Arecaceae family, which is the family of palm trees. *S. zalacca* is commonly referred to as a snake fruit (SF) globally because its skin resembles that of a snake. The species is referred to as salak in Malaysia and Indonesia, while it is referred to as sala in Thailand. This fruit may be eaten fresh, as well as processed into products, such as jams, pickles, sweets, fruit salads, fruit juice, and wines. These tropical fruits are not only enjoyed in Asian countries; SF consumption has become more common in both North America and Europe (Mazumdar et al., 2019; Supapvanich et al., 2011).

Numerous investigations have been conducted on the phytochemicals in SF, particularly its caffeic acid derivatives and procyanidins (Hlásná Čepková et al., 2021; Kanlayavattanakul et al., 2013). Both chlorogenic acid (CGA) and neochlorogenic acid (Neo) are esters of caffeic and quinic acids, which are two compounds possessing powerful anti-inflammatory and antioxidant activities

(Naveed et al., 2018; Vongsak et al., 2018). Additionally, CGA affects lipid and glucose metabolism in genetically inherited metabolic diseases and has hepatoprotective effects from lipopolysaccharide-induced injury or chemical damage in animals (Hao et al., 2015; Shi et al., 2013; Tsai et al., 2018). Neo contains a wide variety of biological activities, including antibacterial and antioxidative properties, as well as glucose modulation and lipid metabolism in vivo in both healthy individuals and those with hereditary metabolic illnesses (Bajko et al., 2016; Huang et al., 2014; Yu et al., 2021). Procyanidins are polymers that are generated from flavan-3-ol monomers. They have powerful chemical and biological capabilities, including ultraviolet light absorption and bacterial growth inhibition. They also serve as antioxidants (Cruz et al., 2015). Procyanidin B4 (B4) decreases the reactive oxygen species and malondialdehyde content; increases the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase activity; upregulates quinone oxidoreductase 1 and heme oxygenase-1 expression; inhibits cancer cell proliferation; and induces apoptosis via the up-and down-regulation of various genes (Chen et al., 2022; Li et al., 2007).

Markers and analytical methodologies that can effectively track the chemical composition of botanical source materials should be developed concerning the biological activities linked with SF and its constituents. These tools serve as a valuable aid in optimizing the extraction process. The chemical composition of herbal raw materials can be monitored using markers and techniques. Literature reports a high-performance liquid chromatography (HPLC) method to determine Neo and CGA in plant extracts such as *Coffea arabica*, *Pluchea indica*, and *Moringa oleifera* (Kongkiatpaiboon et al., 2018; Oteef, 2022; Vongsak et al., 2014). Green tea, cocoa, and cranberry extracts have also been quantitatively analyzed with HPLC for procyanidins (Gao et al., 2018; Sultana et al., 2008; Toro-Uribe et al., 2020). However, previous HPLC methods are disadvantageous due to low resolution, lengthy analysis times, and complex sample preparation methods. To the best of our knowledge, a technique for concurrently detecting Neo, CGA, and B4 has yet to be described. Thus, this study aims to establish and validate an HPLC methodology to simultaneously determine Neo, CGA, and B4 in diverse SF extracts, following the International Conference on Harmonization (ICH) guidelines (ICH guideline, 2005).

2. MATERIALS AND METHODS

2.1 Materials

Labscan Asia Co., Ltd. in Thailand provided HPLC-grade acetonitrile and ethanol. Chengdu Biopurify Phytochemicals Ltd., with its headquarters in Sichuan, China, provided Neo, CGA, and B4. The purity of the standards was better than 98% according to HPLC measurements. The United Kingdom's Fisher Scientific provided the trifluoroacetic acid. Thailand's Myskinrecipe provided polysorbate 20, polysorbate 80, and PEG-40 hydrogenated castor oil. The remainder of the chemical reagents used were all of analytical purity.

2.2 Plant extracts and preparations

The SFs were procured from Chanthaburi province, Thailand, in 2021. The voucher specimen was deposited at the Faculty of Pharmaceutical Sciences, Burapha University, Chonburi, Thailand. SF skin of SF was subjected to manual separation, cleaning, and drying in a hot air oven at 50 °C. The resulting powder was stored in a cool dry place until commencement of the extraction process.

For ultrasound-assisted extraction, SF peel of 500 mg was individually sonicated in 10 mL of various solvents (1% ethanol, 10% ethanol, 50% ethanol, 99% ethanol, 1% polysorbate 20, 10% polysorbate 80, 1% PEG-40 hydrogenated castor oil, and 10% PEG-40 hydrogenated castor oil) at 30 °C, 50 °C, and 70 °C for 30 min. Each extraction was conducted in triplicate, put through a 0.22-micron pore nylon membrane filter, and then stored at −20 °C in a brown glass container for subsequent HPLC analysis.

Neo, CGA, and B4 were carefully weighed and then diluted with methanol in a volumetric flask to create their standard stock solution. Working standard solutions for these three compounds were created by diluting stock solutions with methanol into 11 different concentrations ranging from 0.29 to 300 µg/mL to create a calibration curve.

2.3 Chromatographic circumstances

A Shimadzu (Kyoto, Japan) HPLC system with a thermostated column compartment (CTO-40C), diode array detector (SPD-M40), and autosampler (SIL-40C) was utilized. Data were acquired using the software from Shimadzu Lab Solutions. Chromatographic separation was accomplished using a C18 guard column and a Luna® C18 column (250 × 4.6 mm, i.d., 5 µm; Phenomenex, California, USA). The mobile phases used gradient elution of 15% B for 10 min, 48%–50% B for 5 min, and 15% B for 5 min. Mobile phases were (A) 0.1% trifluoroacetic acid in water and (B) 0.1% trifluoroacetic acid in water mixed with acetonitrile (6:4). The injection volume was 10 µL, the flow rate was 1.0 mL/min, and the detection wavelength was 279 nm at 35 °C.

2.4 Validation

Accuracy, linearity, precision, the limit of detection (LOD), and the limit of quantitation (LOQ) were evaluated following the ICH Harmonized Tripartite Guideline recommendations (ICH guideline, 2005).

2.4.1 Accuracy

The accuracy of the analytical method was assessed over its specified range using a recovery study and was measured using spike recovery experiments. The determined sample extract was spiked with three different Neo, CGA, and B4 concentrations (100, 200, and 300 µg/mL). The obtained recoveries were calculated by performing a subtraction operation between the values derived from the control (unspiked matrix) and the spiked standard samples, followed by a division operation with the quantities added. Recovery was determined in triplicate for each concentration, and recovery (%) was calculated as Equation 1.

$$\% \text{ recovery} = \frac{\text{Amount found} - \text{Original amount}}{\text{Amount spiked}} \times 100 \quad (1)$$

2.4.2 Precision

Several intra- and inter-day administrations of standard solutions were evaluated to determine the accuracy of the method. The suggested approach was used to analyze seven sample injections (300 µg/mL) within a single day to evaluate intra-day precision and three consecutive days to determine inter-day precision. The precision of this procedure was measured using the relative standard deviation (%RSD).

2.4.3 Linearity

Each chemical underwent a linear relationship study at concentrations between 0.29–300 µg/mL. Three identical injections of each analyte were made at 11 different known concentrations. Calibration curves were created using least-squares regression with the peak area acting as the dependent variable and the number of standards acting as the independent variable.

2.4.4 LOQ and LOD

The signal-to-noise ratio was developed to establish the minimum concentration at which an analyte can be reliably identified under the suggested chromatographic conditions by comparing the signals obtained from samples containing known low analyte concentrations to those obtained from blank samples. This made the signal-to-noise ratio calculation possible. The LOD concentration of an analyte was considered with a signal-to-noise ratio of 3:1, whereas that of the LOQ concentration was 10:1 by injecting a series of dilute solutions with known concentrations based on visual evaluation according to ICH guidelines.

3. RESULTS AND DISCUSSION

Markers are chemically defined by European standards as parts or groups of parts that help manufacture herbal medicines and herbal medicinal products. These parts are referred to as active markers when they support therapeutic activity (European Medicines Agency, 2011). Neo, CGA, and B4 (Figure 1) might be regarded as active markers based on earlier studies using an SF extract (Hlásná epková et al., 2021; Kanlayavattanakul et al., 2013). A gradient approach was created to assess all components in one step, thereby separating these active molecules. The separation was unsuccessful despite attempting various mobile phase combinations, including methanol and water, methanol and water with 0.5% formic acid, and methanol with 1.0% acetic acid. However, the mobile phase system of acetonitrile and water (0.1% trifluoroacetic acid) demonstrated more effective separation performance when acetonitrile was utilized instead of methanol. The best peak resolution for Neo, CGA, and B4 was obtained using lines A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in water mixed with acetonitrile in a 6:4 ratio). Hence, the ideal mobile phase system with acetonitrile and water (0.1% trifluoroacetic acid) was investigated.

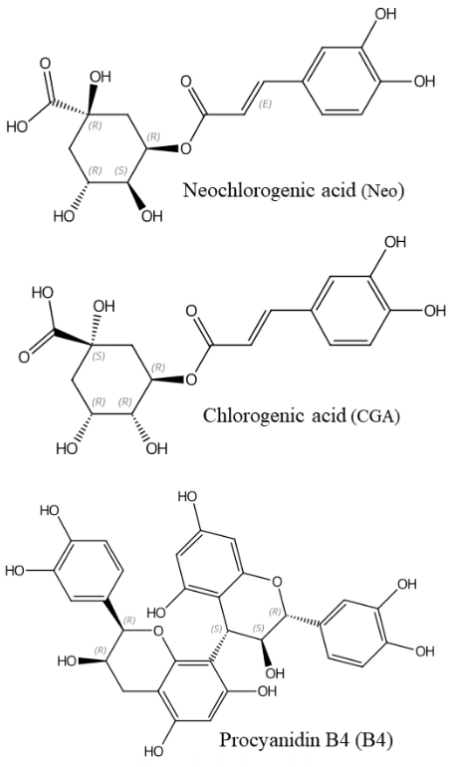


Figure 1. The chemical structures of neochlorogenic acid (Neo), chlorogenic acid (CGA), and procyanidin B4 (B4)

The recommended HPLC validation parameters were sufficient, as shown in Table 1. The calibration curves for Neo, CGA, and B4 were linear at concentrations of 0.29–300, 0.29–300, and 0.59–300 µg/mL, respectively. The HPLC correlation coefficient was >0.9995, indicating a reasonably linear connection. The intra- and inter-day RSD values were <2%. Neo, CGA, and B4 had LODs of 0.05, 0.03, and 0.13 µg/mL, and LOQs of 0.15, 0.10, and 0.39 µg/mL, respectively, for each standard. The developed HPLC method was accurate using the standard addition

technique. The resulting extracts and average recoveries were 99.26 ± 7.66%, 84.91 ± 2.09%, and 97.86 ± 9.61% when Neo, CGA, and B4 were added directly to the aliquots of the examined extract, respectively. Table 2 shows that the chromatographic characteristics of these chemicals, such as capacity factor, resolution, and peak asymmetry, satisfied the requirements for method appropriateness. The resolution and capacity factor values for each peak pair were >1.2. Neo, CGA, and B4 had tailing factors of 1.10, 1.35, and 1.72 and theoretical plate numbers of 18032.67, 26919.88, and 34048.55, respectively. A tailing factor of <2 and >2000 theoretical plates are ideal system suitability characteristics. Hence, the analytical approach demonstrated a respectable level of precision and accuracy, as determined by the validation parameters (Shabir, 2003).

Table 1. The validation parameters of the proposed HPLC method

Parameter	Neochlorogenic acid	Chlorogenic acid	Procyanidin B4
Regression equation	Y =14071X-2181	Y=15005X+3366.8	Y = 4971.2X-1895.8
Correlation coefficient (r^2)	0.9998	0.9998	0.9998
Linear range (µg/mL)	0.29–300	0.29–300	0.59300
Average recovery (%)	99.26 ± 7.66	84.91 ± 2.09	97.86 ± 9.61
Intraday % RSD	0.17	0.10	0.90
Interday % RSD	0.82	0.59	1.47
LOD (µg/mL)	0.05	0.03	0.13
LOQ (µg/mL)	0.15	0.10	0.39

Note: X is the concentration of the compounds; Y is the peak area at 279 nm

Table 2. System-suitability report of neochlorogenic acid, chlorogenic acid, and procyanidin B4

Compound (n = 9)	*k'*	*Rs*	*T*	*N*
Neochlorogenic acid	1.29	4.73	1.10	18032.67
Chlorogenic acid	1.84	2.27	1.35	26919.88
Procyanidin B4	1.98	2.18	1.72	34048.55

Note: *n* is the number of determinations; *k'* is the capacity factor; *Rs* is the USP resolution; *T* is the USP tailing factor; *and N* is the number of theoretical plates

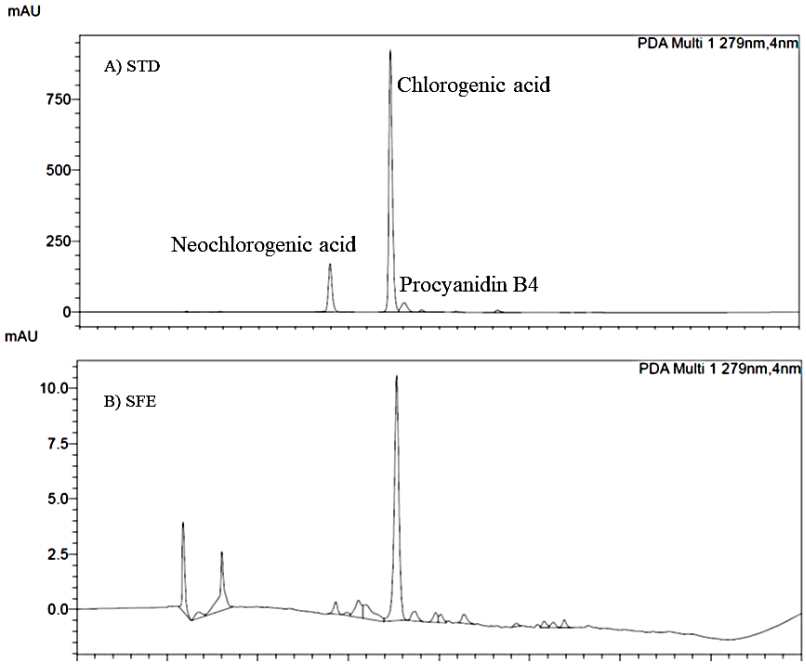
HPLC is the method of choice for qualitative and quantitative analyses of botanical components in pharmaceutical and cosmetic products. This technique effectively accomplished the simultaneous measurement of Neo, CGA, and B4 in SF peel extracts using environmentally friendly extraction solvents such as PEG, polysorbate, and ethanol. Several pharmaceutical excipients or co-solvents can be directly added to formulations as an alternative to traditional solvents for bioactive extraction. Surfactant-mediated extraction is a more ecologically friendly method of extraction since it requires less energy, less solvent, and less time to complete (Chemat et al., 2017; Petchsomrit et al., 2022). Several studies (Kongkiatpaiboon et al., 2018; Li and Jiang, 2007; Petchsomrit et al., 2022; Vongsak et al., 2020) reported that several medicinal plants, including *Litchi chinensis, Pluchea indica*, and *Maclura cochinchinensis*, have active chemicals that are influenced by the extraction process. The extraction of *Echinacea purpurea* using ultrasound-assisted glycerol-water mixtures revealed 70 °C as the ideal temperature for extracting phenolic mixtures, such as caftaric and cichoric acids (Momchev et al., 2020). In addition, a higher temperature (70 °C) may produce a higher concentration of these active compounds. The lowest amounts of these chemicals were found in the ethanol extract, containing 99% ethanol, which was significantly different from the concentrations of 1%, 10%, and 50% ethanol, 1% and 10% polysorbate 20, polysorbate 80, and PEG-40 hydrogenated castor oil. Figure 2 shows the HPLC chromatograms of SF peel extracts and reference standards from different solvents. CGA could be viewed as the most significant bioactive component in all extracts. The CGA concentrations of polysorbate 20, polysorbate 80, PEG-40 hydrogenated castor oil, and ethanol were not substantially different at 70 °C, but they were for 1% polysorbate 80 and 99% ethanol. Additionally, previous HPLC techniques for SF extract analysis have exhibited prolonged analysis durations of approximately 60 min and poor resolution (Kanlayavattanakul et al., 2013). Our study revealed the validated HPLC method to be a rapid and efficient means of analyzing Neo, CGA, and B4 in various extracts.

Table 3. The contents of neochlorogenic acid, chlorogenic acid, and procyanidin B4 in snake fruit peel extracts from different extraction methods

Extract	Content of major compounds (µg/mL)		
	Neo	CGA	B4
At 30 °C			
1% Ethanol	0.43 ± 0.01	3.99 ± 0.37	0.80 ± 0.03
10% Ethanol	0.41 ± 0.01	5.17 ± 0.24	0.94 ± 0.07
50% Ethanol	0.45 ± 0.05	5.51 ± 0.10	1.24 ± 0.01
99% Ethanol	0	0.57 ± 0.01	0.63 ± 0.01

Table 3. (Continued)

Extract	Content of major compounds (µg/mL)		
	Neo	CGA	B4
At 30 °C			
1% Polysorbate 20	0.34 ± 0.01	3.11 ± 0.05	0.68 ± 0.03
10% Polysorbate 20	0.32 ± 0.01	3.33 ± 0.35	0.87 ± 0.06
1% Polysorbate 80	0.33 ± 0.02	2.96 ± 0.27	0.70 ± 0.01
10% Polysorbate 80	0.31 ± 0.01	3.03 ± 0.14	0.88 ± 0.04
1% PEG-40 hydrogenated castor oil	0.34 ± 0.01	3.09 ± 0.15	0.71 ± 0.01
10% PEG-40 hydrogenated castor oil	0.30 ± 0.01	2.77 ± 0.21	0.83 ± 0.04
At 50 °C			
1% Ethanol	0.47 ± 0.03	5.57 ± 0.49	1.13 ± 0.21
10% Ethanol	0.43 ± 0.01	5.09 ± 0.16	1.06 ± 0.00
50% Ethanol	0.47 ± 0.02	5.98 ± 0.34	1.35 ± 0.03
99% Ethanol	0	1.13 ± 0.04	0.78 ± 0.02
1% Polysorbate 20	0.40 ± 0.01	4.35 ± 0.24	1.02 ± 0.03
10% Polysorbate 20	0.40 ± 0.02	4.80 ± 0.31	1.26 ± 0.03
1% Polysorbate 80	0.41 ± 0.03	4.42 ± 0.40	0.93 ± 0.01
10% Polysorbate 80	0.42 ± 0.02	5.02 ± 0.66	1.27 ± 0.07
1% PEG-40 hydrogenated castor oil	0.40 ± 0.03	4.11 ± 0.39	0.89 ± 0.01
10% PEG-40 hydrogenated castor oil	0.37 ± 0.01	3.58 ± 0.77	1.07 ± 0.14
At 70 °C			
1% Ethanol	0.48 ± 0.00	5.63 ± 0.06	1.14 ± 0.03
10% Ethanol	0.48 ± 0.03	5.80 ± 0.34	1.28 ± 0.10
50% Ethanol	0.49 ± 0.01	6.61 ± 0.10	1.46 ± 0.02
99% Ethanol	0	1.76 ± 0.22	0.85 ± 0.02
1% Polysorbate 20	0.48 ± 0.02	5.60 ± 0.22	1.12 ± 0.00
10% Polysorbate 20	0.46 ± 0.01	5.62 ± 0.71	1.43 ± 0.14
1% Polysorbate 80	0.44 ± 0.02	5.18 ± 0.40	0.97 ± 0.04
10% Polysorbate 80	0.45 ± 0.02	5.71 ± 0.36	1.48 ± 0.06
1% PEG-40 hydrogenated castor oil	0.47 ± 0.02	5.58 ± 0.42	1.01 ± 0.05
10% PEG-40 hydrogenated castor oil	0.45 ± 0.01	5.69 ± 0.15	1.47 ± 0.00



Note: Expressed as mean ± SD (n = 3); (Neo) neochlorogenic acid, (CGA) chlorogenic acid, and (B4) procyanidin B4

Figure 2. HPLC chromatogram of (A) neochlorogenic acid, chlorogenic acid, and procyanidin B4 (STD); and (B) snake fruit peel extract from ethanol (SFE); (C) snake fruit peel extract from polysorbate 20 (SFP20); (D) snake fruit peel extract from polysorbate 80 (SFP80); and E) snake fruit peel extract from PEG-40 hydrogenated castor oil (SFPEG)

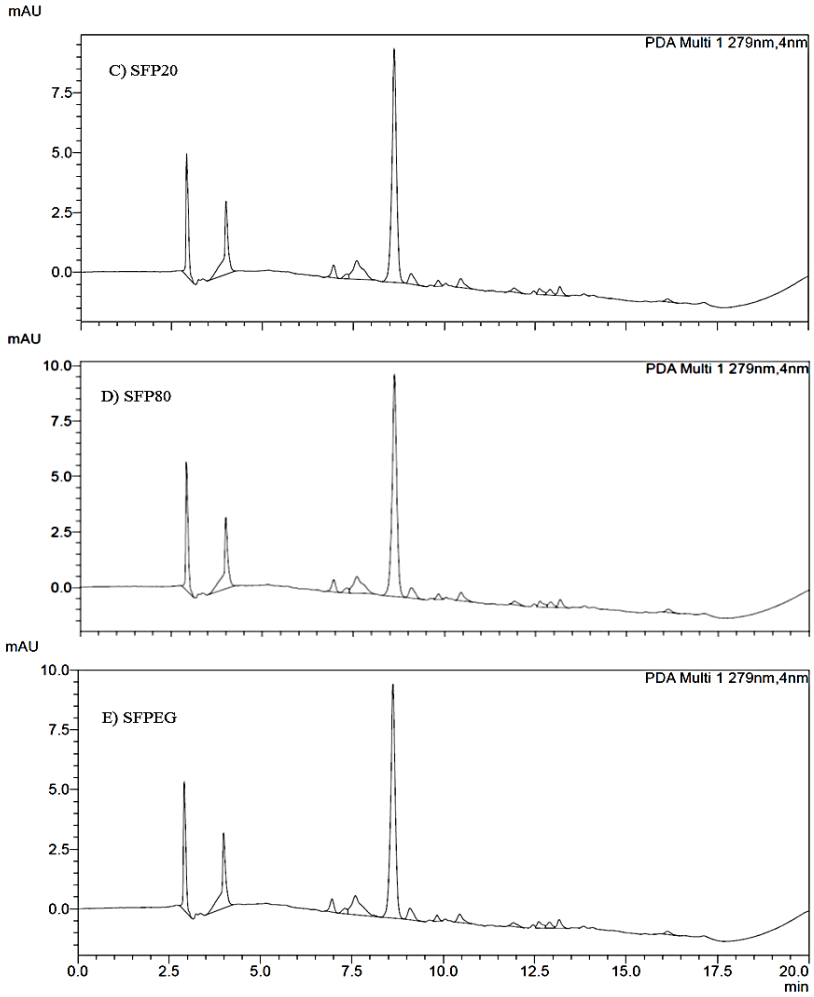


Figure 2. (Continued)

4. CONCLUSION

This quick and straightforward HPLC approach successfully analyzed the amounts of Neo, CGA, and B4 in SF peel extracts. The established approach was verified based on linearity, accuracy, precision, LOQ, and LOD. Additionally, the validated HPLC method perfectly quantified Neo, CGA, and B4 in SF peel extracts produced from various extraction techniques. For optimum quantification, ultrasound-assisted extraction should be used with 50% ethanol at 70 °C for 30 min.

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