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Rapid and simultaneous determination of antioxidant markers and caffeine in commercial teas and dietary supplements by HPLC-DAD

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ABSTRACT

A simple and fast reverse-phase high-performance liquid chromatography procedure coupled with photodiode array detector (RP-HPLC-DAD) was developed and validated for the analysis of major catechins, proanthocyanidin (procyanidin B2) and caffeine in 25 different natural complex matrices containing *Camellia sinensis* L. and/or grape seed extracts, two popular plant extracts that have been widely used as natural antioxidants in various food and beverage applications. Using an isocratic elution system, separation of all compounds was achieved within 12 min. Excellent linearity was observed for all of the standard calibration curves, and the correlation coefficients were above 0.9997. Limits of detection for all of the analyzed compounds ranged between 2.80×10^{-3} and 2.51×10^{-2} $\mu g\ mL^{-1}$; limits of quantitation ranged between 9.30×10^{-3} and 8.36×10^{-2} $\mu g\ mL^{-1}$. The developed method was found to be accurate and sensitive and is ideally suited for rapid, routine analysis of principal components in these well-known natural antioxidants.

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1. Introduction

Antioxidants have become one of the most studied topics in the world; their broad range of effects in biological systems has drawn the attention of many researchers. The strong interest in antioxidants in the scientific community arose from several reasons, including the increasing knowledge of reactive oxygen and nitrogen species, the definition of predictive markers for oxidative damage and new evidence linking chronic diseases to oxidative stress [1].

Most recently, phenolic antioxidants of natural origin have received increasing attention because synthetic antioxidants have been found to have long-term toxicological effects, including carcinogenicity [2]. Currently, special interest has been paid to teas and grape seed extracts because they are two popular plant extracts that have been widely used in various nutraceuticals, beverages, pharmaceuticals, cosmetics and medicinal applications. It is therefore essential to be able to offer consumers a consistent level of polyphenols in such products [3–5].

Tea (*Camellia sinensis*, family Theaceae) is the most widely consumed plant-based beverage in the world [5,6]. White, green, oolong, black, and red (Pu-erh) teas are the major tea types and are categorized based on variations in harvesting, processing, the associated degree of oxidation of polyphenols in fresh tea leaves and the variety of *Camellia sinensis* L. used in their production [6].

Each type of tea has a different polyphenol composition and concentration. The major tea catechins are (−)-epicatechin (EC), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG); these are considered to be responsible for the majority of the biological activity of tea extracts and are usually used as an indicator of quality [7]. Recently, the health benefits of tea consumption have been published and include protective effects against cancer, diabetes, and cardiovascular diseases [6,7]. Furthermore, tea contains other biologically active compounds such as caffeine, which has many effects on the body's metabolism, including stimulation of the central nervous system and short-term increases in blood pressure [8].

Grape (*Vitis vinifera*, family Vitaceae) is considered the world's most prevalent fruit crop [5,9]. Grape seeds are known to be rich in bioactive polyphenols, especially monomeric catechins and proanthocyanidins [5,10]. Commercial preparations of grape seed extract (GSE) are usually standardized on the basis of procyanidin content [11,12].

GSE is a popular dietary supplement that has been reported to possess a broad spectrum of pharmacological and therapeutic effects, such as anti-aging, anti-inflammatory, anti-carcinogenic, and anti-microbial activities, as well as having cardioprotective, hepatoprotective, and neuroprotective effects [9,13]. Most of these beneficial effects are commonly attributed to its antioxidant contents [14]. Furthermore, the antioxidant activity of GSE is superior to other well-known antioxidants such as vitamin C, vitamin E, and β-carotene [15]. Some clinical data have shown that procyanidin oligomers from grape seeds are 20 times more potent antioxidants than vitamin C and 50 times more potent than vitamin E [16].

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Great interest has been shown in investigating the levels of these bioactive compounds in various sample matrices, thus creating a need for reliable analytical methods. High-performance liquid chromatography (HPLC) is the most widely used tool for the analysis of beneficial compounds in TE or GSE [17–26]. Other reported methods include gas chromatography/mass spectrometry

Table 1
Determination of EGC, CAF, EC, B2 and EGCG content in commercial teas using the proposed HPLC-DAD method.

Tea bag no.	Brand	Mean ± S.D.[a]					
		EGCG	EGC	EC	B2	Total phenolics	CAF
Green teas							
1	Green Tea (Ahmad Tea Ltd., London)						
	Proposed HPLC-DAD method	624.70 ± 1.19	264.21 ± 0.79	121.33 ± 0.59	33.93 ± 0.09	1044.17 ± 0.67	247.27 ± 0.68
	Published method [19,26]	622.85 ± 0.95	265.49 ± 0.88	120.12 ± 0.84	33.88 ± 0.10	1042.34 ± 0.96	246.65 ± 0.54
2	Jasmine Green Tea (Ahmad Tea Ltd., London)						
	Proposed HPLC-DAD method	618.13 ± 1.68	667.13 ± 0.68	95.83 ± 0.73	34.07 ± 0.36	1415.16 ± 0.86	267.85 ± 0.91
	Published method [19,26]	619.24 ± 1.36	668.51 ± 0.71	94.99 ± 0.65	33.65 ± 0.19	1416.39 ± 0.73	268.19 ± 0.76
3	Mint Green Tea (Ahmad Tea Ltd., London)						
	Proposed HPLC-DAD method	557.68 ± 0.98	219.33 ± 0.38	74.06 ± 0.15	20.13 ± 0.05	871.20 ± 0.39	199.60 ± 1.18
	Published method [19,26]	556.79 ± 0.92	220.10 ± 0.49	73.78 ± 0.19	20.23 ± 0.07	870.90 ± 0.42	200.19 ± 1.30
4	Pure Green Tea (Twinings of London, England)						
	Proposed HPLC-DAD method	630.67 ± 1.39	358.63 ± 0.49	88.05 ± 0.05	28.79 ± 0.07	1106.14 ± 0.50	257.45 ± 1.01
	Published method [19,26]	631.11 ± 1.34	357.99 ± 0.52	87.97 ± 0.07	28.67 ± 0.06	1105.74 ± 0.49	258.13 ± 1.11
5	Green Tea and Mint (Twinings of London, England)						
	Proposed HPLC-DAD method	627.17 ± 1.51	310.24 ± 0.47	68.28 ± 0.36	26.04 ± 0.03	1031.73 ± 0.59	217.46 ± 1.03
	Published method [19,26]	626.42 ± 1.37	311.11 ± 0.52	69.13 ± 0.44	26.12 ± 0.05	1032.78 ± 0.60	218.12 ± 1.14
6	Dilmah Natural Green Tea						
	Proposed HPLC-DAD method	627.61 ± 0.43	307.30 ± 1.09	61.90 ± 0.25	24.32 ± 0.07	1021.13 ± 0.46	219.04 ± 0.91
	Published method [19,26]	626.72 ± 0.55	308.01 ± 1.12	62.12 ± 0.30	24.19 ± 0.08	1021.04 ± 0.51	220.11 ± 0.75
7	Green Tea (Lipton Tea Co.)						
	Proposed HPLC-DAD method	598.08 ± 0.79	332.88 ± 1.01	82.59 ± 0.15	37.12 ± 0.05	1050.67 ± 0.49	248.14 ± 1.39
	Published method [19,26]	597.39 ± 0.81	333.75 ± 0.98	82.22 ± 0.22	36.99 ± 0.07	1050.35 ± 0.53	247.75 ± 1.21
8	Green Tea Mint (Lipton Tea Co.)						
	Proposed HPLC-DAD method	575.94 ± 0.67	319.62 ± 1.31	76.72 ± 0.26	21.59 ± 0.03	993.87 ± 0.55	228.83 ± 1.19
	Published method [19,26]	576.21 ± 0.34	320.13 ± 1.40	76.15 ± 0.41	21.63 ± 0.05	994.12 ± 0.57	229.24 ± 1.12
9	Rabea Green Tea (AMS Baeshen & Co., Saudi Arabia)						
	Proposed HPLC-DAD method	310.81 ± 1.63	218.33 ± 0.47	42.87 ± 0.54	19.37 ± 0.26	591.38 ± 0.73	164.93 ± 0.51
	Published method [19,26]	311.62 ± 1.35	219.12 ± 0.35	41.66 ± 0.67	18.91 ± 0.30	591.31 ± 0.67	165.22 ± 0.45
10	Green Tea (Isis Co., Egypt)						
	Proposed HPLC-DAD method	611.21 ± 1.46	317.33 ± 1.39	74.26 ± 0.14	22.25 ± 0.06	1025.05 ± 0.76	212.05 ± 1.09
	Published method [19,26]	610.73 ± 1.35	316.88 ± 1.41	73.95 ± 0.16	22.15 ± 0.05	1023.71 ± 0.74	212.46 ± 1.14
11	Green Tea with Mint (Isis Co., Egypt)						
	Proposed HPLC-DAD method	222.89 ± 1.37	151.89 ± 1.21	28.24 ± 0.09	7.66 ± 0.05	410.68 ± 0.53	97.92 ± 0.39
	Published method [19,26]	223.45 ± 1.41	152.14 ± 1.18	28.05 ± 0.11	7.75 ± 0.07	411.39 ± 0.50	97.33 ± 0.39
12	Life Tea (Isis Co., Egypt)						
	Proposed HPLC-DAD method	481.57 ± 0.71	260.80 ± 0.67	51.09 ± 0.16	16.86 ± 0.03	810.32 ± 0.39	149.66 ± 1.05
	Published method [19,26]	482.48 ± 0.80	260.12 ± 0.59	51.43 ± 0.22	16.92 ± 0.05	810.95 ± 0.42	150.10 ± 1.11
13	Royal Green Tea (Royal for Herbs, Egypt)						
	Proposed HPLC-DAD method	383.79 ± 0.54	220.32 ± 0.33	53.07 ± 0.13	21.01 ± 0.26	678.19 ± 0.32	218.60 ± 0.66
	Published method [19,26]	383.11 ± 0.61	220.78 ± 0.21	52.95 ± 0.15	21.50 ± 0.21	678.34 ± 0.30	219.15 ± 0.50
14	Dr.Life Green Tea (Family Pharmacia Co., Egypt)						
	Proposed HPLC-DAD method	154.84 ± 1.71	128.87 ± 0.54	29.18 ± 0.17	10.23 ± 0.06	323.12 ± 0.62	100.86 ± 1.02
	Published method [19,26]	154.35 ± 1.55	129.21 ± 0.65	29.55 ± 0.22	10.36 ± 0.08	323.47 ± 0-63	100.15 ± 1.10
Black teas							
15	English Breakfast (Ahmad Tea Ltd., London)						
	Proposed HPLC-DAD method	65.29 ± 0.46	52.51 ± 0.54	23.15 ± 0.41	17.33 ± 0.03	158.28 ± 0.36	274.88 ± 0.59
	Published method [19,26]	65.96 ± 0.40	53.19 ± 0.63	23.30 ± 0.46	17.40 ± 0.06	159.85 ± 0.39	275.25 ± 0.63
16	Earl Grey (Ahmad Tea Ltd., London)						
	Proposed HPLC-DAD method [19,26]	63.06 ± 0.05	67.49 ± 0.26	27.35 ± 0.05	21.28 ± 0.04	179.18 ± 0.10	259.16 ± 0.30
	Published method [19,26]	63.15 ± 0.07	66.95 ± 0.45	27.45 ± 0.08	21.35 ± 0.06	178.90 ± 0.17	258.85 ± 0.25
17	Dilmah Premium Ceylon Tea						
	Proposed HPLC-DAD method	112.08 ± 0.54	47.43 ± 0.02	29.07 ± 0.03	17.31 ± 0.06	205.89 ± 0.36	261.77 ± 1.38
	Published method [19,26]	112.55 ± 0.35	47.40 ± 0.04	29.11 ± 0.04	17.40 ± 0.07	206.46 ± 0.13	262.15 ± 1.27
18	Earl Grey (Lipton Tea Co.)						
	Proposed HPLC-DAD method	47.43 ± 0.42	48.03 ± 0.17	19.71 ± 0.15	13.98 ± 0.02	129.15 ± 0.32	266.09 ± 0.12
	Published method [19,26]	48.01 ± 0.55	48.44 ± 0.20	20.10 ± 0.21	13.95 ± 0.04	130.50 ± 0.25	266.45 ± 0.20
19	Forest Fruits Tea (Lipton Tea Co.)						
	Proposed HPLC-DAD method	104.89 ± 0.61	51.45 ± 0.26	29.04 ± 0.03	17.56 ± 0.05	202.94 ± 0.24	233.88 ± 1.06
	Published method [19,26]	105.11 ± 0.70	50.99 ± 0.35	29.10 ± 0.04	17.49 ± 0.06	202.69 ± 0.29	234.15 ± 1.20
20	Carrefour Black tea (Carrefour hypermarkets, Egypt)						
	Proposed HPLC-DAD method	85.55 ± 0.42	44.49 ± 0.16	20.50 ± 0.26	16.96 ± 0.04	167.50 ± 0.55	243.24 ± 1.28
	Published method [19,26]	85.90 ± 0.55	44.75 ± 0.13	21.10 ± 0.41	17.10 ± 0.09	168.85 ± 0.65	244.00 ± 1.35
White tea							
21	Pure white Tea (Twinings of London, England)						
	Proposed HPLC-DAD method	575.05 ± 1.71	236.93 ± 0.63	89.12 ± 0.64	21.27 ± 0.02	922.37 ± 0.75	245.94 ± 1.71
	Published method [19,26]	575.70 ± 1.45	236.15 ± 0.71	90.20 ± 0.75	21.35 ± 0.06	922.37 ± 0.74	246.25 ± 1.35

[a] Mean concentration given in μg per tea bag ± S.D. for three determinations.

(GC/MS) [27], capillary electrophoresis (CE) [28], high-performance thin layer chromatography (HPTLC) [29], spectrophotometry [20] and square-wave voltammetry [23].

From the above-cited literature, it is obvious that the majority of recently published analytical HPLC methods have focused on optimizing the separation of catechins or proanthocyanidins. However, no analytical method has been reported for the simultaneous analysis of EGC, CAF, EC, B2 and EGCG in the same formulation(s). Furthermore, most of the previously published methods are both solvent- and time-consuming; thus, there is an urgent need for a more rapid and less complicated method that would enable quantification of the major components present in teas and other natural complex samples.

The main objectives of this work were to develop and validate a new HPLC method for the simultaneous determination of EGC, CAF, EC, B2 and EGCG in a single run, by comparing the levels of these compounds in 21 tea bags extracted with hot water (under conditions that simulate tea prepared by consumers), and by comparing the levels of these compounds in four dietary supplements extracted with aqueous methanol.

2. Materials and methods

2.1. Chemicals and standards

Authentic standards of (-)-Epicatechin (EC), (-)-Epigallocatechin (EGC) and (-)-Epigallocatechin gallate (EGCG) were purchased from ChromaDex™ (California, USA). Procyanidin B2 (B2) and caffeine (CAF) were purchased from Sigma-Aldrich Chemie GmbH D-89555 (Steinheim, Germany). HPLC-grade acetonitrile and methanol were purchased from Carlo Erba Reactifs-SDS SAS (France), and analytical-grade orthophosphoric acid was purchased from BDH Laboratory Supplies (Poole, England).

2.2. Tea samples and botanical dietary supplements

A total of 21 tea samples (14 green, 1 white, and 6 black teas) were purchased from local markets in Egypt as individual tea bags. Detailed information for the tea samples is shown in Table 1. A total of four dietary supplements were analyzed: Multi-Treat® tablets (Arab Co. Pharmaceuticals & Medicinal Plant MEPACO, Egypt) containing 300 mg of green tea dry extract (Camellia sinensis), GREEN TEA® tablets (EL Obour Modern Pharmaceutical Industries Co., for Technomad Group, Nasr City, Cairo, Egypt), labeled as containing 1000 mg of green tea; Grapexon® capsules (The Arab Co. for Gelatin & Pharmaceutical Products [Arab Caps] for Egy Pharma Co.; Nasr City, Cairo, Egypt) containing 300 mg of green tea extract and 30 mg GSE; and Oxyplex® capsules (Arab Caps for Tiba Pharmaceutical Industries, Nasr City, Cairo, Egypt) containing 100 mg of green tea extract and 200 mg GSE.

2.3. HPLC Instrumentation and conditions

HPLC analysis was carried out using a Hitachi LaChrom Elite Liquid Chromatograph L-2000 equipped with a photodiode array detector (model L-2455, Hitachi La Chrom Elite, Tokyo, Japan), an autosampler (model L-2200, Hitachi LaChrom Elite, Tokyo, Japan), a column oven (model L-2300, Hitachi LaChrom Elite, Tokyo, Japan) and a built-in degasser (model L-2130 pump, Hitachi LaChrom Elite, Tokyo, Japan). The column (250 mm × 4.6 mm i.d.) was made of stainless steel and packed with Inertsil ODS-3v (5 μm particle diameter; GL Sciences; Tokyo, Japan). Data acquisition was performed using EZChrom Elite software (Agilent Technologies). The RP-HPLC-DAD assay was carried out using an isocratic elution system with a flow rate of 1.5 mL min^{-1}. The mobile phase consisted of water containing 0.05% orthophosphoric acid:acetonitrile (85:15 v/v). The DAD acquisition wavelength was set to scan from 200 to 400 nm, and all analyses were performed at ambient temperature (25 °C). Before use, the mobile phase was filtered through 0.45-μm membrane filters (Millipore, Milford, MA, USA) and degassed under vacuum.

2.4. Standard solutions and calibration

Stock standard solutions were prepared by dissolving 10 mg of EGC, CAF, B2, EC or EGCG in 10 mL of mobile phase. For calibration, standard solutions were prepared by diluting the stock standard solutions with mobile phase. Prior to analysis, precautions were taken to ensure the stability of EGC, B2, EC and EGCG, due to their sensitivity to light and heat. Immediately after preparation, all solutions were either transferred to amber colored volumetric flasks, or were covered by aluminum foil and stored in a 4 °C refrigerator. The concentration ranges were 1–500 μg mL^{-1} for CAF, 0.1–20 μg mL^{-1} for B2 and 1–100 μg mL^{-1} for EGC, EC and EGCG. Each standard solution was injected in triplicate (20-μL injection volume) and chromatographed under the previously specified conditions. Peak areas were then plotted against the corresponding concentrations to obtain the calibration graph. Linear relationships were obtained for each compound.

2.5. Sample preparation

2.5.1. For tea bags

Water-based extraction of tea was the preferred extraction method because it mimics commonly used consumer tea

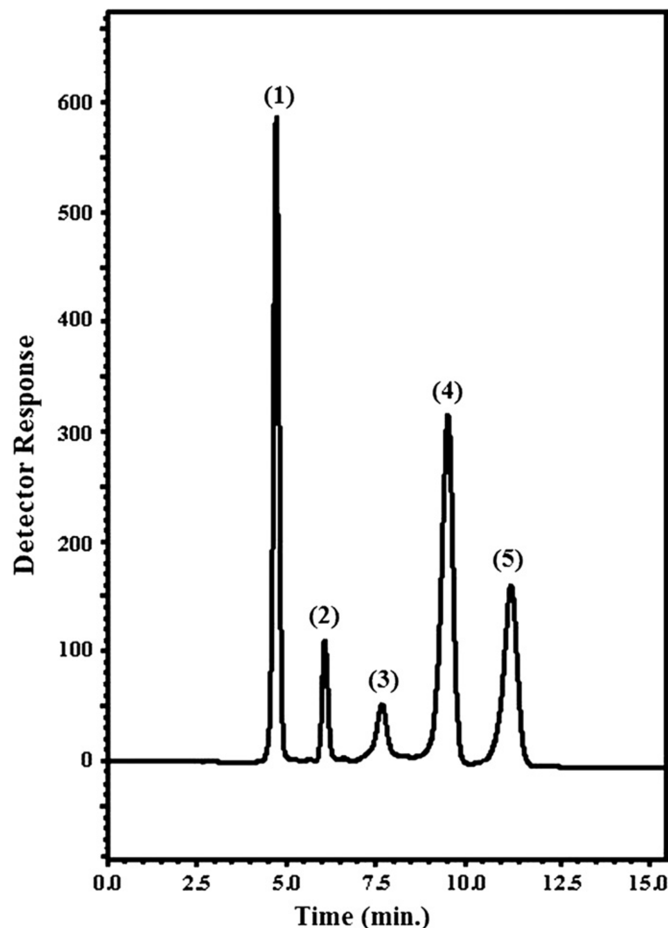

Fig. 1. HPLC chromatogram (20-μL injection volume) of laboratory-prepared mixture of (1) EGC, (2) CAF, (3) B2, (4) EC and (5) EGCG.

preparation. One gram was weighed from each sample and steeped in 25 mL of distilled water at 95 to 100 °C for 5 min. The resulting tea mixtures were filtered using Whatman filter paper, filtered again through 0.45-μm disposable filters and then analyzed directly by HPLC.

2.5.2. For botanical dietary supplements

Alcohol extraction of botanical dietary supplements was performed because it mimics industrial and research conditions [24]. Methanol extracts were obtained using a modification of the previously described procedure [25]. For each phytopharmaceutical preparation, a total of 10 tablets were weighed and made into a fine powder or the contents of 10 capsules were emptied and weighed. An accurately weighed portion of the contents equivalent to one tablet or capsule was extracted with 25 mL of 70% aqueous methanol using a sonicator (WUC-D06H Ultrasonic Sonicator, Daihan Scientific, Korea) for 30 min. The sample solutions were then filtered through 0.45-μm disposable filters. The general procedures for the HPLC method described under calibration (Section 2.4) were followed, and the concentrations of EGC, CAF, EC, B2 and EGCG were calculated.

3. Results and discussion

3.1. Optimization of the HPLC-DAD method

Initial efforts to develop a separation method using a C18 column and an isocratic elution system with a methanol-based mobile phase were unsuccessful; the compounds could not be separated efficiently. Good separation was achieved after screening a series of mobile phases. The chromatographic conditions were studied and optimized as a function of the acetonitrile and orthophosphoric acid composition of the mobile phase. Different quantities of orthophosphoric acid in the mobile phase were tested. The results showed that the most effective amount of orthophosphoric acid in the mobile phase is from 0.04 to 0.1%; these conditions gave optimum resolution, clear baseline separation with reasonable retention time and no tailing of peaks of the studied compounds (Fig. 1). A satisfactory separation was obtained using a mobile phase consisting of water containing 0.05% orthophosphoric acid:acetonitrile (85:15 v/v) at ambient temperature. Increasing the acetonitrile concentration to more than 15% led to inadequate separation and characteristic overlap

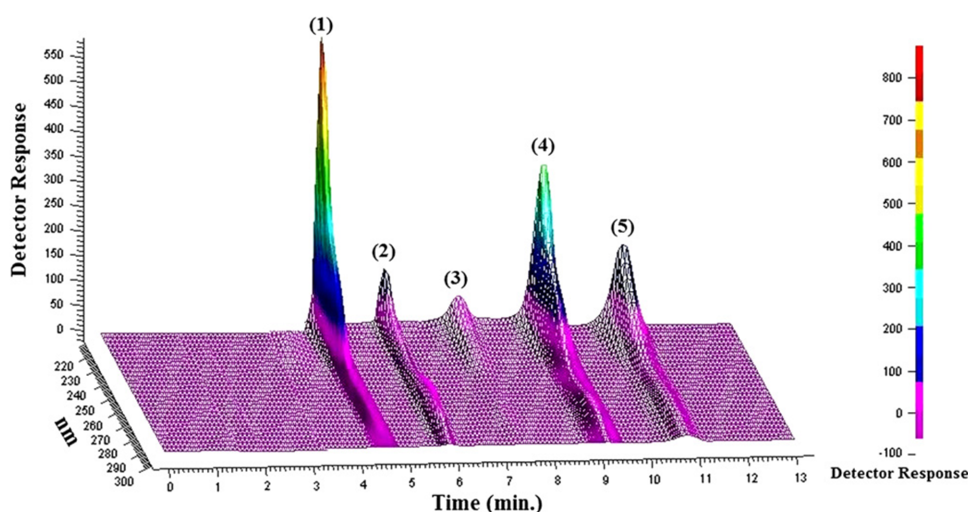

Fig. 2. HPLC-DAD three-dimensional spectra (20-μL injection volume) of laboratory-prepared mixture of (1) EGC, (2) CAF, (3) B2, (4) EC and (5) EGCG.

Table 2
Determination of EGC, CAF, EC, B2 and EGCG content in commercial dietary supplements using the proposed HPLC-DAD method.

Brand	Mean ± S.D.[a]					
	EGCG	EGC	EC	B2	CAF	Total phenolics
Oxyplex® capsules						
Proposed HPLC-DAD method	666.04 ± 1.68	129.78 ± 0.59	106.54 ± 1.16	128.44 ± 0.48	301.44 ± 1.05	1030.80 ± 0.98
Published method [19,26]	667.11 ± 1.45	130.45 ± 0.64	107.16 ± 1.24	127.89 ± 0.39	302.25 ± 1.13	1032.61 ± 0.93
Grapexon® capsules						
Proposed HPLC-DAD method	652.87 ± 0.30	526.72 ± 1.03	368.86 ± 1.03	80.63 ± 0.54	216.54 ± 1.74	1629.08 ± 0.92
Published method [19,26]	653.29 ± 0.45	525.99 ± 1.24	369.22 ± 1.29	81.45 ± 0.69	217.25 ± 1.45	1629.95 ± 0.73
GREEN TEA® tablets						
Proposed HPLC-DAD method	212.16 ± 0.55	38.81 ± 0.06	32.48 ± 0.13	35.46 ± 0.02	124.78 ± 0.59	318.91 ± 0.19
Published method [19,26]	212.85 ± 0.70	39.00 ± 0.11	32.25 ± 0.10	35.45 ± 0.04	125.37 ± 0.76	319.55 ± 0.45
Multi-Treat® tablets						
Proposed HPLC-DAD method	696.71 ± 1.05	345.89 ± 1.31	181.79 ± 1.06	66.00 ± 0.18	309.06 ± 1.28	1290.39 ± 1.08
Published method [19,26]	697.15 ± 1.22	346.45 ± 1.40	182.47 ± 1.23	66.75 ± 0.30	309.45 ± 1.32	1292.82 ± 1.13
Recovery %[b]						
For Product 1	99.88 ± 0.37	99.87 ± 0.59	99.76 ± 0.28	99.95 ± 0.42	99.31 ± 0.12	
For Product 2	100.60 ± 0.18	100.20 ± 0.28	99.81 ± 0.59	100.60 ± 0.61	99.88 ± 0.38	
For Product 3	100.20 ± 0.56	99.76 ± 0.33	99.72 ± 0.50	99.72 ± 0.50	100.25 ± 0.59	
For Product 4	99.42 ± 1.71	100.08 ± 1.03	99.12 ± 1.09	100.08 ± 0.47	99.33 ± 0.18	

[a] Mean concentration given in μg per capsule or tablet ± S.D. for three determinations.
[b] For standard addition of different concentrations of EGC, CAF, EC, B2 and EGCG.

of the studied compounds. At lower acetonitrile concentrations (< 13%), separation was achieved but with excessive tailing and long retention times for the studied compounds.

The three-dimensional UV absorption spectra of the studied compounds (Fig. 2) show that 210 nm is the wavelength of maximum absorbance for the compounds of interest. Orthophosphoric acid also has an absorbance at 210 nm; it was also noted that the baseline of the chromatograms shifted according to the concentration of acetonitrile at shorter wavelengths. Thus, the potential for interference by other chemicals in complex mixtures could not be ignored, and quantitation based on peak area was achieved at 212 nm. The specificity of the HPLC method is illustrated in Fig. 1, showing achievement of complete resolution of the five compounds. The average retention times $\pm$ standard deviations for EGC, CAF, B2, EC and EGCG were found to be 4.58 ± 0.03, 5.82 ± 0.05, 7.45 ± 0.06, 9.21 ± 0.08 and 10.95 ± 0.07 min, respectively, for seven replicates. Characteristic parameters for the regression equations of the method and correlation coefficients were obtained by least squares treatments of the results.

3.2. Analysis of herbal samples

The proposed HPLC-DAD method was applied to the simultaneous determination of EGC, CAF, EC, B2 and EGCG in 25 samples, including 21 commercial tea bags and four dietary supplements, without interference of the other active components/excipients present in the herbal samples. Each sample was analyzed in triplicate. Assays of the samples are shown in Tables 1 and 2 and in Figs. 3 and 4. The results obtained for EGC, CAF, EC and EGCG were statistically compared with those obtained from the reported HPLC method [26], and the results obtained for B2 were statistically compared with those obtained from the reported HPLC method [19]. The results obtained by the proposed method were in agreement with the results of reported HPLC methods [19,26].

3.3. Validation of the HPLC-DAD method

3.3.1. Linearity and range

The linearity of the HPLC method was evaluated by analyzing seven concentrations of CAF, B2, EGC, EC, and EGCG (ranging between 1 and 500 $\mu g\,mL^{-1}$ for CAF, 0.1 and 20 $\mu g\,mL^{-1}$ for B2, and 1 and 100 $\mu g\,mL^{-1}$ for EGC, EC and EGCG). Analysis at each concentration was repeated in triplicate. The assays were performed according to previously established experimental conditions. The calibration graph was constructed by plotting the peak area against the corresponding concentrations of EGC, CAF, EC, B2 and EGCG.

Peak areas and concentrations were subjected to least squares linear regression analysis to calculate the calibration equations and correlation coefficients. The calibration plots for the CAF, B2, EGC, EC, and EGCG assays were linear over the calibration range of 1–500 $\mu g\,mL^{-1}$ for CAF, 0.1–20 $\mu g\,mL^{-1}$ for B2 and 1–100 $\mu g\,mL^{-1}$ for EGC, EC and EGCG. The linearity of the calibration curves was validated by the high value of the correlation coefficients of the regression.

3.3.2. Precision

Each compound was assayed at three concentration levels, as described under the general analytical procedure. Assays were repeated three times within the same day to determine the repeatability (intra-day precision) and three times on three different days to determine the intermediate precision (inter-day precision) of the method. Intra-day repeatability for the three concentration levels ranged between 0.07 and 0.69% RSD and the inter-day precision ranged between 0.21 and 1.01% RSD; these results indicate the high precision of the method.

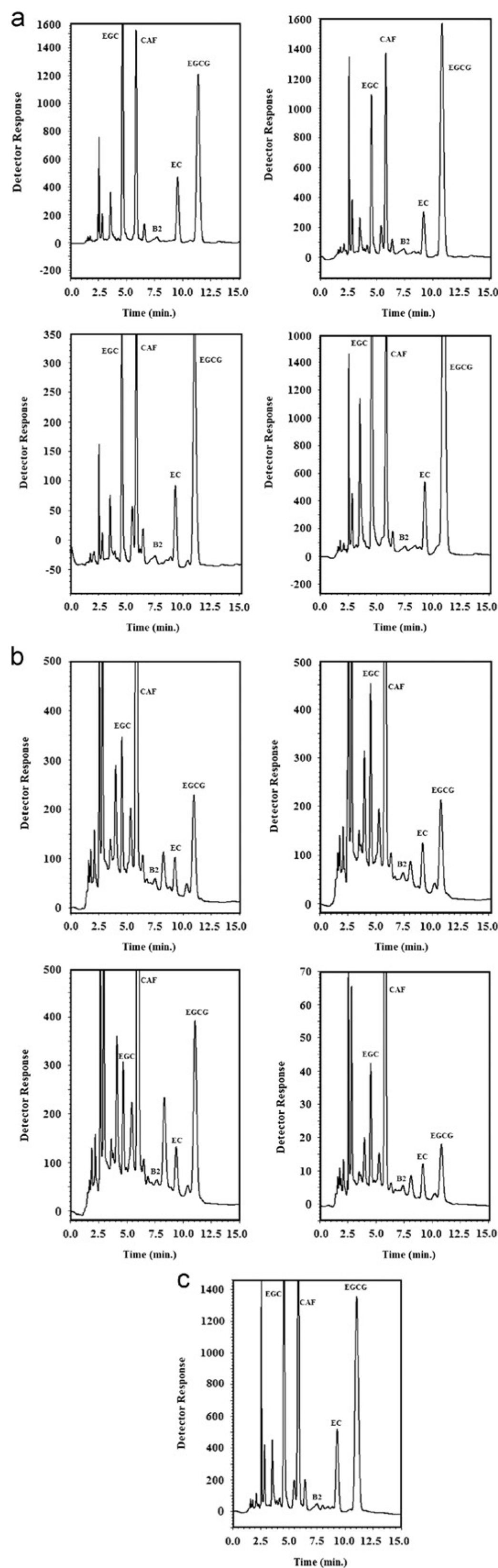

Fig. 3. Typical HPLC chromatograms (20-μL injection volume) of (a) Green tea products, (b) Black tea products and (c) White tea products (commercial teas).

3.3.3. Limits of detection (LOD) and quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the current ICH guidelines as the

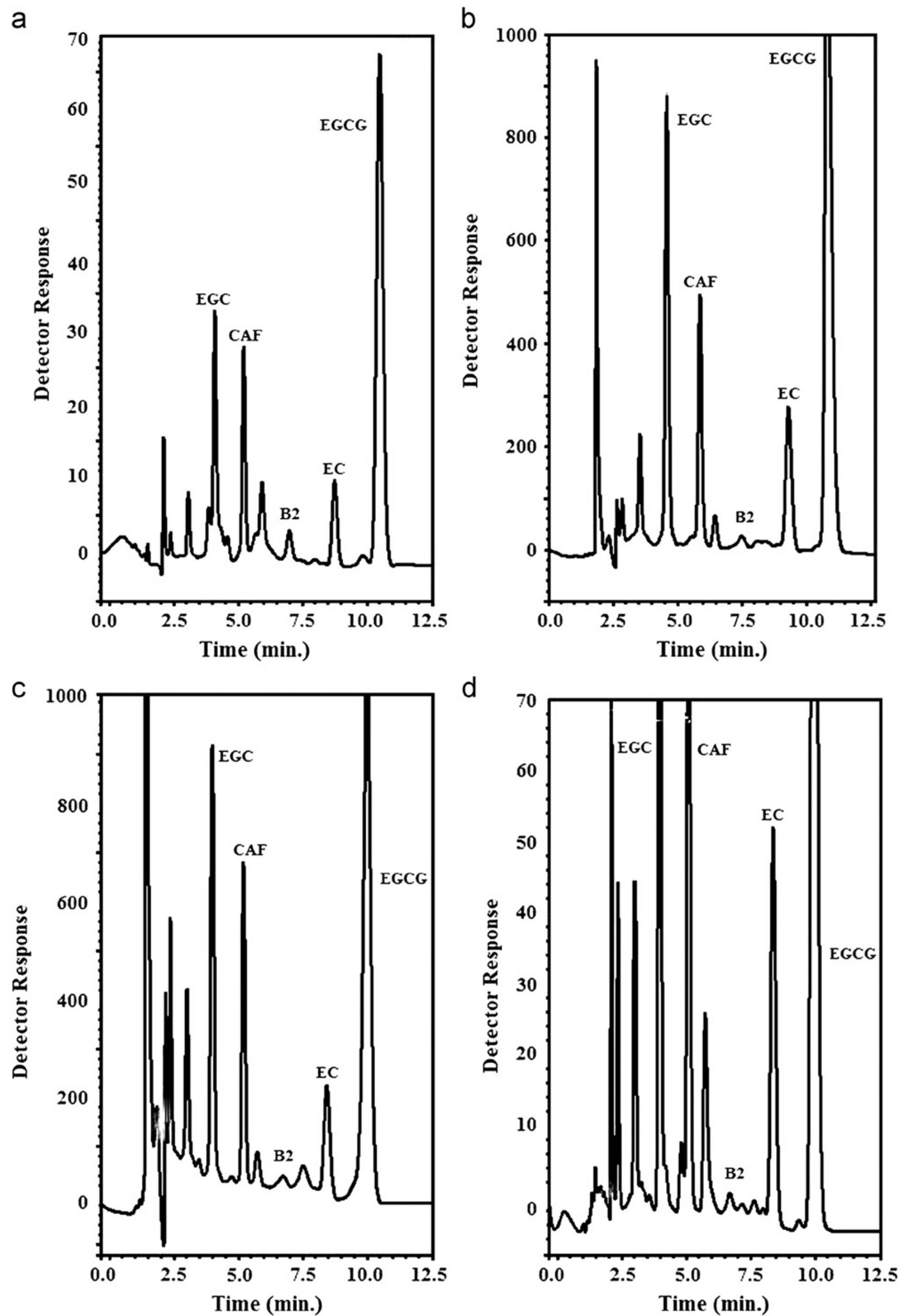

Fig. 4. Typical HPLC chromatograms (20-μL injection volume) of (a) product 1, (b) product 2, (c) product 3 and (d) product 4 (dietary supplements).

ratio of 3.3 and 10 standard deviations of the blank (n=7), respectively, against the slope of the calibration line [30].

The detection limits for EGC, CAF, EC, B2 and EGCG were 6.85×10^{-3}, 2.80×10^{-3}, 5.50×10^{-3}, 2.51×10^{-2} and 6.17×10^{-3} μg mL^{-1}, respectively. The quantitation limits for EGC, CAF, EC, B2 and EGCG were 2.28×10^{-2}, 9.30×10^{-3}, 1.83×10^{-2}, 8.36×10^{-2} and 2.06×10^{-2} μg mL^{-1}, respectively.

3.3.4. Selectivity

Method selectivity was assessed by preparing different mixtures of EGC, CAF, EC, B2 and EGCG at concentrations within the linearity range and analyzing the preparations by the proposed method. Satisfactory results were obtained, indicating the high selectivity of the proposed method for the simultaneous determination of EGC, CAF, EC, B2 and EGCG.

3.3.5. Accuracy

Accuracy is assessed by the proximity of the obtained value to the ‘true’ value and can be reported in terms of recovery by adding known amounts of the studied compounds to a known concentration of the commercial products (standard addition method). The resulting mixtures were assayed, and the mean

percentage recoveries and their standard deviation for three replicates were calculated. According to the results, good accuracy was observed for this method (Table 2).

3.3.6. Analytical solution stability

The stability of standard solutions and of phytopharmaceutical sample solutions during analysis was evaluated by leaving the solutions in tightly capped volumetric flasks protected from light on a laboratory bench or in a refrigerator. The solutions were stable for about a month when the standards of these compounds were prepared with methanol and kept in a freezer at $-20\,^{\circ}C$. Furthermore, the studied compound solutions exhibited no chromatographic changes when stored for two hours at room temperature (25 °C) or for 24 h when stored at 4 °C.

4. Conclusion

The proposed RP-HPLC-DAD method allows for accurate quantitation of EGC, CAF, EC, B2 and EGCG in both their pure form and in commercial formulations, without interference from other constituents/excipients, performed in a single step requiring less than 12 min. The method is isocratic, uses an uncomplicated mobile phase, requires minimal sample preparation, and has rapid assay procedures and reduced solvent consumption. This method is thus ideally suited for routine analyses with short run times of EGC, CAF, EC, B2 and EGCG in crude plant samples and formulations containing *Camellia sinensis* L. and/or grape seed extracts. Furthermore, the developed method can serve as an important reference for the quality control of teas and could help consumers to select tea brands with the highest content of beneficial compounds. Because the nature and amounts of active constituents in teas are prone to variation due to environmental factors and manufacturing conditions, we suggest that labeling teas with the individual active constituent contents would be of benefit to tea consumers, producers of dietary tea supplements, and researchers of information on health-promoting tea compounds, as well as being useful for issues related to dosage and frequency of dosing.

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