

## QUANTIFICATION OF PHENOLIC COMPOUNDS IN DIETARY SUPPLEMENTS AND ANTIOXIDANT ACTIVITIES

Beray Kızılkaya<sup>1</sup>, Reyhan Akpınar<sup>1</sup>, Gizem Yıldırım Baştemur<sup>2</sup> and Sabriye Perçin Özkorucuklu<sup>2\*</sup>

<sup>1</sup>Programme of Molecular Biotechnology and Genetics, Istanbul University, Istanbul, Turkey

<sup>2</sup>Department of Molecular Biology and Genetics, Istanbul University, Istanbul, Turkey

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**ABSTRACT.** Dietary supplements (DS) are products that are recommended for the treatment of various health issues, especially during the global pandemic, and the accuracy of the labeling information of these supplements plays a significant role in human health. In this study, a novel high performance liquid chromatography-diode array detector (HPLC-DAD) method was developed and validated to determine the accuracy of the labeling information of commercial dietary supplements containing phenolic compounds (rutin, quercetin, and resveratrol). The mobile phase, flow rate, and column temperature were optimized. Validation studies were carried out to prove the validity of the developed method. The limit of detection (LOD) and limit of quantification (LOQ) values of rutin, quercetin, and resveratrol were found in the range of 4.83-6.62 ng/mL and 16.09-22.07 ng/mL, respectively. Recoveries% were calculated in the range of 99.52-100.19% and 99.78-100.34% in the intra-day and inter-day analysis, respectively. The IC<sub>50</sub> values of the dietary supplement extracts obtained by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method ranged from 0.08 to 0.76 mg/mL. Total Antioxidant Capacity (TAC) values of DS<sub>1</sub>, DS<sub>2</sub>, DS<sub>3</sub>, DS<sub>4</sub>, DS<sub>5</sub>, measured employing the Cupric Reducing Antioxidant Capacity (CUPRAC) method, were found to be 0.09, 0.03, 0.10, 0.10 and 0.40 mmol trolox per gram of extract, respectively.

**KEY WORDS:** Dietary supplement, HPLC-DAD, Phenolic compound, Antioxidant activity

### INTRODUCTION

Dietary supplements involve a wide range of products containing vitamins, minerals, amino acids, essential fatty acids, various herbs, and bioactive compounds, such as phenolic compounds. These supplements are available in various forms, including capsules, lozenges, tablets, pills, powders, liquid ampoules, and dropper bottles. Over the years, the number of products available in the market and the individuals using dietary supplements has been steadily increasing. For the reliability of products, they need to have accurate and adequate labeling. The information on the label is necessary to ensure that the consumer who buys the dietary supplement uses it consciously, correctly, and reliably. This labeling plays a significant role in consumers health [1].

Dietary supplements encompass various ingredients, including phenolic compounds known for their antioxidant properties. As one of these flavonoids, quercetin is a powerful antioxidant with a 3-hydroxyflavone backbone. Quercetin prevents oxidative stress and plays an important role in cholesterol imbalances, kidney diseases, asthma, diabetes, schizophrenia, Alzheimer's, and cardiovascular diseases. Moreover, recent and pivotal research has indicated its potential application in the treatment of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), underscoring its emerging significance in the field of healthcare [2-5]. Another flavonoid which is rutin (quercetin-3-O-rutinoside) provides cardioprotective advantages by enhancing neurodegenerative processes and regulating cardiac remodeling. Furthermore, it effectively suppresses the production of nitric oxide and reactive oxygen species [6-9]. Resveratrol (trans-3,5,4'-trihydroxy-stilbene) is a polyphenolic compound abundant in various plant sources. It exerts preventive effects against oxidant-antioxidant imbalance, thereby retarding the aging

\*Corresponding authors. E-mail: [sabriyeo@istanbul.edu.tr](mailto:sabriyeo@istanbul.edu.tr)

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process and exhibiting efficacy against aging-related diseases. Notably, Resveratrol has demonstrated a favorable impact on blood pressure during pregnancy and has shown promising outcomes in patients with Type-1 diabetes. Furthermore, its proven benefits extend to enhancing immunity and modulating the inflammatory response [10-14].

Various analytical methods have been utilized for the determination and separation of these phenolic compounds, including gas chromatography–mass spectrometry (GC-MS) [15], capillary electrophoresis (CE) [16] and high-performance liquid chromatography with different detectors (ultraviolet (UV), diode array detector (DAD), fluorescence detector (FD)) [17-22].

In previous studies, it has been reported that various methods such as DPPH and CUPRAC have been used to determine the antioxidant activities of dietary supplements [23-26]. DPPH is a stable radical owing to its aromatic ring structure, readily capable of capturing other radicals. Transitioning from a dark purple-violet hue to colourless-light yellow when the radical is reduced. This reduction is observed at 515-520 nm with UV-Vis spectrometer [27]. Trolox, L-ascorbic acid, quercetin and catechin, which are standard antioxidants, are commonly used in DPPH assays [28-32]. The CUPRAC method, developed by Apak *et al.* [23], allows the determination of antioxidant capacity for various substances, including flavonoids, by utilizing the Cu(II)-neocuproine (Nc) reagent. This method offers advantages such as cost-effectiveness, widespread availability and responsiveness to both hydrophilic and lipophilic antioxidants. The Cu(II)-Nc chelate reaches its maximum absorbance at 450 nm and undergoes reduction to form the Cu(I)-Nc complex. As the reaction progresses, the initially light blue solution turns into a yellow colour. Trolox is typically employed as the standard antioxidant and its molar absorption coefficient is used for calibration purposes [23, 33].

The objective of this work was to analyze the phenolic compounds content of commercial dietary supplements tablets using the HPLC-DAD method and to evaluate the antioxidant activities using the DPPH and CUPRAC methods. The optimum conditions for chromatographic separations of the compounds were determined by examining the percentage of phosphoric acid in the A mobile phase, flow rate, and column temperature. The analytical method was validated with system suitability, linearity, precision, sensitivity and recovery tests. The new method developed was used for the dual determination of phenolic compounds (rutin, quercetin, and resveratrol) in the content of five different commercial dietary supplements.

## EXPERIMENTAL

### *Standards and reagents chemicals*

Methanol, ethanol, thiourea ( $t_0$ ), DPPH (1,1-diphenyl-2-picrylhydrazyl), L-ascorbic and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), copper(II) chloride, neocuproine, ammonium acetate, resveratrol and rutin were purchased from Sigma-Aldrich. Quercetin and orthophosphoric acid were procured from the USP Reference Standard and Merck, respectively. Milli-Q water purification system was used to purify ultrapure water. (Millipore, Bedford, MA, USA). Five different commercial dietary supplements (DS), named as DS<sub>1</sub>, DS<sub>2</sub>, DS<sub>3</sub>, DS<sub>4</sub> and DS<sub>5</sub>, were purchased from local pharmacy.

### *HPLC instruments and conditions*

The liquid chromatographic behavior of phenolic compounds in dietary supplements was examined using a Shimadzu high-performance liquid chromatography (HPLC) system, manufactured by Shimadzu Technologies in Kyoto, Japan. In the HPLC system, pump (LC-10AD VP), autosampler (SIL-20A), column oven (CTO-10AS), degassing unit (DGU-20A) and diode array detector (SPD-M20A). A SUPELCOSIL LC-18 column (25 cm x 4.6 mm, 5  $\mu$ m) was used

in the study with a gradient program. Chromatographic separation of phenolic compounds was made using deionized water with 0.5% (v/v) orthophosphoric acid as mobile phase A and methanol as mobile phase B. The time-volume change of the gradient program was given as: 0-2 min, 40% B; 2-4 min, 40-50% B; 4-10 min, 50-56% B; 10-15 min, 56-40% B. The chromatographic separation was carried out at a flow rate of 1.2 mL/min and a column temperature of 20 °C. The injection time was adjusted to 15 min. The DAD detector was set at 230 nm and the injection volume was determined to be 20 µL.

#### *Preparation of solutions*

For the quantification of the samples, two replicates of each sample were crushed and 0.1 g of sample powder, equivalent to 1 tablet, were weighed. Subsequently, 10 mL of methanol was added to the powder and dissolved using an ultrasonic bath. The solution was then filtered through a 0.45 µm filter. The dilutions were prepared using methanol from the stock solution with a concentration of 10 mg/mL.

For stock solutions, standard compounds (resveratrol, quercetin and rutin) were prepared with methanol at a concentration of 250 µg/mL. In order to increase the accuracy and precision during the analysis, the internal standard method was used. A certain amount of internal standard was added to the sample and calibration standards. The internal standard concentration was always kept constant. In order for the method to be successful, far ( $R_s > 1.5$ ) was preferred in the sample peaks [34]. In the analysis of phenolic compounds, parameters such as separation power, retention time, peak symmetry were examined and catechin was chosen as the internal standard (IS). Catechin were dissolved in the methanol to prepare 10 µg/mL solution. Thiourea compound was chosen as  $t_0$  for determining the capacity factors of phenolic compounds and this compound was prepared in methanol at a concentration of 20 µg/mL. All prepared solutions were filtered through a syringe filter (0.45-µm pour) and properly stored at 4 °C.

#### *In vitro antioxidant assay*

The DPPH method was used for evaluating the antioxidant activities of dietary supplements. The DPPH solution with a concentration of 0.2 mM and Trolox and L-ascorbic acid solution (2.5 mg/mL) which were the controls were dissolved methanol, ethanol, and deionized water, respectively. DPPH solution at +4 °C and the controls at room temperatures were stored in the dark.

The antioxidant activity was assessed using the method described by Akpınar *et al.* [35], 40 µL of each dietary supplement solution, prepared at seven different concentrations, was mixed with 160 µL of 0.2 mM DPPH solution in methanol and the mixture was transferred to a 96-well microplate. As a blank, methanol, ethanol or deionized water was used instead of the dietary supplement. To determine the absorbance caused by the colour of the extracts, 160 µL of methanol was added to 40 µL of the sample. The microplates were incubated on a shaker for 10 min and the absorbance values were then measured at 520 nm.

In the copper(II) Ion Reducing Capacity (CUPRAC) method,  $1.0 \times 10^{-2}$  M  $\text{CuCl}_2$  solution in deionized water,  $7.5 \times 10^{-3}$  M neocuproine solution in ethanol, 1 M ammonium acetate solution in deionized water and 5 mM trolox solution in ethanol were prepared. All except the ammonium acetate solution were stored in the dark.

The method developed by Apak *et al.* [23] was modified: 250 µL of  $1.0 \times 10^{-2}$  M  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  solution, 250 µL of  $7.5 \times 10^{-3}$  M neocuproine solution, 250 µL of 1 M ammonium acetate (pH = 7) buffer solution mixed with 25 µL of extract (1 mg/mL) and 250 µL of deionized water were added. The 24-well microplate was incubated for 1 hour at room temperature. At the end of the period, absorbances were measured against methanol for dietary supplements and ethanol for trolox at 450 nm. At least 3 repeated measurements were taken for each concentration.

## RESULTS AND DISCUSSION

### Optimization of HPLC-DAD method

An optimum separation, the peaks should have sharp and symmetrical peaks [36]. For the dual determination of phenolic compounds in dietary supplement tablets and capsules, a new HPLC-DAD method with high separation efficiency, practical and economical has been developed. In the method, the percentage of phosphoric acid in mobile phase A, flow rate and column temperature were optimized.

In the gradient system, methanol was preferred as mobile phase B and deionized water-phosphoric acid mixture was preferred in mobile phase A. Phosphoric acid in the mobile phase A mixture was selected in five different ratios (0.0, 0.25, 0.5, 0.75, 1.0%) and compared. In the flow rate optimization, the standard mixture was injected at four different flow rates (0.8, 1.0, 1.2, 1.5 mL/min). High column temperature leads to degradation of compounds and disruption of peak symmetry. Therefore, four different column temperatures (20, 25, 30, 35 °C) were studied without increasing the high column temperature, resolution factor, peak symmetry, selectivity factor, analysis time, retention time parameters were considered for optimum conditions [37]. As a result, 0.5% phosphoric acid-deionized water mixture in mobile phase A, 1.2 mL/min flow rate and 20 °C column temperature were decided. The chromatographic data of the compounds under optimum conditions are shown in Table 1.

Table 1. The values of capacity factors, selectivity and resolution factor pertaining to the compounds.

Compounds	$k_2$	$\alpha$	$(\alpha-1)/\alpha$	$(k_2)/(k_2+1)$	$1/4 \sqrt{N}$	$R_s$
Rutin	2.02	2.31	0.57	0.67	34.74	13.20
Resveratrol	2.02	2.36	0.58	0.67	38.92	15.10
Quercetin	3.22	1.59	0.37	0.76	36.45	10.37

### Validation of HPLC-DAD method

Method validation aims to statistically prove the high sensitivity and precision of the method. For method validation, system suitability tests, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and recovery parameters were studied.

System suitability tests are performed to determine the suitability of the developed method. Retention time (RT) in the standard mixture of the compounds (min), capacity factor (k), selectivity ( $\alpha$ ), tailing factor ( $T_f$ ), theoretical plate (N), resolution factor ( $R_s$ ) and the compliance of the percentage relative standard deviation of the retention time (RSD%) with the recommended values was determined [38, 39]. In calculating the capacity factors of the compounds, thiourea was chosen as  $t_0$  and the  $t_0$  value was 2.56. System suitability test results are shown in Table 2.

Table 2. System suitability test results.

Compounds	RT (min)	k	$\alpha$	N	$T_f$	$R_s$	RSD% (min)
Recommended values	-	1-10	$\geq 1.15$	$> 2000$	$\leq 2$	$\geq 1.5$	$\leq 1$
I.S.	4.79	0.87	-	3778	1.17	-	0.38
Rutin	7.72	2.02	2.31	19305	0.83	13.20	0.08
Resveratrol	7.74	2.03	2.21	24476	0.84	14.36	0.26
Quercetin	10.78	3.22	1.59	21719	0.90	10.40	0.18

The linearity of the method was assessed using the internal standard technique to obtain values within a certain range corresponding to the substance concentration in the sample solution. Calibration curves were generated for all compounds in the concentration range of 0.025-25

$\mu\text{g/mL}$ . The correlation coefficients for rutin, resveratrol and quercetin were determined as 0.9998, 0.9999 and 0.9997, respectively.

The lowest amount that can be detected in a sample but cannot be calculated quantitatively is called limits of detection (LOD). The lowest amount that can be calculated with accuracy and precision in the sample is called limits of quantification (LOQ). The LOD and LOQ values for rutin, resveratrol and quercetin were calculated according to equations below. The symbols SD and  $m$  in these equations represent the standard error of the intercept and slope of the calibration curve equation for the compound, respectively [40-42]. LOD and LOQ value was calculated according to the following formula:

$$LOD = \frac{3 \times SD}{m}$$

$$LOQ = \frac{10 \times SD}{m}$$

The LOD values for rutin, resveratrol and quercetin were found to be 6.47, 4.83, 6.62  $\text{ng/mL}$  and the LOQ values were determined as 21.58, 16.09, 22.07  $\text{ng/mL}$ , respectively. In a study by Artem *et al.* [21] utilizing a wide range of phenolic compounds, the LOD and LOQ values were found to be 0.09–0.21 and 0.20–0.76  $\text{mg/L}$ , respectively. According to the findings of Vinas *et al.* [43], the LOD values for rutin, resveratrol, and quercetin were determined as 0.11, 0.06, and 0.16  $\mu\text{g/mL}$ , respectively, while the LOQ values were determined as 0.35, 0.22, and 0.55  $\mu\text{g/mL}$ . The LOD values for rutin, resveratrol, and quercetin were determined as 0.93, 0.39, and 0.35  $\text{mg/L}$ , respectively, in the study conducted by Anlı *et al.* [44]. In the analysis undertaken by Sakkiadi *et al.* [45], the LOD values for rutin, resveratrol, and quercetin were found to be 0.85, 0.50, and 0.35  $\text{mg/L}$ , respectively. Chafer *et al.* [46] reported the LOD values for rutin, resveratrol, and quercetin to be 2.6, 2.3, and 2.9, respectively, while the LOQ values were determined to be 8.6, 7.7, and 9.8, in the same order. In their determination with various phenolic compounds, Rodríguez-Bernaldo de Quirós *et al.* [47] found the LOD values for rutin, resveratrol, and quercetin to be 0.03, 0.02, and 0.2  $\mu\text{g/mL}$ , respectively. It was determined that the calculated limit of detection and correlation coefficient values were close to or lower than the literature [21, 43-48].

The fact that the data is similar to each other as a result of many measurements of the same sample is called precision. The precision parameter is calculated by conducting intra-day and inter-day studies [25]. For intra-day precision, at least 9 repetitions were used at 3 different times and two concentrations were taken as reference. Inter-day precision was obtained with three repetitive injections on three different days with reference to two concentrations under the same chromatographic conditions [49]. RSD% values of intra-day and inter-day were found between 0.12–0.69% and 0.33–0.72%, respectively, and given in Table 3.

Table 3. Results of intra-day and inter-day values of compounds.

Compounds	Concentration ( $\mu\text{g/mL}$ )	Intra-day		Inter-day	
		Recovery (%) $\pm$ SD	RSD (%)	Recovery (%) $\pm$ SD	RSD (%)
Rutin	1	99.52 $\pm$ 0.69	0.69	99.78 $\pm$ 0.49	0.49
	10	100.03 $\pm$ 0.47	0.47	100.08 $\pm$ 0.37	0.37
Resveratrol	1	100.07 $\pm$ 0.12	0.12	100.20 $\pm$ 0.72	0.72
	10	100.20 $\pm$ 0.25	0.25	100.28 $\pm$ 0.33	0.33
Quercetin	1	99.81 $\pm$ 0.47	0.47	100.34 $\pm$ 0.67	0.67
	10	100.02 $\pm$ 0.52	0.52	100.02 $\pm$ 0.55	0.55

#### Quantitative determination of flavonoids in dietary supplements

A novel HPLC-DAD method was developed and validated for the quantitative analysis of phenolic compounds contained in five different dietary supplement tablets/capsules. The amounts

of phenolic compounds were calculated for each compound with the help of the regression equation obtained from the calibration curves and these calculated quantities are given in Table 4. According to Table 4, the amounts of rutin and quercetin in DS<sub>4</sub> are in accordance with the label; however, the amounts of resveratrol and quercetin found in the other samples are not consistent with the labelled values.

Table 4. The amounts of phenolic compounds in dietary supplement tablets/capsules and their values on the label (mg).

	Rutin		Resveratrol		Quercetin	
	Analysis result	Label	Analysis result	Label	Analysis result	Label
DS <sub>1</sub> ± SD		NP*	158.85 ± 0.07	200	67.50 ± 0.08	200
DS <sub>2</sub> ± SD		NP*	7.29 ± 0.03	100	78.14 ± 0.06	80
DS <sub>3</sub> ± SD		NP*	6.76 ± 0.11	100	74.37 ± 0.08	80
DS <sub>4</sub> ± SD	11.08 ± 0.04	10		NP*	509.81 ± 0.04	500
DS <sub>5</sub> ± SD		NP*	15.46 ± 0.08	100	178.90 ± 0.13	500

NP\*: not present in the content.

#### Antioxidant activity

DPPH free radical scavenging activity of dietary supplements were determined. The percent inhibition values of different concentrations were given in Table 5. Linear % inhibition graphs were drawn at the indicated concentrations for dietary supplements and positive controls (L-ascorbic acid and trolox). The IC<sub>50</sub> values, which represent the concentration at which 50% DPPH radical inhibition occurs, were calculated using the exponential equations of the graphs [50]. The low IC<sub>50</sub> value indicates high antioxidant activity [26]. In the study, DPPH free radical scavenging activity was evaluated according to IC<sub>50</sub> values against reference to two positive controls. The IC<sub>50</sub> values of DS<sub>1</sub>, DS<sub>2</sub>, DS<sub>3</sub>, DS<sub>4</sub>, DS<sub>5</sub> supplements, L-ascorbic acid and trolox were detected as 0.35, 0.76, 0.19, 0.42, 0.08, 0.03, 0.05 mg/mL, respectively. When compared to the trolox, IC<sub>50</sub> values of DS<sub>1</sub>, DS<sub>2</sub>, DS<sub>3</sub> and DS<sub>5</sub> exhibit significant antioxidant activity ( $p < 0.0001$ ) whereas DS<sub>4</sub> demonstrated a significantly higher antioxidant effect compared to the others ( $p < 0.05$ ). No significant difference was observed among the positive control groups (ns) (Figure 1). Graphs were constructed by Graph Pad Prism® 9 software.

Table 5. Antioxidant activities of dietary supplements determined by DPPH radical scavenging.

Concentration (mg/mL)	DPPH free radical scavenging activity (%)				
	DS <sub>1</sub>	DS <sub>2</sub>	DS <sub>3</sub>	DS <sub>4</sub>	DS <sub>5</sub>
2.5	97.63	91.89	94.09	95.82	97.37
1	97.25	69.35	94.35	96.13	95.26
0.5	70.05	28.58	94.33	96.15	59.07
0.25	38.90	11.27	69.45	61.26	32.87
0.1	17.29	2.46	23.50	26.29	11.77
0.05	8.75	0.02	9.18	13.86	5.34
0.025	5.23	-3.70	2.17	5.05	3.10

The copper(II) ion reducing capacity was determined at 1.0 mg/mL concentration of all dietary supplements. Trolox was used as the standard antioxidant and concentration-absorbance graph was drawn at six different concentrations. The molar absorption coefficient ( $\epsilon$ ) of trolox was calculated as  $8.48 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ , based on the slope of the graph. For the dietary supplements DS<sub>1</sub>, DS<sub>2</sub>, DS<sub>3</sub>, DS<sub>4</sub> and DS<sub>5</sub> the total antioxidant capacity (TAC) values were determined as follows: 0.09, 0.03, 0.10, 0.10 and 0.40 mmol trolox per gram of dietary supplement, respectively. When the TAC values were evaluated, it was determined that DS<sub>4</sub> exhibited higher antioxidant activity compared to the other dietary supplements.

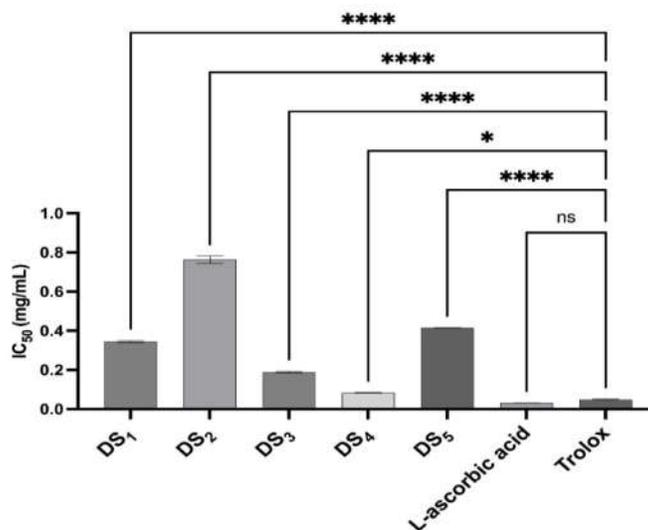


Figure 1. Relative content of various major compounds based on IC<sub>50</sub> values. Values are presented as mean  $\pm$  SD. Error bars represent the standard deviation of the mean. <sup>ns</sup>p  $\geq$  0.05; \*p < 0.05; \*\*\*\*p < 0.0001 levels of p indicate significant statistical differences of dietary supplements versus positive controls.

## CONCLUSION

In this study, a simple, reliable, sensitive, and rapid HPLC-DAD method was developed and validated for the determination of phenolic compounds in five different commercial dietary supplements. Taking into account parameters such as resolution factor, peak symmetry, selectivity factor, analysis time, and retention time, optimization was performed, demonstrating the reliability of the developed method in terms of system suitability, linearity, LOD, LOQ, precision, and recovery parameters. The new method developed was successfully applied for the analysis of phenolic compounds from commercial dietary supplement tablets. At the conclusion of the evaluation, it was determined qualitatively that DS<sub>4</sub> contained rutin and quercetin as stated on the labels and DS<sub>1</sub>, DS<sub>2</sub>, DS<sub>3</sub>, and DS<sub>5</sub> contained resveratrol and quercetin, whereas quantitatively, it was found that DS<sub>4</sub> dietary supplement showed conformity with the numerical values stated on the label. Furthermore, the antioxidant activities of all dietary supplements were investigated by utilizing two different methods that DPPH and CUPRAC and DS<sub>4</sub> was exhibited more effective antioxidant activity than the other samples in both methods. The developed new HPLC-DAD method can be easily applicable for the analysis of phenolic compounds in various industrial products as well as in the fields of medicine, pharmacy, chemistry, and food. Moreover, this method can confidently be recommended for the verification of the accuracy of label contents of dietary supplements.

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