Front. Agric. China 2010, 4(3): 334–340
DOI 10.1007/s11703-010-0007-0

RESEARCH ARTICLE

Ye LI, Huiling LI, Tong CUI

Thiolysis-HPLC analysis of proanthocyanidins in health foods and their materials

Abstract Proantocyanidins (PCs) found in plant products, are reported to be beneficial to human health. A thiolysis-HPLC method was developed to determine PC in health foods and the basic PC material, grape seed extract (GSE). PCs were quantitatively resolved by acid-catalysis in the presence of benzyl mercaptan as nucleophiles. The reaction solutions of actual purchased products were injected into an HPLC and the gallic acid (GA), catechin (CT), epicatechin (EC), epicatechin gallate (ECG), epicatechin benzyl sulfide (EC-S), and epicatechin gallate benzyl sulfide (ECG-S) were separated and detected. The HPLC was performed on a Hypersil BDS C_{18} (4.6 mm id $\times$ 250 mm, 5 μm) column at 35°C, formic acid (0.05%) was employed as mobile phase A, and methanol : acetonitrile = 1:2 (v/v) was employed as mobile phase B. The flow rate of gradient elution was 0.8 mL·min^{-1}, and the DAD detection was carried out at 280 nm. The relative standard deviation of CA, CT, EC, ECG, EGS and ECG-S followed by repeated reactions and injections were between 0.4%–4.2%, and the PC levels in six health foods were between 6.9–161.3 mg·g^{-1} with the mean degree of polymerization (mDP) 2.2–4.2. In a GSE product, the PC content was 531.4 mg·g^{-1}, and the mDP was 9.0, which although higher than other products tested, was far below the labeled, claimed, concentration. Data showed this method to be accurate and sensitive and can be used for the quality evaluation of actual health products containing PC components.

Keywords proanthocyanidins, high performance liquid chromatography (HPLC), health food, thiolysis, grape seed extract

Received December 6, 2009; accepted January 29, 2010

Ye LI, Huiling LI, Tong CUI (✉)
College of Food Science and Technology, Agricultural University of Hebei, Baoding 071001, China
E-mail: cuitong98@yahoo.com.cn

1 Introduction

Proanthocyanidins (PCs) are a group of polymeric phenolic secondary metabolites that use flavanols as the structural units. They are distributed in many edible plants and foods, such as grape, hawthorn, apple, tea, wine, cocoa, beer, and cider (Rigaud et al., 1993). The unit compositions and their degree of polymerization often differ due to the differences between the sources of PC molecules. The most common flavanol units are catechin (CT), epicatechin (EC), as well as their esters formed with gallic acid (GA), such as epicatechin gallate (ECG). Substantial research shows that PCs are an excellent natural anti-oxidants and free radical scavengers, both properties offering important physiological effects on health care including heart and brain artery protection, inflammatory and radiation protection, and the ability to reduce the risk of cancer (Hertog et al., 1995; Kozikowski et al., 2000; Ou et al., 2002; Knekt et al., 2002; Yi et al., 2005; Wojdyło et al., 2008). It has become one of the important materials for the development of health foods and functional cosmetics. Because PCs are very complex chemicals, the quantitative analysis of the active ingredients in various health products containing PC is always an inevitable technical issue.

The method that is widely used currently is spectrophotometry, such as Vanillin-hydrochloric acid method, Bate-Smith method (Bate-Smith, 1975), and Porter method (Porter et al., 1986). The drawback of these methods is that varying compositions of the PC differ in color performance. Therefore, selecting a specific reference substance as the standard is bound to introduce some systematic error. In addition, the coexistence of polyphenols in the sample would also interfere with the color reaction. Although the use of high performance liquid chromatography (HPLC) (Svedstrom et al., 2002), as well as the LC-MS (Fulcrand et al., 1999; Vidal et al., 2004), can directly separate and quantify some components of PC, yet due to the complexity of PC components, in fact, it is impossible to obtain all of the reference substances of the PC

components and their decomposition products. Therefore, it is difficult for either method to give reliable results of the total content and the mean degree of polymerization (mDP) of the PC components.

Thompson and his collaborators found that PC can be degraded when heated under acidic conditions and in the presence of nucleophiles, such as benzyl mercaptan (Thompson et al., 1972). The under unit of a PC molecule will give a corresponding flavanol molecule, while the upper parts of the remaining structural elements are generated into corresponding thioether derivatives. This reaction can be quantitatively completed and thus would be able to make a very complex mixture of PC produce only a limited number of degradation products after the thiolysis reaction. Recently, a number of HPLC analyses upon this reaction have been developed (Labarbe et al.,1999; Guyot et al., 2001; Kennedy and Jones, 2001; Gu et al., 2002; Dueñas et al., 2003; Karonen et al., 2007). However, these methods do not use the thiolysis derivatives as the reference substance, and the samples to be analyzed are often single plant materials. There is limited information on the quantitative analyses of thiolysis PC composition in health foods. In the present work, we prepared and purified the epicatechin benzyl sulfide (EC-S) and epicatechin gallate benzyl sulfide (ECG-S), two important thioether derivatives of PC as reference substances and developed a thiolysis-HPLC method, which can be adapted to a variety of samples so as to analyze and evaluate the PC content, unit composition, and the mean degree of polymerization (mDP) in health foods and their basic materials.

2 Materials and methods

2.1 Instrument

The quantitative analyses of HPLC were carried out by using an Agilent 1200 series (Palo, Alto, CA, USA) equipped with a degasser (G1322A), quaternary pump (Quat Pump G1311A), and diode array detection (DAD), and all were controlled by an Agilent's ChemStation, combined with a Hypersil BDS C_{18} (250 mm × 4.6 mm id, 5 μm) column and a CO-3010 column thermostat control box made by America Varitek Technology Co. Ltd. (Tianjin, China). The preparative HPLC made by Elite Scientific Instrument Co. Ltd. (Dalian, China) was equipped with a SinoChrom ODS-BP column (250 mm × 20 mm id, 10 μm), a P260 pump, an UV detector, and an Echrom 98 chromatographic workstation. The LC-MS analyses were employed with an LTQ type LC-MS system made of Thermo Fisher Co. (New York City, USA).

2.2 Materials

Five kinds of commercially available grape seed extract (GSE) health foods were purchased from the Lerentang Pharmacy (Shijiazhuang city, China): 'Bai He Kang Grape Seed and Soybean Extract Softgel' (Biotechnology Co. Ltd. Rongcheng lily production. Rongcheng, China), 'Pu Ling An Soft Capsule' (San Ye Biological Technology Co. Ltd. Shenzhen, China), 'Grape seed extract capsule' (Tong Ren Tang Pharmaceutical Co. Ltd. Beijing, China), 'Grape Seed' (Science and Technology Development Co. Ltd. Shenzhen, China), and 'Red Grape Seed' (By-Health Biotechnology Co. Ltd. Guangdong, China), A dietary supplement 'OPC Antioxidant Nature's' was purchased from Nature's Valueinc Co. Ltd. (USA). As a base material, we used a grape seed extract (labeled content > 95%), purchased from Xi'an Biotechnology Co. Ltd. (Xi'an, China).

2.3 Chemicals

Catechin (CT) and epicatechin (EC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); gallic acid (GA) was purchased from Chumbo Bio-Engineering Co. Ltd. (Shanghai, China); epicatechin gallate (ECG) was purchased from Tongtian Biotechnology Co. Ltd. (Shanghai, China); benzyl mercaptan was purchased from Merck and Co. Inc. (Hohenbrunn, Germany); acetonitrile and methanol (HPLC grade) used for HPLC mobile phase were purchased from Honeywell Burdick and Jackson Inc. (Tacoma, WA, USA); water was purified using an XGJ-series of high-water system. Epicatechin-4-benzyl sulfide (EC-S) and epicatechin gallate-4-benzyl sulfide (ECG-S) were prepared and purified according to the following method, 2 g GSE was suspended in 3 mL methanol. Five hundred μL of the supernatant, 500 μL ethanol solution containing 30% benzyl mercaptan, and 250 μL solution of glacial acetic acid: 1 M hydrochloric acid (50∶1, v/v) were mixed and reacted at 90°C for 60 min. Then, the mixture was separated by the preparative HPLC repeatedly using a SinoChrom ODS-BP column (250 mm × 20 mm id, 10 μm). The mobile phase was 70% (v/v) methanol containing 0.05% (v/v) formic acid with a flow rate 10 $mL \cdot min^{-1}$; UV detection was at 280 nm. Fractions A and B were collected, respectively, and the solution was vaporized at 40°C and extracted by ethyl acetate. Then, the extract was evaporated to 1 mL and was repurified by using a SinoChrom SiO_2 column (250 mm × 20 mm id, 10 μm), and the mobile phase of the ethyl acetate: petroleum ether was 60∶40 v/v. Both compounds were confirmed on the basis of spectra data mainly MS, ^{1}H, and ^{13}C-NMR after comparison with published data (Gu et al., 2002; Vidal et al., 2004; Karonen et al., 2007) which confirmed EC-S and ECG-S.

2.4 Preparation of sample solutions and thiolysis reaction

Twenty capsules of each item were shelled and mixed. Two grams of the content was degreased three times with petroleum ether in order to remove the lipophilic

compounds. The residue was dissolved by 95% ethanol up to 10 mL. Likewise, 20 tablets were crushed with a mortar, and 2 g of the powder was dissolved in 10 mL ethanol. Moreover, 50 mg GSE was dissolved in 10 mL ethanol. One hundred μL supernatant of the sample solution, 100 μL ethanol solution containing 5% benzyl mercaptan, and 50 μL glacial acetic acid containing 20 mM HCl were mixed, and the reaction flask was sealed and incubated at 90°C for 60 min.

2.5 Quantitative HPLC conditions

Formic acid at a concentration of 0.05% (v/v) was employed as mobile phase A and methanol: acetonitrile = 1:2 (v/v) as B. The gradient procedure was 0–8 min, 13% B (isocratic); 8–16 min, 13%–40% B (linear gradient); 16–24 min, 40%–50% B (linear gradient); 24–27 min, 50%–100% B (linear gradient); 27–34 min, 100% B (isocratic); 34–37 min, 100%–13% B (linear gradient); 37–45 min, 13% B (isocratic). The flow rate was 0.8 $mL \cdot min^{-1}$. The column temperature was 35°C, detection wavelength was 280 nm, and the scanning range was 190–400 nm, and it was performed automatically at the apex of each peak. Quantitative analyses were based upon the peak area by the external standard method.

2.6 LC-MS analysis

The HPLC conditions were as described in Section 2.5. The mass spectra were acquired in ESI mode using nonshunt detection at a source temperature of 350°C and a capillary voltage of 33 V. The mass spectrometer was operated in the positive mode, the photomultiplier voltage was −1560 V, the Tube Lens was 95 V, and the scanning range was m/z 50–1000.

3 Results

3.1 Preparation of thiolysis derivatives and LC-MS analysis

The thiolysis reaction and HPLC analyses were operated under the conditions described in the sections 2.4 and 2.5, respectively. Four peaks in the reactive solution were identified as GA, CT, EC, and ECG based on retention times and UV spectra. Other two peaks, which emerged after 25 min, were isolated and purified by using the methods described in Section 2.3 (chromatogram omitted) and detected by LC-MS. The peak corresponding to composition 1 possessed MS peaks at m/z = 413 and 289 (Fig. 1(a)), which was consistent with the $[M + H]^+$ of EC-S and its fragment ion $[EC\text{-}H]^+$. Additionally, the peak corresponding to composition 2 possessed MS peaks at m/z = 565 and 441 (Fig. 1(b)), which is consistent with $[M + H]^+$ of ECG-S and its fragment ions $[ECG\text{-}H]^+$. Therefore, the results of the MS analysis support the hypothesis that compounds 1 and 2 are indeed EC-S and ECG-S. They corresponded to the structures illustrated in Figs. 1(c) and (d), respectively.

3.2 HPLC analysis of reference standards

Six reference standards, GA, CT, EC, ECG, EC-S, and ECG-S, were dissolved in methanol and prepared in a mixed stock standard solution. The concentrations was 100 $\mu g \cdot mL^{-1}$, 1000 $\mu g \cdot mL^{-1}$, 1000 $\mu g \cdot mL^{-1}$, 200 $\mu g \cdot mL^{-1}$, 1000 $\mu g \cdot mL^{-1}$, and 500 $\mu g \cdot mL^{-1}$, respectively. The mixed standard solution was diluted 10 times with 50% methanol (v/v) and was detected according to the HPLC method described in Section 2.5. The profile is shown in Fig. 2. Under this HPLC condition, not only can the six main monomers be separated clearly but also other flavanol oligomers and their thiolysis derivatives can be separated (data omitted).

3.3 Evaluations of the regression equation

By dilution of the reference substance stock solution and subsequent analysis by HPLC according to the conditions described above, the compound's concentration is shown in Table 1. By taking the concentration ($\mu g \cdot mL^{-1}$) as the abscissa and peak area ($mv \cdot s$) as the ordinate, the data of linear range and detection limit (defined with the S/N of 3:1) are also shown in Table 1.

3.4 Evaluation of the precision

Validation of the HPLC method was performed by determining of the intra-day and inter-day precision (relative standard deviation, RSD) of the five analyses, and the results are shown in Table 2. We can see that the intra-day precisions of the six components are acceptable, except for RSD of ECG-S that was slightly larger. The inter-day precisions were from 2.5% to 5.3%.

3.5 Recovery

The method accuracy was validated by the recovery. We dissolved 40 mg GSE sample in 10 mL methanol solution. The thiolysis reactions were conducted repeatedly ($n = 3$) according to the conditions described in Section 2.4, and the HPLC detection was carried out according to the conditions described in Section 2.6. Then, we measured the contents of GA, CT, EC, and ECG in the thiolysis reaction solution. Then, the standards of GA, CT, EC, and ECG were added into the GSE samples at the levels of 5, 65, 20, and 10 $mg \cdot g^{-1}$ dry weight, respectively. Then, the thiolysis reaction and HPLC determination were performed repeatedly ($n = 5$). The average recoveries and RSD were shown in Table 3. In Table 3, we can see that

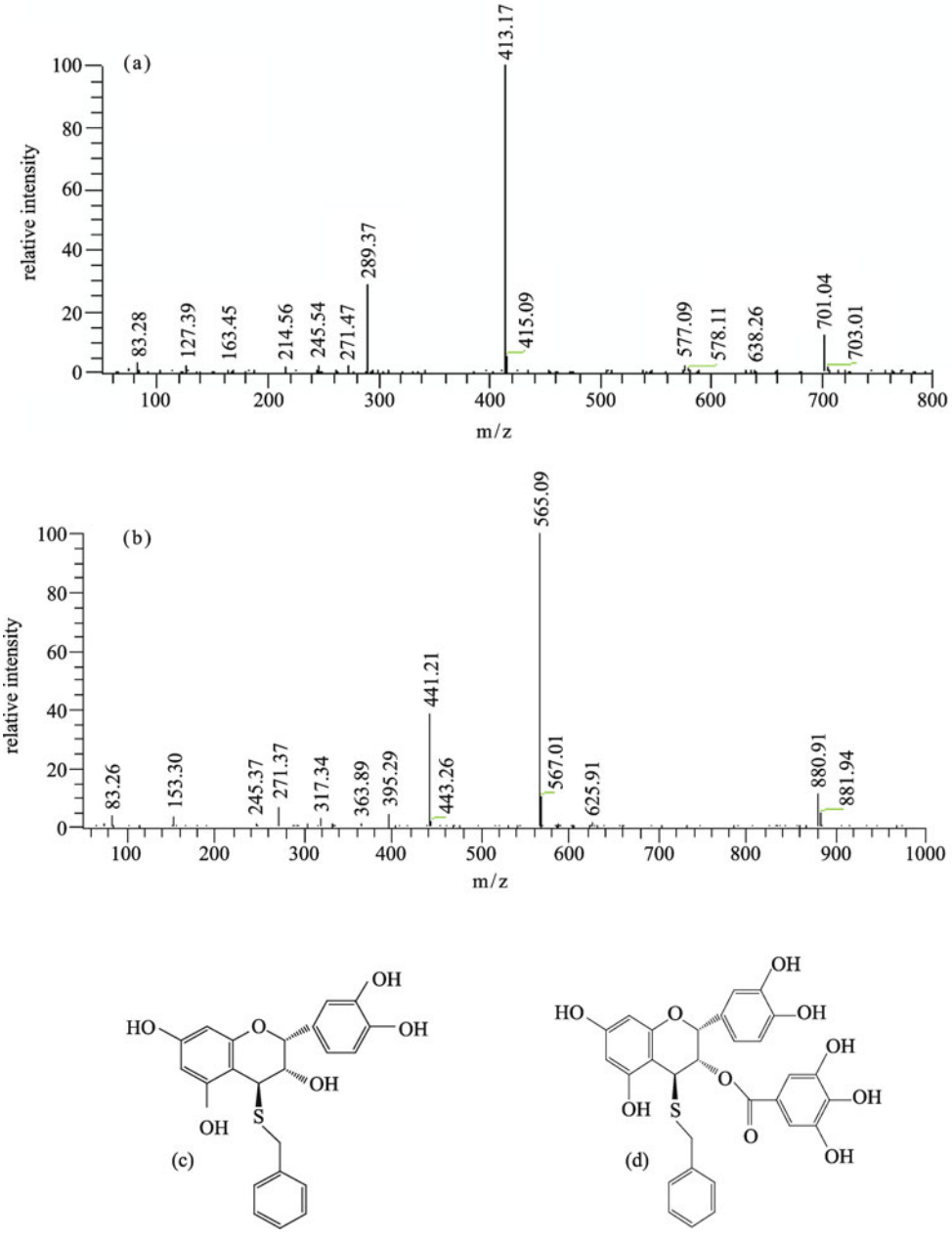

Fig. 1 LC-MS spectra of two thiolysis derivatives

Note: (a) MS spectrum of epicatechin-4-benzyl sulfide, (b) MS spectrum of epicatechin gallate-4-benzyl sulfide, (c) structure of epicatechin benzyl sulfide (EC-S), (d) structure of epicatechin gallate benzyl sulfide (ECG-S).

CT, EC, and ECG got higher recoveries, while the recovery of GA was low, for it is more reactive, and there may be some influencing factors in the thiolysis reaction.

3.6 Analysis of the samples

Six commercially available health food samples were treated, reacted, and detected according to the methods described in Sections 2.4 and 2.5. A typical chromatogram of sample analysis is shown in Fig. 3. In the profile, the chromatography of a GSE became very simple. The polymers gathered and eluted around 20–25 minutes, which had been previously had been split into CT, EC, ECG, EC-S, and ECG-S. This process makes the PC quantification accurate and reliable, and the comparative flavanol composition of PC between different sources of plant materials now had become more credible. We have employed this approach to analyze many different materials including apple, hawthorn, and grape. The results showed that PCs from different plant sources have quite different styles of flavanol composition. For example, the PC of hawthorn does not contain GA, CT, or

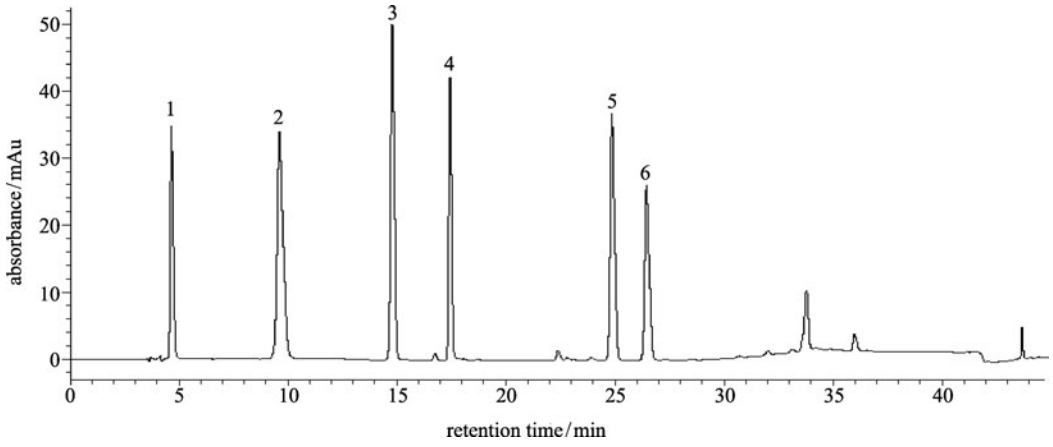

Fig. 2 HPLC profile (detected at 280 nm) of reference standards including gallic acid, flavan-3-ols, and their relational thiolysis derivatives
Note: 1–6 stand for gallic acid, catechin, epicatechin, epicatechin gallate, epicatechin-4-benzyl sulfide, and epicatechin gallate-4-benzyl sulfide, respectively. For the details of the HPLC conditions, see Section 2.5.

Table 1 Regression equation, correlation coefficient, linear range, and the minimum detection limit

composition	regression equation	correlation coefficient	linear range/ng	minimum detection limit*/ng
GA	$Y = 27.396X + 3.3653$	0.9999	0.8–400	0.4
CT	$Y = 6.4661X - 4.9762$	0.9999	8–4000	4.0
EP	$Y = 7.3972X - 0.2753$	0.9999	6.4–3200	3.2
ECG	$Y = 21.159X + 7.6892$	0.9997	1.6–800	0.8
EP-S	$Y = 5.2046X - 9.6089$	0.9999	8–4000	4.0
ECG-S	$Y = 8.9804X - 26.576$	0.9990	4–2000	2.0

Note: * means values are defined with a signal-to-noise (S/N) ratio of 3∶1.

Table 2 Precision evaluation on intra-day and inter-day analyses ($n = 5$)

composition	intra-days RSD/%	inter-day RSD/%
GA	0.800	3.70
CT	0.400	2.90
EC	0.700	2.80
ECG	3.10	3.30
EC-S	0.900	2.50
ECG-S	4.20	5.30

Table 3 Recoveries of GA, CT, EC, and ECG

composition	recovery rate/%	RSD/%
GA	77.8	3.80
CT	89.5	3.00
EC	92.0	3.10
ECG	94.6	3.70

ECG and therefore has a simple chromatogram (data omitted).

The analytic results of the seven actual samples are shown in Table 4. From the data, we can know that the GSE product, a base material of the dietary supplements, has the highest content of the six components, the total content reaching 53%. However, it was still significantly lower than "$>95\%$" as claimed by the manufacturer and marked on the package. Possible reasons may be that the GSE (Sample No.7) also contains other PC components besides the selected six monomers in our method; another possible reason is that the GSE (Sample No.7) product may contain some non-PC components, which can elevate the colorimetric analysis. The results in the table also showed that all of the six commercially available health foods contained the six kinds of monomer ingredients, and among them, CT and EC had higher concentrations, and the ratio between the two monomers was nearly 1∶1. The concentration of ECG was comparatively lower. Similarly,

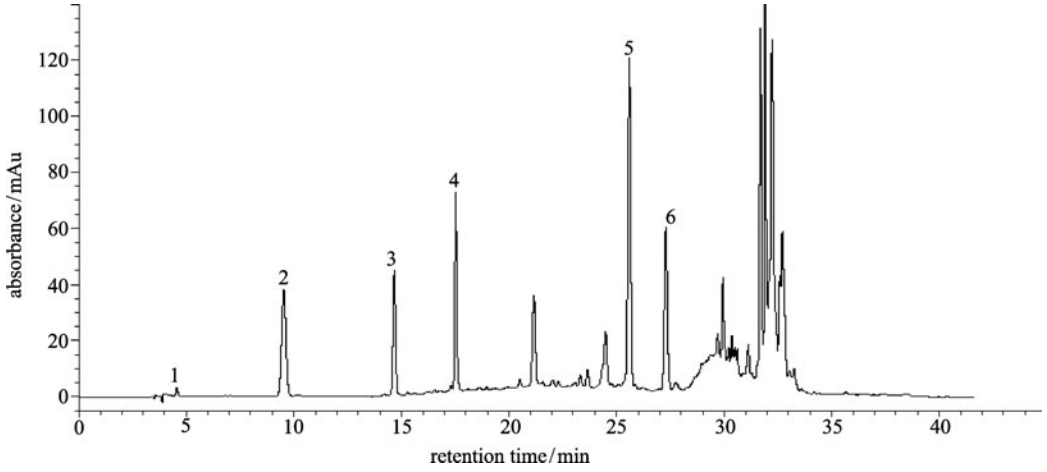

Fig. 3 HPLC profile of "Tablets of Red Grape Seed" after thiolysis reaction (detected at 280 nm)
Note: 1–6 stand for gallic acid, catechin, epicatechin, epicatechin gallate, epicatechin-4-benzyl sulfide, epicatechin gallate-4-benzyl sulfide, respectively. For HPLC conditions, see the text.

Table 4 HPLC analytic results of health food products and basic materials after thiolysis

sample*	GA	CT	EC	ECG	EC-S	ECG-S	SUM /(mg·g^{-1})
1	0.030±0.001	1.11±0.040	0.870±0.030	0.150±0.005	4.50±0.150	0.320±0.010	6.89
2	0.090±0.010	0.580±0.010	0.610±0.010	0.170±0.010	5.24±0.100	0.800±0.030	7.49
3	0.190±0.010	12.4±0.450	7.84±0.290	3.54±0.120	32.9±0.880	5.45±0.170	62.32
4	0.050±0.002	2.33±0.070	1.84±0.060	0.810±0.020	12.5±0.330	2.65±0.060	20.18
5	0.660±0.030	12.1±0.400	13.2±0.340	7.58±0.270	106±2.13	21.8±0.750	161.34
6	0.140±0.030	0.860±0.020	0.660±0.020	0.330±0.010	6.29±0.210	1.51±0.030	9.79
7	0.970±0.100	64.2±2.10	26.3±0.970	13.3±0.430	360±12.8	66.5±2.80	531.35

Note: * 1–7 of the samples represent Bai He Kang Grape Seed and Soybean Extract Softgel, Pu Ling An Soft Capsule, Grape Seed Extract Capsule, Grape Seed, Red Grape Seed, OPC Antioxidant; and 7 Grape Seed Extract respectively.

the proportion between the two thioether derivatives also showed that the EC-S was more abundant than ECG-S in the GSE thiolysis reaction solution. From the data, it can be seen that, in the five kinds of capsule products, the total content was quite different. The maximum content was 62.32 mg·g^{-1}, and the lowest was 6.89 mg·g^{-1}, a difference of nearly 10 times. Therefore, we believe that it is essential to develop a reliable analytical method for the quality control of the commercial health products containing PC components.

3.7 Calculation of mDP of PC

According to the PC thiolysis reaction principle, flavanols are from the under units of the PC molecules. Therefore, in the reaction solution, the sum of the flavanol concentrations including CT, EC, and ECG is actually equal to the PC molar concentration in solution before reaction. The total of the concentrations of the flavanols and their derivatives (EC-S and ECG-S) does reflect the molar concentration of all flavanol units contained in PC components. Thus, we can calculate the mDP using the equation:

$$\mathrm{mDP} = (C_{CT} + C_{EC} + C_{ECG} + C_{EC\text{-}S} + C_{ECG\text{-}S}) / (C_{CT} + C_{EC} + C_{ECG})$$

By the analytic results shown in Table 4, we obtained the PC mDP of the six kinds of health products, which are 2.6, 4.2, 2.2, 3.2, 3.8, 4.0, and 9.0, respectively. Previous researches found that only PC monomers and dimers could be absorbed through the human small intestine cells, while the PC polymer of more than trimeric could not be absorbed directly. Therefore, the mDP of PC components should be an important indicator in the quality evaluation of PC productions.

4 Discussion

The major health-care active factor in the GSE is proanthocyanidins. The total content and mDP are the most important indicators reflecting PC quality. Since PC components are complex, there are shortages in some

former analytical methods for the evaluation of the two indicators. In recent years, although there are some reports using thiolysis-HPLC to analyze the PC content, these methods have not provided the purified thioether derivatives as the reference standards for the quantitative analyses. In the present research, two major flavanol thioether derivatives were isolated and purified, and a new thiolysis-HPLC analytical method was developed to analyze PC content and their mDP. The results show that the sensitivity and accuracy of this method can be adapted to the requirements of PC components analysis. Our method provides the information of total content, flavanol unit composition, and the mDP of the PC components. This reliable analytical method can be used for the quality evaluation of actual health products containing PC components.

References

Bate-Smith E C (1975). Phytochemistry of proanthocyanidins. Phytochemistry, 14(4): 1107–1113

Dueñas M, Sun B, Hernández T, Estrella I, Spranger M I (2003). Proanthocyanidin composition in the seed coat of lentils (*Lens culinaris* L.). J Agric Food Chem, 51(27): 7999–8004

Fulcrand H, Remy S, Ouguet J M (1999). Study of wine tannin oligomers by on-line liquid chromatography electro-spray ionization mass spectrometry. J Agric Food Chem, 47(3): 1023–1028

Gu L, Kelm M, Hammerstone J F, Beecher G, Cunningham D, Vannozzi S, Prior R L (2002). Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. J Agric Food Chem, 50(17): 4852–4860

Guyot S, Marnet N, Drilleau J (2001). Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. J Agric Food Chem, 49(1): 14–20

Hertog M G, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedljkovic S (1995). Flavonoid intake and long-term risk of coronary heart disease and cancer in seven countries study. Arch Intern Med, 155(4): 381–386

Karonen M, Leikas A, Loponen J, Sinkkonen J, Ossipov V, Pihlaja K (2007). Reversed-phase HPLC-ESI/MS analysis of birch leaf proanthocyanidins after their acidic degradation in the presence of nucleophiles. Phytochem Anal, 18(5): 378–386

Kennedy J A, Jones G P (2001). Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. J Agric Food Chem, 49(4): 1740–1746

Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, Habulinen T, Aromaa A (2002). Flavonoid intake and risk of chronic disease. Am J Clin Nutr, 76(3): 560–568

Kozikowski A P, Tuckmantel W, George C (2000). Studies in polyphenol chemistry and bioactivity. 2. Establishment of interflavan linkage regio- and stereochemistry by oxidative degradation of an O-alkylation derivative of procyanidin B_2 to (R)-(-)-2,4-diphenylbutyric acid. J Org Chem, 65(17): 5371–5381

Labarbe B, Cheynier V, Brossaud F, Souquet J M, Moutounet M (1999). Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. J Agric Food Chem, 47(7): 2719–2723

Ou B, Hampsch-Woodill M, Flanagan J, Deemer E K, Prior R L, Huang D (2002). Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. J Agric Food Chem, 50(10): 2772–2777

Porter L J, Hrstich L N, Chan B G (1986). The conversion of procyanidins and prodelphinidins to cyanidin and delphindin. Phytochemistry, 25(1): 223–230

Rigaud J, Escribano-Bailon M T, Prieur C, Souquet J M, Cheynier V (1993). Normal-phase high-performance liquid chromatographic separation of procyanidin from cacao beans and grape seeds. J Chromatogr A, 654(2): 255–260

Svedstrom U, Vuorela H, Kostiainen R (2002). High-performance liquid chromatographic determination of oligomeric procyanidins from dimers up to the hexamer in hawthorn. J Chromatogra A, 968(1–2): 53–60

Thompson R S, Jacques D, Haslam E, Tanner R J N (1972). Plant proanthocyanidins. part I. Introduction, the isolation, structure, and distribution in nature of plant procyanidins. J Chem Soc Perkin Trans, 1: 1387–1399

Vidal S, Meudec E, Cheynier V, Skouroumounis G, Hayasaka Y (2004). Mass spectrometric evidence for the existence of oligomeric anthocyanins in grape skins. J Agric Food Chem, 52(23): 7144–7151

Wojdyło A, Oszmiański J, Laskowski P (2008). Polyphenolic compounds and antioxidant activity of new and old apple varieties. J Agric Food Chem, 56(15): 6520–6530