Journal of Food and Drug Analysis, Vol. 10, No. 3, 2002, Pages 178-182 藥物食品分析 第十卷 第三期

Estimation of Total Flavonoid Content in Propolis by Two Complementary Colorimetric Methods

CHIA-CHI CHANG[1], MING-HUA YANG[2]*, HWEI-MEI WEN[1] AND JIING-CHUAN CHERN[1]

[1.] Department of Food Science, National Pingtung University of Science and Technology (NPUST), 1 Hseuh-Fu Road, Neipu Hsiang, Pingtung Hsien 912, Taiwan, Republic of China

[2.] Department of Food and Nutrition, Hung-Kuang Institute of Technology, 34 Chung-Chi Road, Shalu, Taichung Hsien 433, Taiwan, Republic of China

(Received: January 25, 2002; Accepted: May 16, 2002)

ABSTRACT

Flavonoids, with various biological activities, are considered as key compounds in propolis. In this study, quantitative determinations of flavonoids in propolis were conducted by two complementary colorimetric methods, aluminum chloride method and 2,4-dinitrophenylhydrazine method. Results suggested that the sum of flavonoid contents determined by the above two individual methods may represent the real content of total flavonoids. In this work, six raw propolis samples were investigated and the total contents of flavonoids ranged from 10.38 ± 0.14% to 24.91 ± 0.53%. As for the 12 commercial propolis products examined, the levels of total flavonoids in tinctures were all below 7% and those in powdery products varied from 2.97 ± 0.05% to 22.73 ± 0.72%.

Key words: Propolis, flavonoids, flavones, flavonols, flavanones, flavananols, quantitative determination, colorimetric method, aluminum chloride reaction, 2,4-dinitrophenylhydrazine reaction

INTRODUCTION

Propolis, the material used by bees to protect their hives, is a glue-like substance composed of plant resins, bee waxes and pollens. Since various biological activities of propolis such as antibacterial, antiviral, anti-inflammatory and anaesthetic properties were found[1-3], it is used as a health food. The chemical composition of propolis is quite complicated and over 150 components have been identified[2,4]. Among these compounds flavonoids were suggested to be responsible for the biological activities[5,6]. Therefore, the content of flavonoids is considered as an important index for evaluating propolis quality.

The analysis of flavonoids in propolis has been done by colorimetric methods[7-10], thin layer chromatography[9,11], gas chromatography[12,13], gas chromatography-mass spectrometry[14-16] and high performance liquid chromatography[7,15,17,18]. Although chromatographic techniques in combination with absorption spectrum analysis and mass spectrometry provide definitive information for identification and quantification of flavonoids in propolis, these methods usually require advanced instruments, various authentic standards and are time-consuming. On the other hand, colorimetric methods targeting flavonoids of similar structures are convenient and appropriate for routine analyses. However, none of the colorimetric methods can detect all kinds of flavonoids. For instance, within four major groups of flavonoids in propolis, only flavones and flavonols were found to complex stably with aluminum chloride[19], while flavanones and flavanonols reacted better with 2,4-dinitrophenylhydrazine[8].

Considering the need of criteria for both consumers and government agencies to evaluate numerous commercial propolis products, we propose to determine the content of total flavonoids in propolis complementarily by aluminum chloride and 2,4-dinitrophenylhydrazine reactions. In this work, six raw propolis samples and 12 commercial products were investigated to compare the differences of results from each colorimetric analysis.

MATERIALS AND METHODS

I. *Materials*

Six raw propolis samples named Taiwan-1, Taiwan-2, Taiwan-3, Brazil-1, China-1 and China-2 were provided by Miaoli District Agricultural Improvement Station (Miaoli, Taiwan). Twelve commercial propolis products were obtained from local retailers and the description is shown in Table 1. All samples were stored at room temperature until analysis. Fifteen flavonoid standards including chrysin (catalog number C-3018), apigenin (A-3145), luteolin (L-9283), rutin (R-5143), morin (M-4008), quercetin (Q-0125), myricetin (M-6760), kaempferol (K-0133), quercitrin (Q-3001), galangin (06829HS), naringin (N-1376), (±)-naringenin (N-5893), hesperetin (H-4125), daidzein (D-7802) and genistein (G-6776) were purchased from Sigma-Aldrich (St. Louis, MO) with product numbers in parentheses. The systematic names of 15 flavonoid standards are listed in Table 2. All reagents used were of analytical grade. 2,4-

* Author for correspondence. Tel: 886-4-26318652 ext. 5032
Fax: 886-4-26319176; Email: mhyang@sunrise.hkc.edu.tw

Table 1. The description of 12 commercial propolis products used in the present study

Sample	Form	Color	Country of Production
A	powder	light brown	Brazil
B	powder	light brown	Brazil
C	powder	light brown	Brazil
D	powder	light brown	Brazil
E	liquid	yellowish brown	Brazil
F	liquid	dark brown	Brazil
G	liquid	dark brown	England
H	liquid	dark green	Australia
I	liquid	reddish brown	New Zealand
J	liquid	light yellowish green	Brazil
K	liquid	yellowish brown	Brazil
L	liquid	yellowish green	Brazil

Table 2. Systematic names of the 15 flavonoid standards used in the present study

Flavonoids	Systematic name
Flavones	
chrysin	5,7-dihydroxyflavone
apigenin	4',5,7 -trihydroxyflavone
luteolin	3',4',5,7 -tetrahydroxyflavone
Flavonols	
rutin	3,3',4',5,7-pentahydroxyflavone-3-rutinoside
morin	2',3,4',5,7-pentahydroxyflavone
quercetin	3,3',4',5,7-pentahydroxyflavone
myricetin	3,3',4',5,5',7-hexahydroxyflavone
kaempferol	3,4',5,7-tetrahydroxyflavone
quercitrin	3,3',4',5,7-pentahydroxyflavone-3-L-rhamnopyranoside
galangin	3,5,7-trihydroxyflavone
Flavanones	
naringin	4',5,7-trihydroxyflavanone-7-rhamnoglucoside
(±)-naringenin	4',5,7-trihydroxyflavanone
hesperetin	3',5,7-trihydroxy-4'-methoxyflavanone
Isoflavones	
daidzein	4',7-dihydroxyisoflavone
genistein	4',5,7-trihydroxyisoflavone

Dinitrophenylhydrazine reagent, abbreviated 2,4-D, was prepared by dissolving 1 g of 2,4-dinitrophenylhydrazine in 2 mL of 96% sulfuric acid and then diluting to 100 mL with methanol.

II. *Extraction of Flavonoids from Propolis*

About 1 g (accurately weighed to 0.0001 g) of raw propolis was extracted with 25 mL of 95% ethanol under 200 rpm shaking for 24 hr. After filtration, the filtrate was adjusted to 25 mL with 80% ethanol and stored in an amber bottle.

For commercial propolis products in solid form, 0.1 to 1 g (accurately weighed to 0.0001 g) was first dissolved with 10 mL of 80% ethanol. After centrifugation at 1,000 x g for 10 min, the supernatant was collected and the precipitate was then extracted with 5 mL of 80% ethanol twice. Finally, the supernatant was combined with previous supernatant and adjusted to 25 mL with 80% ethanol. Liquid propolis products were directly diluted with 80% ethanol to the concentrations appropriate for colorimetric analysis.

III. *Estimation of Total Flavonoid Content*

(I) *Aluminum Chloride Colorimetric Method*

The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatino[10]. Quercetin was used to make the calibration curve. Ten milligrams of quercetin was dissolved in 80% ethanol and then diluted to 25, 50 and 100 μg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 mL of ethanol extracts or 15 flavonoid standard solutions (100 ppm) were reacted with aluminum chloride for determination of flavonoid content as described above.

(II) *2,4-Dinitrophenylhydrazine Colorimetric Method*

The current method was modified from the procedure described by Nagy and Grancai[8]. (±)-Naringenin was used as the reference standard. Twenty milligrams of (±)-naringenin was dissolved in methanol and then diluted to 500, 1000 and 2000 μg/mL. One milliliter of each of the diluted standard solutions was separately reacted with 2 mL of 1% 2,4-dinitrophenylhydrazine reagent and 2 mL of methanol at 50˚C for 50 min. After cooling to room temperature, the reaction mixture was mixed with 5 mL of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. Then, 1 mL of the mixture was taken, mixed with 5 mL of methanol and centrifuged at 1,000 x g for 10 min to remove the precipitate. The supernatant was collected and adjusted to 25 mL. The absorbance of the supernatant was measured at 495 nm. The ethanol extracts of propolis and 15 flavonoid standard solutions (1000 ppm) were similarly reacted with 2,4-dinitrophenylhydrazine for determination of flavonoid content as described above.

RESULTS AND DISCUSSION

I. *Aluminum Chloride Colorimetric Method*

The principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids[19]. In preliminary experiments, the wavelength scans of the complexes of 15 standards with aluminum chloride showed that

the complexes formed by flavonols with C-3 and C-5 hydroxyl groups, such as galangin, morin and kaempferol, as well as those with extra ortho-dihydroxyl groups, such as rutin, quercetin, quercitrin and myricetin, had maximum absorbance at 415-440 nm (data not shown). However, the λ_{max} of the complexes formed by chrysin and apigenin which have only the C-5 hydroxyl and C-4 keto groups were at 395 and 385 nm, respectively. Another flavone compound investigated, luteolin, which has the C-5 hydroxyl group and the ortho-dihydroxyl groups in B ring formed a complex that showed a strong absorption at 415 nm. In compromise, therefore, the wavelength 415 nm was chosen for absorbance measurement.

Among the 15 flavonoid standards investigated, chrysin, apigenin and luteolin belong to flavones, while rutin, morin, quercetin, myricetin, kaempferol, quercitrin and galangin belong to flavonols. As expected, except for chrysin and apigenin, the absorbance readings of the complexes formed by flavone and flavonol compounds were much higher than those formed by flavanones and isoflavones as shown in Table 3. On the other hand, although the presence of C-5 hydroxyl group in flavanones such as naringin, (±)-naringenin, hesperetin and genistein helped these compounds complex with aluminum chloride, the absorbance at 415 nm was too low to make meaningful contribution to total absorbance.

Generally speaking, the aluminum chloride complexes of compounds with more functional groups absorbed stronger at 415 nm and showed the absorption maximum at longer wavelength (data not shown). For instance, we observed the λ_{max} of the complexes of chrysin and apigenin having only the C-5 hydroxyl group and that of luteolin having the C-5, C-3' and C-4' hydroxyl groups showed maximum absorbance at 395, 385 and 415 nm, respectively, in the absence of acid. Comparing the data of chrysin and apigenin with those of other flavones and flavonols shown in Table 3, we also found that the extra complexes formed between aluminum chloride and the adjacent hydroxyl groups in B ring not only influenced the absorption spectra but also increased the absorbanc at 415 nm.

To select a standard for calibration, factors such as the absorption maxima and absorbance at 415 nm were considered. Although apigenin, kaempferol and quercetin were all widely found in propolis[7], the absorbance of apigenin after reaction with aluminum chloride was too low to be measured at concentrations lower than 100 ppm. Quercetin which gave the second high absorbance reading among the 15 standards (Table 3) was used as the reference compound by Woisky and Salatino[10]. Therefore, we used quercetin solutions at concentrations ranging from 0 to 100 ppm to build up the calibration curve. The coefficients of determination (R^2) obtained were all higher than 0.99.

II. *2,4-Dinitrophenylhydrazine Colorimetric Method*

The principle of this method is that 2,4-dinitrophenylhydrazine reacts with ketones and aldehydes to form 2,4-dinitrophenylhydrazones. Interestingly, we found that flavones, flavonols and isoflavones with the C_2-C_3 double bond could not react with 2,4-dinitrophenylhydrazine, while the hydrazones of all flavanone standards, i.e. naringin, (±)-naringenin and hesperetin, showed maximum absorbance at 495 nm (data not shown). Therefore, the wavelength 495 nm was selected for all measurements in the 2,4-dinitrophenylhydrazine reaction. The absorbance readings of flavonoid standards reacted with 2,4-dinitrophenylhydrazine were shown in Table 3.

Pinocembrin was the most abundant flavanone found in various propolis samples[7] and used as the reference compound in quantitative determination of flavonoid content of propolis by Nagy and Grancai[8]. However, we could not obtain commercial pinocembrin. Therefore, instead of pinocembrin, we chose (±)-naringenin with one more hydroxyl group at the 4' position to make the calibration curve. The coefficients of determination (R^2) obtained were all above 0.999.

Similarly, there was no commercial flavanonol available. To investigate more flavanoid compounds, we used two isoflavones, daidzein and genistein, to react with aluminum chloride and 2,4-dinitrophenylhydrazine separately. Results showed that these isoflavone compounds did not form hydrazone product with 2,4-dinitrophenylhydrazine, while only slight absorbance was obtained when genistein with the C-3 hydroxyl group complexed with aluminum chloride (Table 3).

III. *Flavonoid Contents of Propolis Samples*

Flavonoid contents of six raw propolis samples deter-

Table 3. The absorbance of 15 flavonoid standards determined by aluminum chloride and 2,4-dinitrophenylhydrazine colorimetric methods

Flavonoids[a]	$AlCl_3$	2,4-D
	Abs (415 nm)[b]	Abs (495 nm)[b]
Flavones		
Chrysin	0.032 ± 0.000	0.000 ± 0.000
Apigenin	0.037 ± 0.001	0.000 ± 0.000
Luteolin	0.391 ± 0.004	0.024 ± 0.001
Flavonols		
Rutin	0.191 ± 0.004	0.000 ± 0.000
Morin	0.167 ± 0.004	0.000 ± 0.000
Quercetin	0.451 ± 0.006	0.000 ± 0.000
Myricetin	0.471 ± 0.007	0.000 ± 0.000
Kaempferol	0.427 ± 0.002	0.000 ± 0.000
Quercitrin	0.291 ± 0.005	0.000 ± 0.000
Galangin	0.358 ± 0.005	0.000 ± 0.000
Flavanones		
Naringin	0.016 ± 0.000	0.113 ± 0.005
(±)Naringenin	0.016 ± 0.000	0.240 ± 0.002
Hesperetin	0.016 ± 0.001	0.258 ± 0.013
Isoflavones		
Daidzein	0.000 ± 0.000	0.000 ± 0.000
Genistein	0.023 ± 0.000	0.000 ± 0.000

a: The concentrations of each authentic standard are 100 ppm in aluminum chloride reaction and 1000ppm in 2,4-dinitrophenylhydrazine reaction.

b: Results were shown as mean ± SD (n=3).

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mined by aluminum chloride colorimetric method were generally lower than those determined by 2,4-dinitrophenylhydrazine colorimetric method (Table 4). The formers ranged from 2.82 ± 0.02% to 7.73 ± 0.19 %, while the latters ranged from 7.12 ± 0.02% to 21.84 ± 0.34 %. Similarly, flavonoid contents of commercial propolis samples, except for tinctures E, F and G, were found to be lower by aluminum chloride reaction than those obtained by 2,4-dinitrophenylhydrazine reaction (Table 5). The results seem to be opposite to our general knowledge that the major flavonoid species of propolis from the temperate zone are flavones and flavonols. According to Serra Bonvehi *et al.*[7], who analyzed 15 propolis samples from Brazil, Uruguay and China by HPLC, the contents of flavanones were only 6-40% of total flavonoids in raw propolis and 4-9% in commercial propolis samples. In fact, the amounts of flavonoids determined by HPLC were greatly influenced by the selected authentic standards. Sometimes, limited by the availability of authentic standards, the identification of flavonoid peaks in chromatograms may be incomplete. Fortunately, modern technology of mass spectrometry facilitates the identification of suspicious peaks. Markham *et al.*[16] identified 10 major peaks in the HPLC chromatogram of a New Zealand propolis tincture by comparing the absorption spectra and using GC-mass spectrometry. They found that the total amounts of flavanones including pinobanksin, pinocembrin, pinobanksin 3-acetate and pinocembrin 7-methyl ether were 2.22 to 3.14 times more than those of flavones and flavonols including chrysin, galangin, chrysin 7-methyl ether and galangin 7-methyl ether in eight tincture samples. Our results were consistent with their finding and indicated the importance of the quantitative determination by 2,4-dinitrophenylhydrazine reaction.

Table 4. The flavonoid contents of six raw propolis samples determined by aluminum chloride and 2,4-dinitrophenylhydrazine colorimetric methods

Sample	Flavonoid content (%)[a]		
	$AlCl_3$[b]	2,4-D[c]	Total
Taiwan-1	2.82 ± 0.02	18.50 ± 0.17	21.35 ± 0.19
Taiwan-2	3.07 ± 0.01	21.84 ± 0.34	24.91 ± 0.35
Taiwan-3	2.95 ± 0.07	17.65 ± 0.39	20.60 ± 0.46
Brazil-1	3.26 ± 0.12	7.12 ± 0.02	10.38 ± 0.14
China-1	5.37 ± 0.05	12.12 ± 0.02	17.49 ± 0.07
China-2	7.73 ± 0.19	10.03 ± 0.05	17.76 ± 0.24

a: Results were presented as mean ± SD (n=3).

b: Flavonoid content (%) = quercetin equivalent (μg/mL) × total volume of ethanol extract (mL) ÷ sample weight (g) × dilution factor × 10^{-6} (g/μg) × 100.

c: Flavonoid content (%) = naringenin equivalent (μg/mL) × total volume of ethanol extract (mL) ÷ sample weight (g) × dilution factor × 10^{-6} (g/μg) × 100.

Table 5. The flavonoid contents of 12 commercial propolis products determined by aluminum chloride and 2,4-dinitrophenylhydrazine colorimetric methods

Sample	Flavonoid content (%)[a]		
	$AlCl_3$[b]	2,4-D[c]	Total
A	0.67 ± 0.00	4.12 ± 0.16	4.79 ± 0.16
B	1.43 ± 0.03	21.30 ± 0.69	22.73 ± 0.72
C	3.95 ± 0.17	6.86 ± 0.06	10.81 ± 0.23
D	1.30 ± 0.02	1.67 ± 0.03	2.97 ± 0.05
E	1.02 ± 0.00	0.84 ± 0.02	1.86 ± 0.02
F	1.45 ± 0.06	0.29 ± 0.00	1.74 ± 0.06
G	1.47 ± 0.05	0.82 ± 0.05	2.29 ± 0.10
H	1.00 ± 0.02	1.47 ± 0.01	2.47 ± 0.03
I	1.96 ± 0.03	4.02 ± 0.04	5.98 ± 0.07
J	0.55 ± 0.00	0.58 ± 0.01	1.13 ± 0.01
K	1.82 ± 0.08	3.34 ± 0.02	5.16 ± 0.10
L	1.26 ± 0.02	5.22 ± 0.14	6.48 ± 0.16

a: All results were presented as mean ± SD (n=3).
Flavonoid content (%) of solid samples = flavonoid (μg/mL) × total volume of ethanol extract (mL) ÷ sample weight (g) × dilution factor × 10^{-6} (g/μg) × 100. Flavonoid contents (%) of tinctures = flavonoid (μg/mL) × dilution factor × 10^{-6} (g/μg) × 100.

b: Levels calculated as quercetin equivalents.

c: Levels calculated as naringenin equivalents.

To avoid bias, we conducted the quantitative determination of flavonoid contents in various propolis samples by 2,4-dinitrophenylhydrazine method in addition to aluminum chloride method. Since the flavones, flavonols and isoflavones investigated formed complexes only with aluminum chloride, while flavanones strongly reacted only with 2,4-dinitrophenylhydrazine, the contents determined by the two methods were added up to evaluate the total content of flavonoids. Results showed that, among the six raw propolis samples, Brazil propolis contained the lowest level of total flavonoids (10.38 ± 0.14%), while the three samples obtained from Taiwan contained higher levels of total flavonoids (20.60 –24.91%) (Table 4). However, there was tremendous variation in total flavonoid contents of commercial propolis products (Table 5), indicating that the quality of commercial products does require verification.

CONCLUSIONS

With various biological activities, flavonoids are as key candidate compounds for evaluating the quality of propolis products. However, the convenient colorimetric method utilizing aluminum chloride reaction to determine flavonoid contents was proved to be specific only for flavones and flavonols, while another colorimetric method utilizing 2,4-dinitrophenylhydrazine reaction was specific for flavanones. Therefore, we suggest both analyses be conducted so that the sum of the results may better represent the real content of total flavonoids.

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以兩種互補的呈色法測定蜂膠中總類黃酮物質含量

張嘉琪[1]　楊明華[2*]　溫惠美[1]　陳景川[1]

1. 國立屏東科技大學食品科學系　屏東縣內埔鄉學府路1號
2. 弘光技術學院食品營養系　台中縣沙鹿鎮中棲路34號

（收稿：January 25, 2002；接受：May 16, 2002）

摘　要

類黃酮具有多種生理活性，咸認是蜂膠中的關鍵成分。本研究利用氯化鋁反應及2,4-雙硝基苯肼反應這兩種互補的呈色法，針對蜂膠中的類黃酮物質進行定量分析。結果顯示將兩種方法分別測得的含量相加後的總和可能較接近類黃酮物質的真正總含量。6件原膠中的類黃酮總含量在10.38 ± 0.14%至24.91 ± 0.53%之間，而就12件市售蜂膠產品所作的分析則發現，液態（酒精溶液）商品中的類黃酮總含量都在7%以下，粉末狀商品中的類黃酮總含量則在2.97 ± 0.05至22.73 ± 0.72%之間。

關鍵詞：蜂膠，類黃酮，黃酮，黃酮醇，黃烷酮，黃烷醇，定量分析，呈色法，氯化鋁反應，2,4-雙硝基苯肼反應