行政院國家科學委員會專題研究計畫　成果報告

化妝品與皮膚色素淡化製品中黃酮類測定方法之研究

計畫類別：個別型計畫
計畫編號：NSC93-2113-M-041-002-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：嘉南藥理科技大學醫藥化學系

計畫主持人：王來好

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處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中　華　民　國 94 年 9 月 8 日

行政院國家科學委員會補助專題研究計畫ˇ□ 成 果 報 告
□期中進度報告

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中文摘要

利用高效能液相層析儀連結光二極體列陣偵測器，測定藥用植物與化妝品原料中類黃酮之成份。探討波長、移動相組成、溶劑選擇與萃取等樣品處理對分離之影響。以Phenomenex C_{18} (25 cm × 4.6 mm i.d.)管柱，甲醇-磷酸 (pH 2.0) 移動相，可在短時間內同時分離十二種類黃酮。

Abstract

The separation of flavonoids in medicinal plants and raw cosmetics was described by high performance liquid chromatography with photodiode array detector. The influence of wavelength, mobile phase component, sample treatment including solvent selection and extraction and other conditions affecting the separation were optimized. A methanol / phosphoric acid (pH 2.0) with Phenomenex C_{18} (25 cm × 4.6 mm i.d.) column allowed to separate twelve of flavonoids in medicinal plants and raw cosmetics with high resolution and short analysis time.

報告內容

前言

Flavonoids are based upon a fifteen-carbon skeleton. The skeleton consists of two benzene rings linked via a heterocyclic pyrane ring that can be divided into a variety of classes [1-2]. The general structures of flavones such as flavone, apigenin and luteolin, flavonols such as quercetin, kaempferol, myricetin and fisetin and flavanones such as flavanone, hesperetin, naringenin are shown in Fig. 1. Flavonoids have antioxidant properties depending on the hydroxylation of the benzene rings. This property may explain an anticarcinogenic effect [1-7]. Nevertheless, the mutagenicity of flavonoids in bacteria has been found to be dependent on the presence of a hydroxy group at position 3 and the double bond between position 2 and 3 [8-9].

研究目的

Many methods for the determination of flavonoids have been reported, including spectrofluorimetry [10], high-performance liquid chromatography with ultraviolet [11-15], mass spectrometric detections [16-21] and capillary electrochromatographic techniques [22], and voltammetry [23-24]. Reports on the determination of flavonoids in urine and serum were with gas chromatography / mass spectrometry [25-26], liquid chromatography / ultraviolet and electrochemical detection [27-31]. However, these methods have previously been used for the

characterization and quantification of limited constituents in one or two kinds of fruits and vegetables.

Photodiode array detectors make it possible to obtain the spectrum of an unknown flavonoid during a single chromatographic run. The purpose of the present work was to develop a screening method for the simultaneous detection of flavonoids in medicinal plants. Sample preparation, stability, and reproducibility were targeted in the current research.

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研究方法

Evaluation of extraction efficiency

Extraction was performed by adding 0.25g pulverized medicinal plants to a mixture of methanol, 50 % aqueous methanol and water (20 ml), followed by stirring and refluxing at 70℃ in water bath for 30 min. The extract was separated from the medicinal plant powder by centrifugation at 6000 rpm for 30 min. The supernatant volume was extracted with 15 ml hexane, ether, ethyl acetate, chloroform and dichloromethanol / chloroform (20: 80, *v/v*) and evaporated to dryness with a stream of nitrogen, respectively. The above procedure was repeated three times. Methanol (1 ml) was immediately added to the residue, and after mixing with vortex-mixer for 5 min. The final solution was filter through 0.45 μm and 0.2 μm membrane filters before LC analysis.

Determination by liquid chromatography

Reverse- phase LC was on a Phenomenex C_{18} (25 cm × 4.6 mm i.d.) column. The separation was performed using two gradients. The mobile phase and gradient conditions: gradient I solvent A: methanol; solvent B: 1 M phosphoric acid, adjusted to pH 2.0 with deionized water. The gradient curve was

set at G-five. A-B (30: 70) was used as initial condition. The methanol concentration was increased from 30 % to 52 % A in 3.5 min, then to 61 % A in 4 min, to 67 % in 4 min, and finally to 40 % in 4 min. After 6 min at 40 %, the gradient was reversed to initial condition in 35 min and equilibrated for an additional 12 min before the next sample was injected. Gradient II: This gradient was developed for the separation and quantitation of flavonoids from a mixture. A-B (30: 70) was used as initial condition. The methanol concentration was linearly (G-three) increased from 30 % to 55 % in 5 min, and finally to 68 % in 4 min. The mobile phase flow rate was 0.9 ml min^{-1} The absorption was measured either as full spectrum (190 – 400 nm), at 350 nm for most constituents, or at 300 nm for flavone.

結果與討論

Table 1 Flavonoids assayed absorption by HPLC with photodiode array.

Standards	Retention time (min)	Absorption maxima (nm)
Catechin	5.92	230, 276
Rutin	10.43	225, 255, 352
Myricetin	11.97	225, 251, 304, 370
Fisetin	12.69	221, 246, 317, 358
Quercetin	15.92	226, 253, 302, 367
Naringenin	16.56	225, 287, 331
Hesperetin	17.60	228, 285, 334
Luteolin	18.16	223, 251, 265, 287, 344
Kaempferol	21.23	223, 245, 263, 317, 364
Apigenin	22.05	223, 265, 334
Flavone	27.60	213, 250, 294
Flavanone	29.07	213, 251, 321

Table 2 Statistical evaluation of flavonoids the calibration data obtained by HPLC with UV-DAD.

Flavonoids	Best wavelength (nm)	y = a + bx*	r¥	Range of linearity£ (mg l^{-1})	LOD§ (mg l^{-1})
Catechin	236	y = 10.18 + 312.7 x	0.9999	1.0- 160	0.01
Rutin	255	y = 339.7 + 1148 x	0.9999	0.5- 160	0.03
Myricetin	367	y = – 205.6 +1334 x	0.9995	0.5- 160	0.06
Fisetin	358	y = 470.7 + 2258 x	0.9999	0.5- 160	0.03
Quercetin	255	y = – 174.0 + 1403 x	0.9999	1.0- 160	0.02
Naringenin	287	y = – 156.1 + 1712 x	0.9999	0.5- 160	0.02
Hesperetin	285	y = – 584.7 + 1530 x	0.9999	0.5- 160	0.09
Luteolin	344	y = – 158.2 + 1267 x	0.9998	0.5- 160	0.03
Kaempferol	364	y = 55.41 + 1747 x	0.9997	0.5- 160	0.03
Apigenin	334	y = 1339 + 2092 x	0.9998	0.5- 160	0.03
Flavone	296	y = – 765.8 + 2300 x	0.9999	0.5- 160	0.02
Flavanone	251	y = – 713.8 + 2864 x	1.0000	1.0- 160	0.04

*a = intercept on the ordinate;

b = slope

¥r = correlation coefficient

£mg l^{-1}, which corresponds to amounts injected from 10 to 3200 ng in 20 µl

§Limit of detection (mg l^{-1}) at a signal-to-noise ratio of 3

Table 3 Concentration of major constituents found (1) acacia catechu willd and (2) white nettle in the different organic solvent.

Solvent	Concentration (mg l^{-1}) [a]			
	Acacia catechu willd		White nettle	
	Quercetin	Kaempferol	Myricetin	Catechin
Methanol	268.4	131.3	9.803	135.3
Methanol / water	292.3	101.8	63.01	381.4
Water	197.3	57.10	35.61	464.8

[a] Number of determination (n = 3)

Table 4 Recovery of flavonoids for medical plants.

Flavonoids		Sample		
		Chinese medicines		
		Added (mg l^{-1})	Found (mg l^{-1})	Recovery (%, n = 3[a])
Quercetin	1	12.00	12.02	100 (0.4%)[b]
	2	24.00	24.10	101 (0.3%)
Naringenin	1	12.00	11.90	99 (0.2%)
	2	48.00	47.12	98 (0.1%)
Kaempferol	1	24.00	24.04	100 (0.03%)
	2	48.00	48.00	100 (0.2%)
Luteolin	1	12.00	12.07	101 (0.6%)
	2	24.00	23.50	98 (0.2%)
Apigenin	1	24.00	23.95	100 (0.1%)
	2	24.00	23.99	100 (0.2%)
Flavone	1	24.00	24.04	100 (0.5%)
	2	48.00	47.77	100 (0.2%)
Flavanone	1	12.00	12.03	100 (0.5%)
	2	48.00	47.48	99 (0.2%)

[a] Number of determination

[b] R. S. D., relative standard deviation

Table 5 Analytical results of determination of twenty-four medical plants and propolis by HPLC.

Family／Species	Concentration (mg l^{-1}) [a]									
	Quercetin	Naringenin	Kaempferol	Myricetin	Luteolin	Apigenin	Catechin	Rutin	Flavone	Hesperetin
LEGUMINOSAE										
Trigonella foenum-graecum L.	—	14.20 (20%) [b]	—[c]	—	—	—	—	—	—	—
Acacia catechu willd	—	—	—	—	13.16 (0.18%)	—	—	—	—	—
Sophora japonica L.	213.9 (2.0%)	—	165.4 (1.3%)	—	—	—	—	179.8 (0.85%)	—	—
Glycyrrhiza uralensis Fisch.	—	52.62 (3.0%)	—	—	—	—	—	—	—	—
ZINGIBERACEAE										
Languas galanga (L.) stuntz75	—	—	591.2 (3.0%)	104.1 (6.2%)	—	—	—	—	—	—
Alpinia officinarum Hance	—	—	63.94 (2.4%)	—	—	—	—	—	68.60 (2.5%)	—
THEACEAE										
Camelia sinensis O. Ktze.	—	—	6.425 (0.97%)	—	7.954 (4.4%)	—	—	210.2 (1.0%)	—	—
GINKGOACEAE										
Ginkgo biloba L.	58.48 (6.9%)	—	—	—	—	48.33 (0.6%)	—	—	—	—

Table (Contd.)

Family／Species	Concentration (mg l^{-1})									
	Quercetin	Naringenin	Kaempferol	Myricetin	Luteolin	Apigenin	Catechin	Rutin	Flavone	Hesperetin
ROSACEAE										
Malus pumila Mill (apple skin)	—	—	—	—	—	—	—	69.03 (2.9%)	—	—
Prunus persica batsch	—	—	—	—	—	27.53 (3.7%)	—	—	—	22.85 (3.2%)
UMBELLIFERAE										
Daucus carota L. var. sativa DC.	1 —	—	—	15.44 (3.1%)	—	4.618 (5.0%)	—	—	—	—
(radish)	2 ?.435 (6.2%)	—	—	—	—	—	—	17.91 (1.6%)	—	—
SAPINDACEAE										
Sapindus mukorossi Gaertn.	—	—	—	—	—	21.51 (2.7%)	—	—	—	—
FAMILY APIDAE										
Apis cerana Fabricius	26.14 (1.9%)	37.83 (10%)	—[c]	20.83 (2.2%)	—	18.08 (3.3%)	—	—	—	—
FAMILY APIDAE										
Apis cerana Fabricius	26.14 (1.9%)	37.83 (10%)	—[c]	20.83 (2.2%)	—	18.08 (3.3%)	—	—	—	—
SAPINDACEAE										
Sapindus mukorossi Gaertn.	—	—	—	—	—	21.51 (2.7%)	—	—	—	—

Table (Contd.)

Family／Species	Concentration (mg l^{-1})									
	Quercetin	Naringenin	Kaempferol	Myricetin	Luteolin	Apigenin	Catechin	Rutin	Flavone	Hesperetin
LAURACEAE										
Cinnamomum japonicum Sieb.	—	2694 (3.1%)	—	—	—	—	2515 (4.7%)	—	—	—
CUPRESSACEAE										
Biota orientalis (L.) Endl.	23.95 (0.9%)	28.39 (0.0%)	—	3123 (2.3%)	—	—	—	—	—	—
NYMPHAEACEAE										
Nelumbo nucifera Gaertn.	27.14 (1.1%)	10.84 (2.0%)	143.2 (1.8%)	—	—	—	—	—	—	—
MAGNOLIACEAE										
Magnolia lilifloraa Desr.	—	—	—	—	—	—	—	408.2 (3.6%)	—	—
EQUISETACEAE										
Equisetum debile Roxb. (horsetail)	—	1.499 (30%)	—	—	—	—	—	—	—	—
LILIACEAE										
Allium cepa L. (onion)	—	—	—	—	—	—	—	6.253 (4.3%)	—	—

[a] Number of determination (n = 3)

[b] R.S.D., relative standard deviation

[c] — : not indicate

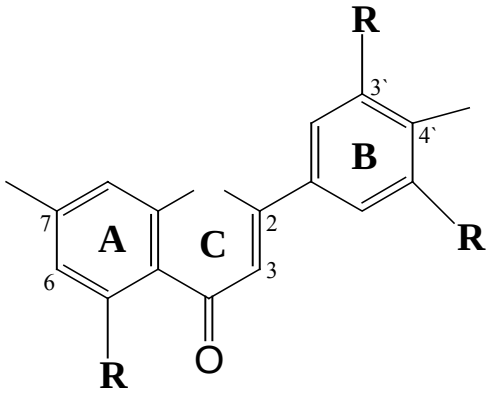
Table 6 Analytical results of determination of raw cosmetics by HPLC.

Family／Species	Concentration (mg l^{-1})[a]					
	Quercetin	Kaempferol	Myricetin	Luteolin	Apigenin	Rutin
COMPOSITAE						
phytexcell, calendula	242.2 (1.5%)[b]	—[c]	—	—	—	32.85 (3.8%)
phytexcell chamomile	—	—	—	13.90 (1.6%)	13.92 (0.7%)	—
GINKGOACEAE						
phytexcall ginkgo biloba	20.68 (0.23%)	0.6786 (1.9%)	52.31(0.98%)	—	—	—
URTICACEAE						
phytexcell white nettle	10.06 (2.5%)	0.6187 (2.8%)	28.24 (1.7%)	—	—	—

[a] Number of determination (n = 3)

[b] R.S.D., relative standard deviation

[c] — : not indicate

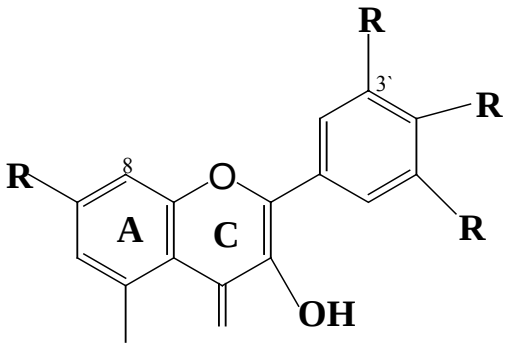


Flavone R_3`, $R_4$`, R_5 , R_5` , R_7 = H

Luteolin R_3`, $R_4$`, R_5 , R_7 = OH, R_5` = H

Apigenin R_4` , $R_5$ , $R_7$ = OH, $R_3$` , R_5` = H

Flavones



Myricetin R_3` , $R_4$`, R_5 , R_5`, R_7 = OH

Quercetin R_3 , R_3` , $R_4$` , R_5 , R_7 = OH,

R_5` = H

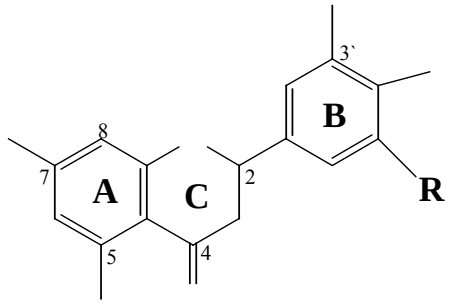
Fisetin R_3 , R_3`, $R_4$`, R_7 = OH, R_5 , R_5` = H

Kaempferol R_3 , R_4`, R_5 , R_7 = OH,

R_3`, $R_5$` = H

Rutin R_3` , $R_4$` , R_5 , R_5` , R_7 = H

Flavonols



Flavanone R_3`, $R_4$` , R_5 , R_5`, R_7 = H

Naringenin R_4`, $R_5$ , $R_7$ = OH, $R_3$` , R_5` = H

Hesperetin R_4` = $OCH_3$, $R_3$` , R_5 , R_7 = OH

R_5` = H

Catechin R_3 , R_3`, $R_4$`, R_5 , R_7 = OH

Flavanones

Fig. 1 chemical structures of the flavanoids studied

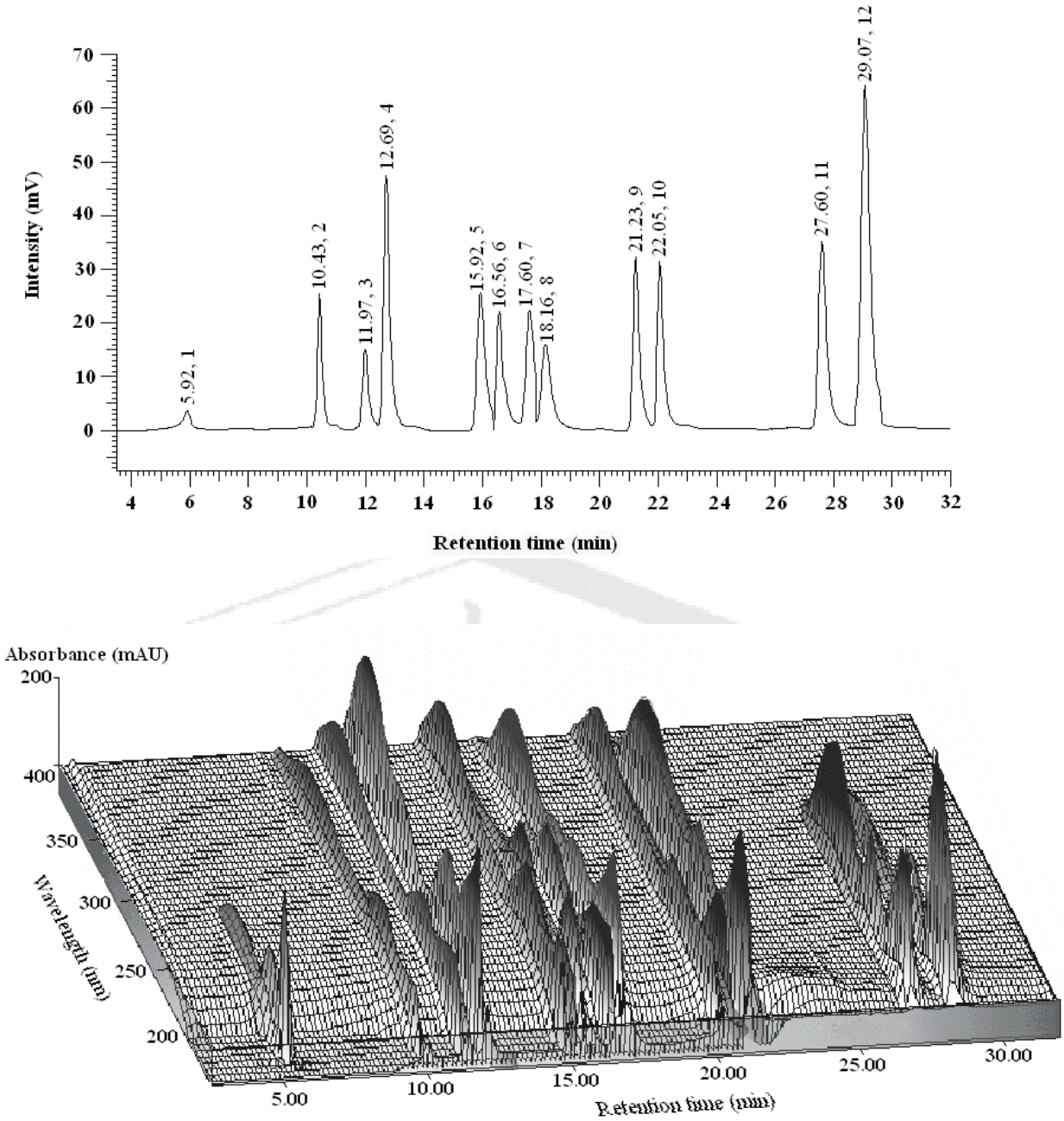


Fig. 2 Chromatogram and three-dimensional spectrochromatogram of flavonoids standards. Column on Phenomenex C_{18} (2) (25 cm × 4.6 mm). Gradient: 30 % CH_3OH / 70% H_2O (phosphoric acid, pH = 2) to 55 % CH_3OH / 45 % H_2O to 68 % CH_3OH / 32% H_2O. Flow rate was 0.9 mL min^{-1}. Detection: UV-DAD. Peak identification: **1** = Catechin, **2** = Rutin, **3** = Myricetin, **4** = Fisetin, **5** = Quercetin **6** = Naringenin, **7** = Hesperetin, **8** = Luteolin, **9** = Kaempferol, **10** = Apigenin, **11** = Flavone, **12** = Flavanone.

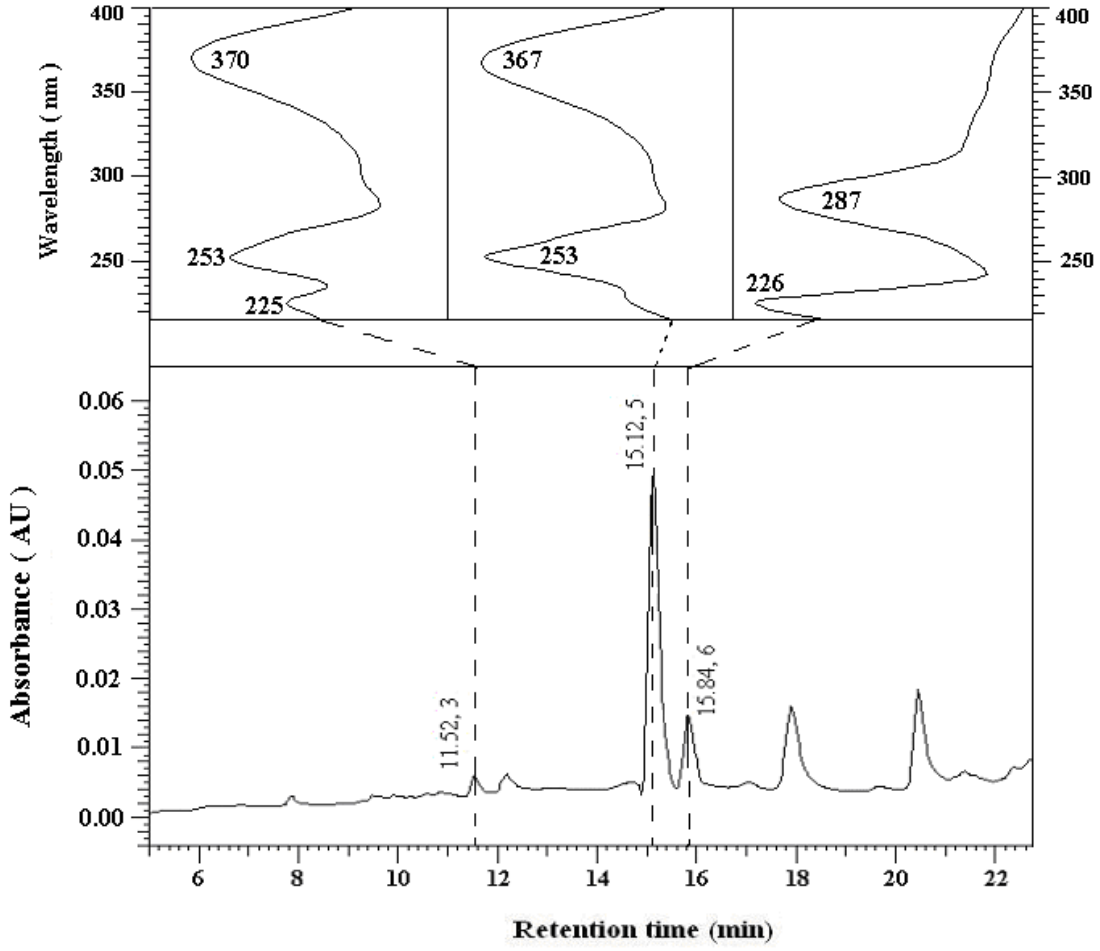


Fig. 3 Flavanoids profile of a commercial propolis. Detection: UV-DAD. Revesed-phase HPLC and UV-VIS spectra of a methanol-water extract of propolis. Assignment of peak number according to Fig 2.