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GENERAL METHOD FOR DETERMINING FLAVONOIDS IN MEDICINAL PLANTS AND RAW COSMETICS USING HPLC WITH A PHOTODIODE ARRAY DETECTOR

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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. Elution with methanol/phosphoric acid (pH 2.0) in a 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 time of analysis.

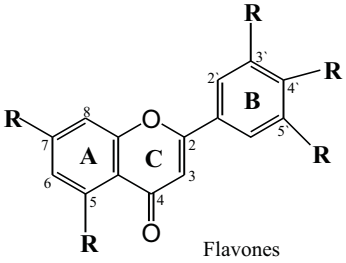
Flavonoids are based upon a fifteen-carbon skeleton, which comprises two benzene rings linked via a heterocyclic pyrane ring that can be divided into a variety of classes [1, 2]. The general structures of flavones (e.g., flavone, apigenin, and luteolin), flavonols (e.g., quercetin, kaempferol, myricetin and fisetin), and flavanones (e.g., flavanone, hesperetin, naringenin) are shown in Fig. 1. Flavonoids exhibit antioxidant properties, which depend on the hydroxylation of the benzene rings. This activity may explain the anticarcinogenic effect [1 – 7] of these compounds. At the same time, the mutagenicity of flavonoids in bacteria has been found to depend on the presence of a hydroxy group at position 3 and the double bond between positions 2 and 3 [8, 9]. Numerous methods for the determination of flavonoids have been reported, including spectrofluorimetry [10], high-performance liquid chromatography (HPLC) with ultraviolet [11 – 15], mass spectrometric [16 – 21], capillary electrochromatographic [22], and voltammetric [23, 24] detection. The determination of flavonoids in urine and serum was also performed with gas chromatography/mass spectrometry (GC/MS) [25, 26], liquid chromatography/ultraviolet (LC/UV), and electrochemical detection [27 – 31]. However, these methods have previously been used for the characterization and quantification of a limited number of components in a few kinds of fruits and vegetables.

PADs make it possible to obtain the spectrum of unknown flavonoids 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. This paper reports on the sample preparation, stability of results, and reproducibility of measurements.

EXPERIMENTAL PART

Instrumentation. HPLC was performed with a system comprising a Hitachi Model L-7100 pump, a Model 7125 injector equipped with a 20μl sample loop, and a Model L-7455 photodiode array detector. Chromatograms were acquired and peak areas calculated by means of D-7000 Chromatographic Data Integrator.

Reagents and materials. We have used a series of reference flavonoids, including fisetin, quercetin, hesperetin, luteolin, apigenin, flavanone (from Lancaster, Eastgate, White Lund, Morecambe, England), catechin, naringenin (Aldrich, Milwaukee, WI 53201, USA), myricetin, flavone (Acros, Geel, Belgium), rutin (Alfa Aesar, Ward



Flavones

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

Flavonols

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

Flavanones

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

Fig. 1. Chemical structures of flavonoids.

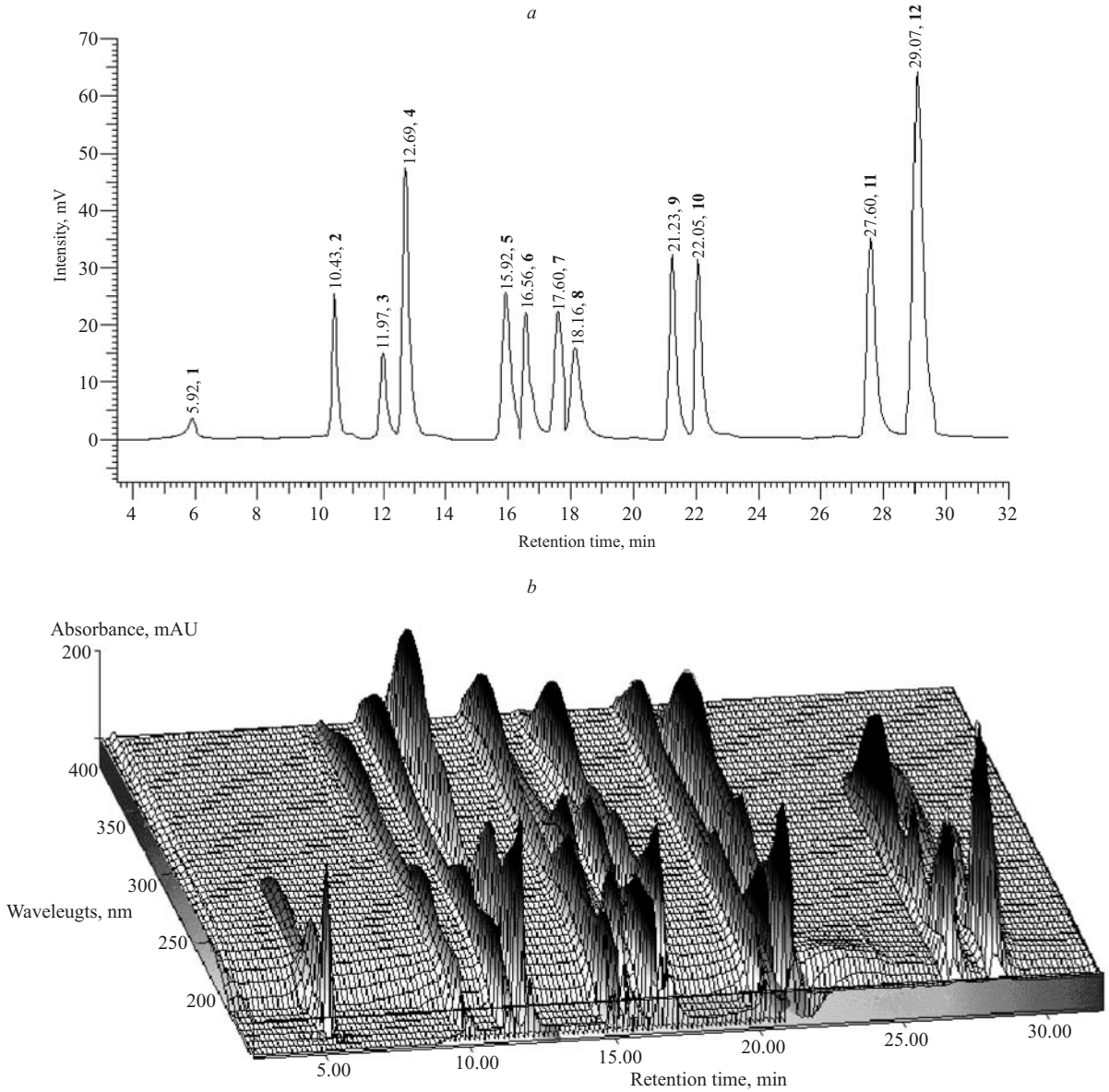


Fig. 2. Typical (*a*) chromatogram and (*b*) three-dimensional spectrochromatogram of reference flavonoids. Column: Phenomenex C_{18} (2) (25 cm × 4.6 mm); gradient (%): 30 CH_3OH/70 H_2O (phosphoric acid, pH 2) to 55 CH_3OH/45 H_2O to 68 CH_3OH/32 H_2O; flow rate, 0.9 ml/min; detection: UV-PAD. Peak 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.

Hill, USA), and kaempferol (TCI, Tokyo Kasei, Kogyo Co., Japan). Cosmetic raw materials included white nettle, chamomile, horsetail, calendula, and ginkgo biloba (from Phytexcell Crodarom, France). Samples of medicinal plants were bought from a number of retail outlets in the south of Taiwan.

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 were as follows. Gradient I: solvent A (methanol) and solvent B (1 M phosphoric acid adjusted at pH 2.0 with deionized water). The gradient curve was set at G-five; A – B (30 : 70) was used as the initial condition. 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 keeping at 40% for 6 min, the gradient was reversed to the initial

Table 1

Retention Times and Absorption Peaks of Flavonoids Assayed by HPLC with Photodiode Array Detector

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 and Calibration Data obtained by HPLC with UV Photodiode Array Detector

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

Note: * a is the intercept on the ordinate; b is the slope; r•• is the correlation coefficient; ** Range of linearity: concentrations (mg/liter) corresponding to amounts (from 10 to 3200 ng) injected in 20 μl; *** LODS is the limit of detection (mg/liter) at a signal-to-noise ratio of 3.

condition in 35 min and then 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. 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 always 0.9 ml/min. The absorption was measured either as a full spectrum (within 190 – 400 nm), at 350 nm for most constituents, or at 300 nm for flavone.

Evaluating the extraction efficiency. The Extraction was performed by adding 0.25 g pulverized medicinal plants to a mixture of methanol, 50% aqueous methanol, and water (20 ml), followed by stirring and refluxing at 70°C on a 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 dichloromethane – chloroform mixture (20 : 80, v/v), and evaporated to dryness in a flow of nitrogen. 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 sequentially filtered through 0.45 μm and 0.2 μm membrane filters prior to the LC analysis.

Table 3

Concentrations of Major Constituents Found in Acacia Catechu and White Nettle Extracted with Different Organic Solvents

Solvent	Concentration (mg/liter)[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

Note: [a] Number of determinations, $n = 3$.

RESULTS AND DISCUSSION

Optimization of the HPLC separation conditions. Since medicinal plants consist of many compounds with different properties, some components being highly polar (e.g., myricetin) and some polar (e.g., flavanone), the separation was very complicated. Different proportions of organic solvents (e.g., methanol – water or acetonitrile – water mixtures) were tried but the separation was still unsatisfactory. With methanol – water (30 : 70, v/v) as mobile phase, the elution time of apigenin, flavone and flavanone was prolonged and their separation was improved. However, the peaks of some early eluted polar flavonoids such as quercetin, luteolin, fisetin and naringenin were still overlapped. For the separation and quantitation of flavonoids by HPLC, several methanol – 8 mM phosphate gradients were employed. Two of these gradients that

Table 4

Recovery of Flavonoids from Chinese Medical Plant Preparations

Flavonoids		Sample		
		Chinese medicines		
		Added (mg/liter)	Found (mg/liter)	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)

Note: [a] Number of determinations; [b] percentage mean recovery (percentage RSD).

Table 5

Results of Flavonoid Determination in 24 Medicinal Plants and Propolis by HPLC

Family Species	Concentration (mg/liter)[a]									
	Quercetin	Naringenin	Kaempferol	Myricetin	Luteolin	Apigenin	Catechin	Rutin	Flavone	Hesperetin
LEGUMINOSAE										
Trigonella foenum-graecum L.	–	14.20 (2.0%) [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%)	–	–	–	–
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.	–	–	–	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%)	–	–	–	–
SAPINDACEAE										
Sapindus mukorossi Gaertn.	–	–	–	–	–	21.51 (2.7%)	–	–	–	–
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%)	–	–

Note: [a] number of determinations, $n = 3$; [b] mean value (RSD); [c] No data.

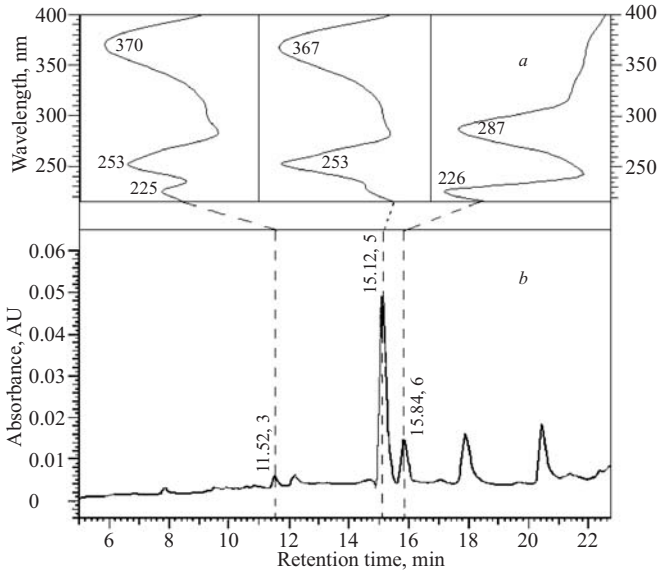


Fig. 3. Flavonoid profiles of commercial propolis (detector, UV-PAD): (*a*) UV-VIS spectra of a methanol – water extract and (*b*) reverse-phase HPLC pattern (assignment of peaks according to Fig 2).

separated the mixture most effectively (gradients I and II) were described above. A mixture consisting of twelve components was resolved using gradient I. However, quercetin, naringenin, hesperitin and luteolin did not completely resolve on gradient I. Then, gradient II was developed for separating these four components from the mixture as shown in Fig. 2. Table 1 lists the retention times and absorption maxima of all compounds recognized in the plant extracts used in this study. The simultaneous determination of these compounds with marked differences in polarity requires the use of a gradient elution program. The identity of registered peaks was confirmed, first, by comparing the observed retention times of the peaks with those of the individual standard solutions. Further peak characterization was performed by means of a PAD.

Linearly and limit of detection. The calibration plots for all twelve compounds were determined at both 300 and 350 nm. Their slopes, intercepts, correlation coefficients, and the limits of detection are listed in Table 2. The intensity of UV absorption was linearly dependent on the concentrations of flavonoids over a wide range up to 0.5 mg/l. The limit of detection (LOD) was calculated using the equation LOD = KS_0/S, where K is a numerical factor (chosen according to the desired confidence level), S_0 is the standard deviation of the blank measurement (n = 3), and S was the sensitivity of the calibration plot. In this work, we used K = 3; the LOD values obtained in our experiments are presented in Table 2.

Extraction efficiency. Data on the extraction efficiency are summarized in Table 3. The extraction was performed with hot solvent; then the mixture was centrifuged and each supernatant was re-extracted with organic solvents of various polarities. Methanol – water (50 : 50) was the best first extractant; an extract obtained with this solvent was immiscible with chloroform and dichloromethane, so that the organic and aqueous layers could be separated. Because of its polarity and relatively high cost, aqueous methanol is the solvent of choice for commercial medicinal plants and propolis preparations.

Validation: reproducibility and recovery. Reproducibility of the extraction procedure was assessed by measuring the peak area variation for seven major peaks in three replicate analyses. Data on the relative standard deviation (RSD) values of flavonoids determined in medicinal plant preparations are shown in Table 4. As can be seen from this table, the RSD values for seven major peaks were less than 1%, which is quite acceptable for quantification. By spiking the standard solutions of flavonoids, the recoveries of these components were measured. The amount of a spiked flavonoids was calculated by subtracting the total amount of flavonoids after spiking from the amount found in the plant before spiking. The spiking experiments were repeated three times for two different concentrations to obtain the mean total amount of flavonoids after spiking.

Stability of compounds. Samples were stored in the dark for 24 h at 4, 21, and 45°C and then the chromatograms were recorded. The peak area remained almost constant upon storage at 4°C and increased by approximately 20% at 45°C. To determine the effect of light on the stability of flavonoids, two batches were prepared. One batch was protected from the light, the other was not, and both were kept at room temperature for 1, 24, 48, and 72 h.

Table 6

Results of Flavonoid Determination in Raw Cosmetics by HPLC

Family Species	Concentration (mg/liter)[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

There was no substantial difference between 24 h and 48 h, except that the peak areas for apigenin and flavone increased by 10% for 48 h. Peak areas increased by approximately 24% for flavonoids, whereas the values for catechin and luteolin were almost constant for 72 h.

Sample analysis. The proposed gradient HPLC procedure was applied to the analysis of flavonoids in medicinal plants and raw cosmetics. The primary purpose was to demonstrate the applicability of the extraction and analysis procedure described above for the analysis of flavonoids present in these samples. With the aid of authentic reference samples of flavonoids, the amount of these twelve compounds in the plants and propolis were determined. Table 5 and 6 show the data for several components present in the plants and raw cosmetics, respectively. As can be seen from Table 5, six main flavonoids in *ginkgoaceae*, *leguminosea* and *zingiberaceae* plants were flavones (apigenin, luteolin), flavonols (quercetin, myricetin, kaempferol), and flavanones (naringenin). Figure 3 shows the typical HPLC patterns of flavonoids extracted from propolis samples and chromatographed using methanol – water mixtures.

In conclusion, the use of a PAD detector in combination with reverse-phase gradient HPLC provides information-rich detection. Highly polar and polar flavonoids, which generally occur in plants, can be reliably separated and analyzed in a single run within 30 min.

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ОПРЕДЕЛЕНИЕ ФЛАВОНОИДОВ В ЛЕКАРСТВЕННЫХ РАСТЕНИЯХ И ОСНОВАХ КОСМЕТИЧЕСКИХ ПРЕПАРАТОВ МЕТОДОМ ВЫСОКОЭФФЕКТИВНОЙ ЖИДКОЙ ХРОМАТОГРАФИИ С ФОТОДИОДНЫМ МАТРИЧНЫМ ДЕТЕКТОРОМ

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Описан анализ флавоноидов в лекарственных растениях и основах косметических препаратов методом высокоэффективной жидкой хроматографии с фотодиодным матричным детектором (позволяющим получить спектр поглощения во всей исследуемой области за один цикл). Подобраны оптимальные условия экстракции и анализа (растворители, состав подвижной фазы, длина волны). С использованием колонки Phenomenex C_{18} (25 см × 4.6 мм) и смеси метанола и ортофосфорной кислоты (pH 2.0) проведено надежное и быстрое разделение 12 флавоноидов в лекарственных растениях различных семейств и косметических препаратах на их основе.