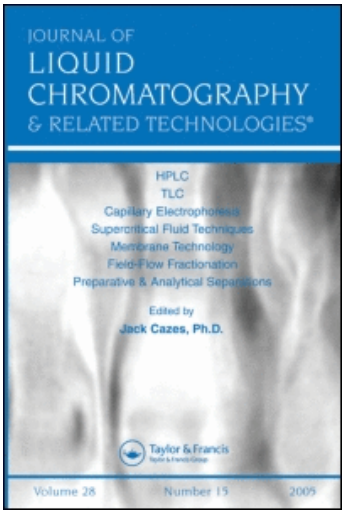

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Phenolic Compounds from *Geranium sanguineum* by HPLC

S. Leucuta[a]; L. Vlase[a]; S. Gocan[b]; L. Radu[b]; C. Fodorea[c]

[a] Faculty of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, 'Iuliu Hateganu' University of Medicine and Pharmacy, Cluj Napoca, Romania [b] Faculty of Chemistry and Chemical Engineering, Analytical Chemistry Department, 'Babes-Bolyai' University, Cluj Napoca, Romania [c] Faculty of Pharmacy, Pharmaceutical Botany Department, 'Iuliu Hateganu' University of Medicine and Pharmacy, Napoca, Romania

To cite this Article Leucuta, S. , Vlase, L. , Gocan, S. , Radu, L. and Fodorea, C.(2005) 'Determination of Phenolic Compounds from *Geranium sanguineum* by HPLC', Journal of Liquid Chromatography & Related Technologies, 28: 19, 3109 — 3117

To link to this Article: DOI: 10.1080/10826070500295211

URL: http://dx.doi.org/10.1080/10826070500295211

Journal of Liquid Chromatography & Related Technologies®, 28: 3109–3117, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500295211

Determination of Phenolic Compounds from *Geranium sanguineum* by HPLC

S. Leucuta and L. Vlase
Faculty of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, 'Iuliu Hateganu' University of Medicine and Pharmacy, Cluj Napoca, Romania

S. Gocan and L. Radu
Faculty of Chemistry and Chemical Engineering, Analytical Chemistry Department, 'Babes-Bolyai' University, Cluj Napoca, Romania

C. Fodorea
Faculty of Pharmacy, Pharmaceutical Botany Department, 'Iuliu Hateganu' University of Medicine and Pharmacy, Napoca, Romania

Abstract: A high performance liquid chromatographic (HPLC) method has been developed for simultaneous determination of seventeen phenolic compounds (one phenolic acid, seven cinnamic acid derivatives, four quercetin glycosides, five flavonol, and flavone aglycones) from plant material. Separation of all examined compounds was carried out in 35 minutes on a Zorbax SB-C_{18} analytical column (100 × 3.0 mm, 3.5 μm) with methanol-KH_2PO_4 buffer (40 mM, pH 2.3) as mobile phase in a linear gradient. The linearity of calibration curves for all compounds was very good ($R2 > 0.999$). The detection limits for the determined compounds were in the range of 18.4 to 91.9 ng/mL. The method was successfully applied for the determination of phenolic compounds from *Geranium sanguineum* dried herb, the following phenolic compounds being identified: caftaric acid (1.30 mg/g), caffeic acid (2.41 mg/g), hyperoside (1.64 mg/g), isoquercitrin (2.58 mg/g), rutin (1.71 mg/g), quercitrin (0.42 mg/g), quercetin (0.82 mg/g), kaempferol (0.19 mg/g). After acid hydrolysis treatment of the plant material, when all glycosides were hydrolyzed to aglycones, the type of unidentified flavonoids present in *Geranium sanguineum* were determined as being kaempferol glycosides.

Keywords: Column liquid-chromatography, Phenolic compounds, Acid hydrolysis, Geranium sanguineum, Identification and quantification

Address correspondence to S. Gocan, Faculty of Chemistry and Chemical Engineering, Analytical Chemistry Department, 'Babes-Bolyai' University, 11 Arany Janos Street, Cluj Napoca, Romania. E-mail: radulaura@yahoo.com

INTRODUCTION

Phenolic or polyphenolic compounds constitute, by far, the largest and most widespread group of secondary plant products. They display a great variety of structures, ranging from simple compounds containing a single aromatic ring to highly complex polymeric substances such as tannins and lignin. The phenolic compounds, which occur commonly in plant material, may be classified into three groups, namely, simple phenols and phenolic acids, hydroxycinnamic acid derivatives, and flavonoids.

Phenolic compounds have been reported to have multiple biological effects, including antioxidant activity, as they can scavenge free hydroxyl and peroxyl radicals.[1–3] According to epidemiological studies the intake of flavonoids is inversely correlated with the risk of coronary heart disease and cancer.[4] Plant extracts, rich in phenolics, are of increasing interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food.[5]

Several methods have been described for the determination and quantitation of phenolic compounds in food and plant material, most of them involved reversed phase HPLC.[6–8] A recent review[9] has focused on the developments of high performance liquid chromatographic (HPLC) analysis of plant material during the last 10 years.

The main objective of our study was to establish a simple, rapid analytical system for the simultaneous separation by HPLC of seven cinnamic acid derivatives, four quercetin glycosides, five flavonol and flavone aglycones of plant origin. The structures of these compounds are given in Figure 1. This method was applied for the characterization and quantification of phenolic constituents in *Geranium sanguineum.* For obtaining more accurate data on flavonol and flavone glycosides and aglycones concentration, the plant material was subjected to acid hydrolysis of all glycosides to aglycone. As flavonols and flavones are often present in plants as glycosides, hydrolysis treatment provides useful information about the type of flavonoid and the location of free hydroxyl groups.

EXPERIMENTAL

Chemicals

Methanol of HPLC analytical-grade, 85% orthophosphoric acid, potassium dihydrogen phosphate, and hydrochloric acid of analytical-grade were purchased from Merck (Germany). Chlorogenic acid, p-coumaric acid, caffeic acid, rutin, apigenin, quercetin, isoquercitrin, hyperoside, quercitrin, kaempferol from Sigma (Germany), ferulic acid, sinapic acid, gentisic acid, patuletin, luteolin from Roth (Germany), cichoric acid, caftaric acid from Dalton (USA).

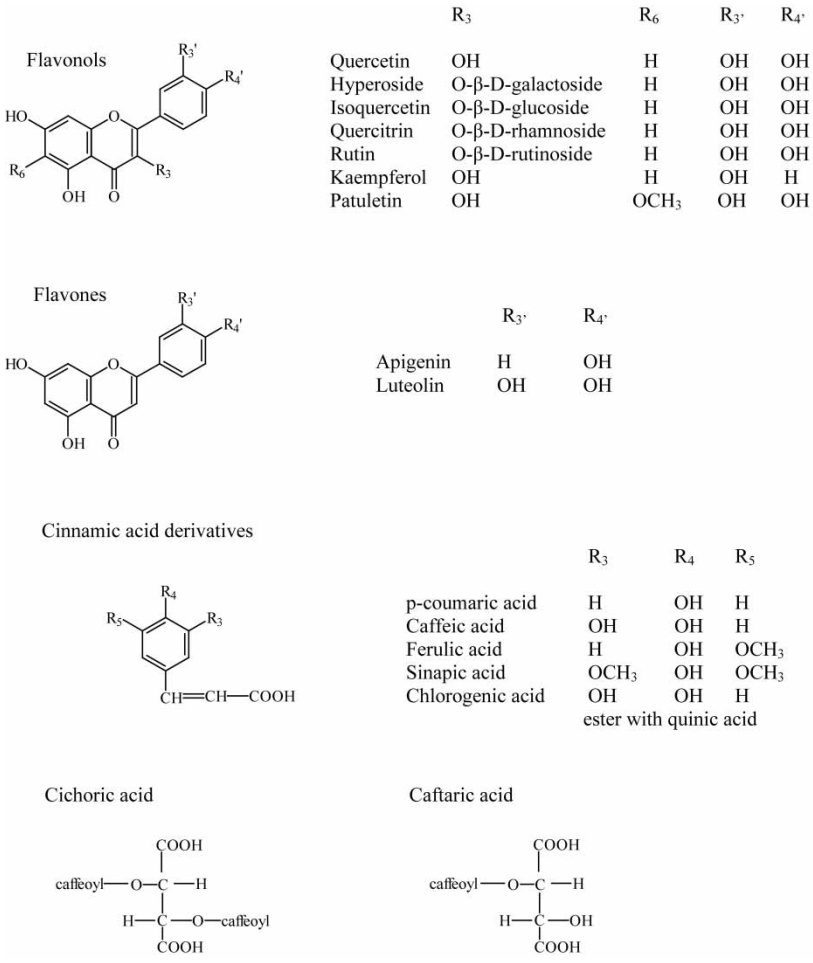

Figure 1. Structures of phenolic compounds.

Preparation of Standard Solutions

Methanolic stock solutions (100 g/mL) of the following pure commercial substances: chlorogenic acid, p-coumaric acid, caffeic acid, rutin, apigenin, quercetin, isoquercitrin, hyperoside, quercitrin, kaempferol ferulic acid, sinapic acid, gentisic acid, patuletin, luteolin, cichoric acid, and caftaric acid were prepared: All stock solutions were stored at 4°C and protected from daylight and before being used as working solutions they were appropriately diluted with water. HPLC grade water was prepared by double distillation.

Plant Material and Sample Preparation

Geranium sanguineum was collected as a wild plant in Romania and was identified in the Department of Pharmaceutical Botany, Faculty of Pharmacy, "Iuliu Hateganu" University of Medicine and Pharmacy, Cluj Napoca.

A sample of 100 mg air dried, powdered herb was placed in a 10 mL centrifuge tube. Extraction mode: (i) For the extraction of phenolic compounds (as glycosides) 2 mL water and 2 mL methanol were added in the centrifuge tube; (ii) for the extraction by simultaneous acid hydrolysis (of free aglycones) 2 mL hydrochloric acid 2M, 2 mL methanol, and 200 mL ascorbic acid solution (100 mg/mL) were added to the centrifuge tube.

Both extractions, with and without acid hydrolysis, were subjected to the same procedure: 30 minutes at 80°C on a water bath, and after 15 minutes of sonication the extractions were continued for another 30 minutes at 80°C. During the heating, from 10 to 10 minutes, 1 mL methanol was added to the extraction mixture to ensure the permanent presence of methanol in the extraction process. After extraction the mixture was centrifuged at 4000 rpm, the supernatant was collected and the remaining solid was extracted two times with 5 mL water using the same procedure. The combined extracts were diluted with water in a 25 mL volumetric flask and filtered through a 0.45 μm filter before injection.

Apparatus and Chromatographic Conditions

The experiment was carried out using an Agilent 1100 HPLC Series system equipped with an autosampler G1311A. For the separation a reversed-phase Zorbax SB-C18 analytical column (100 × 3.0 mm i.d., 3.5 μm particle) was used. The column was operated at 48°C in a G1316A oven. For the gradient elution a degasser, G1322A, and a quaternary gradient pump, G1311A, were used. The detection of the compounds was performed at 330 nm using a G1316A diode array detector system. The chromatographic data were processed using a Chemstation software (Agilent, USA).

The mobile phase was a binary gradient prepared from methanol (solvent A) and buffer solution (solvent B). The buffer solution was prepared by dissolving potassium dihydrogen phosphate (40 mM) in water and the pH of the solution was adjusted to 2.3 with 85% orthophosphoric acid. The gradient, begun with a linear gradient, started at 5% solvent A to 42% solvent A over the first 35 minutes, followed by isocratic elution with 42% solvent A over the next 3 minutes. The flow rate was 1 mL/min and the injection volume was 10 μL. All solvents were filtered through 0.5 μm (Sartorius) filters and degassed in an ultrasonic bath.

RESULTS AND DISCUSSION

This method presents several advantages, such as rapidity, sensitivity, specificity; the gradient elution also allows a good separation and identification of a wide range of phenolic compounds in one run. Figure 2 shows a typical separation of seventeen phenolic compounds of plant origin, from standard solution, with resolution values more than 1.00 in all cases. Retention times were determined with a standard deviation ranging from 0.04 to 0.15 min (Table 1). The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio grater than three. The HPLC method provided LODs in the lower nanogram range.

Quantitative determinations were performed using an external standard method. Good linearity of the calibration curve ($R2 > 0.999$) for a five-point plot was established in the $0.5–50\,\mu g\,mL^{-1}$ range for all phenolic compounds as shown in Table 2.

The intra- and inter-day precision, calculated as RSDs, varied from 0 to 1.2% ($n = 5$) and from 0.2 to 1.8% ($n = 15$), respectively, using concentration levels of $5\,g/mL$ for each phenolic compound.

Accuracy was checked by spiking samples with a solution containing each phenolic compound in a concentration of $10\,\mu g/mL$. The RSDs for accuracy varied from 0.2 to 0.8%. Considering as validation criteria for precision a RSD not higher than 2% and for accuracy a RSD not higher than 1%, the method is precise and accurate.

All compounds were identified in samples by both standard addition and comparison of their retention times, and UV spectra with those of standards in the same chromatographic conditions, as shown in Figure 2.

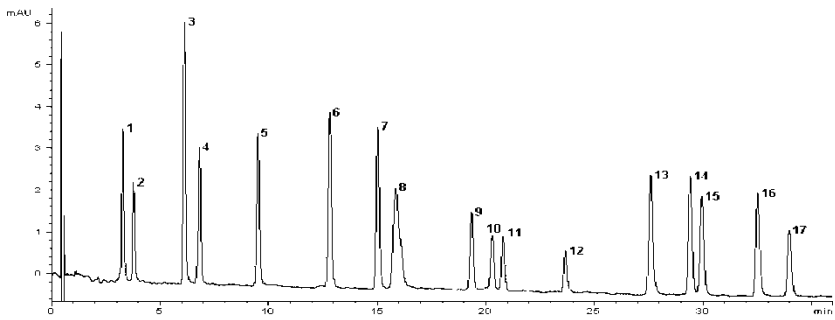

Figure 2. Typical separation of phenolic compounds. Peaks: 1) caftaric acid, 2) gentisic acid, 3) caffeic acid, 4) chlorogenic acid, 5) p-coumaric acid, 6) ferulic acid, 7) sinapic acid, 8) cichoric acid, 9) hype-roside, 10) isoquercitrin, 11) rutin, 12) quercitrin, 13) quercetin, 14) patuletin, 15) luteolin, 16) kaempferol, 17) apigenin. (For chromatographic conditions see Experimental Section.)

Table 1. Peak assignments for phenolic compounds

Peak no.	Phenolic compound	$t_R \pm$ S.D.[a](min)	Relative t_R	Rs
1	Caftaric acid	3.34 ± 0.06	0.17	–
2	Gentisic acid	3.83 ± 0.07	0.20	2.8
3	Caffeic acid	6.12 ± 0.04	0.31	13.0
4	Chlorogenic acid	6.83 ± 0.05	0.35	3.4
5	p-coumaric acid	9.48 ± 0.08	0.49	11.8
6	Ferulic acid	12.8 ± 0.10	0.66	13.0
7	Sinapic acid	15.0 ± 0.10	0.77	8.4
8	Cichoric acid	15.96 ± 0.13	0.83	2.0
9	Hyperoside	19.32 ± 0.12	1	8.4
10	Isoquercitrin	20.29 ± 0.10	1.05	3.3
11	Rutin	20.76 ± 0.15	1.07	1.7
12	Quercitrin	23.64 ± 0.13	1.22	10.0
13	Quercetin	27.55 ± 0.15	1.42	12.7
14	Patuletin	29.41 ± 0.12	1.52	5.7
15	Luteolin	29.64 ± 0.19	1.54	1.7
16	Kaempferol	32.48 ± 0.17	1.68	7.9
17	Apigenin	39.45 ± 0.15	1.76	4.3

[a]t_R = retention time; S.D. = standard deviation.

In order to verify the applicability of the proposed analytical method, qualitative and quantitative determinations of phenolic compounds were carried out on a *Geranium sanguineum* herb. Figure 3 shows the chromatogram of the *Geranium sanguineum* extract for the analysis of phenolic compounds, while Figure 4 presents the chromatogram of *Geranium sanguineum* extract obtained using simultaneous acid hydrolysis.

The calibration curves were used to determine the analyte concentration in plant samples. The concentration of phenolic compounds determined in the

Table 2. The phenolic compounds determined in *Geranium sanguineum*

Peak no	Phenolic compound	Concetration in plant material (mg/g)
1	Caftaric acid	1.30
3	Caffeic acid	2.41
9	Hyperoside	1.64
10	Isoquercitrin	2.58
11	Rutin	1.71
12	Quercitrin	0.42
13	Quercetin	0.82
16	Kaempferol	0.19

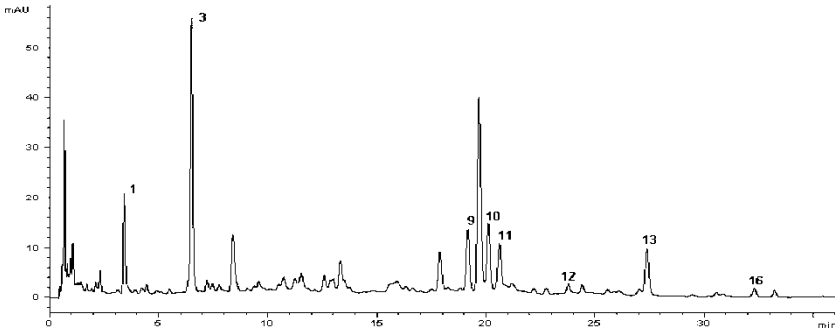

Figure 3. Chromatogram of phenolic compounds from *Geranium sanguineum.* (For chromatographic conditions see Experimental Section.)

Geranium sanguineum extract, without hydrolysis are (presented in Table 3): caftaric acid (1.30 mg/g), caffeic acid (2.41 mg/g), hyperoside (1.64 mg/g), isoquercitrin (2.58 mg/g), rutin (1.71 mg/g), quercitrin (0.42 mg/g), quercetin (0.82 mg/g), kaempferol (0.19 mg/g). After acid hydrolysis, the *Geranium* extract contained no trace of hyperoside, isoquercitrin, rutin and quercitrin, but the concentration of quercetin (4.64 mg/g) and kaempferol (0.78 mg/g) was higher than the concentration of these aglycons which are present unbonded in the herba, suggesting that in *Geranium sanguineum* quercetin and kaempferol glycosides are present, while apigenin, patuletin or luteolin glycosides are absent.

The results obtained with and without acid hydrolysis can help in the determination of the type of unidentified flavonoids. The concentration of bonded quercetin was calculated using the correction factor for conversion of the known quercetin glycosides (rutin, hyperoside, isoquercitrin, quercitrin) to quercetin aglycon. Results are presented in Table 3.

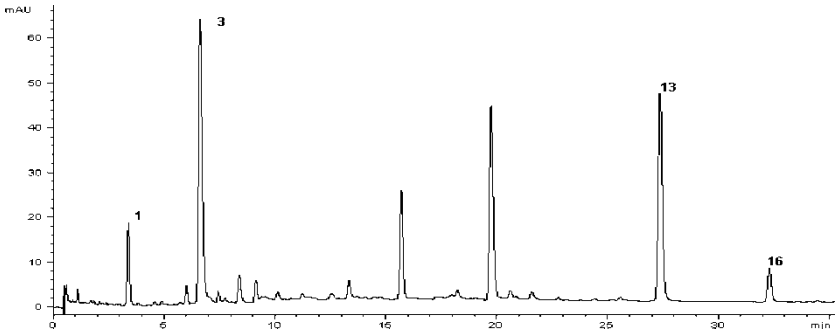

Figure 4. Chromatogram of aglycones after acid hydrolysis of *Geranium sanguineum.* (For chromatographic conditions see Experimental Section.)

Table 3. Bonded and unbonded quercetin content of *Geranium sanguineum*

	Correction factor	Concentration in plant material (mg/g)	Equivalent bonded quercetin (mg/g)
Hyperoside	302.3/464.4 = 0.65	1.64	1.06
Isoquercitrin	302.3/464.4 = 0.65	2.58	1.67
Rutin	302.3/610.5 = 0.49	1.71	0.84
Quercitrin	302.3/448 = 0.67	0.42	0.28
Quercetin	—	0.82	Total 3.85mg/g
		Bonded + unbonded quercetin = 4.67 mg/g	

The sum of bonded and unbonded quercetin from *Geranium sanguineum* was 4.67 mg/g, while the concentration of quercetin determined after extraction with acid hydrolysis was 4.64 mg/g, which leads to the conclusion that in *Geranium sanguineum* no other quercetin glycosides are present than those identified.

The unidentified flavonoids from *Geranium sanguineum* are kaempferol glycosides, as kaempferol concentration increased from 0.19 mg/g in the unbonded form to 0.78 mg/g after hydrolysis.

CONCLUSION

A rapid, highly accurate, and sensitive HPLC method for simultaneous determination of a wide range of phenolic compounds from plant material has been developed. The method was applied for the determination of phenolic compounds from *Geranium sanguineum*, a medicinal plant known for its herpes and influenza virus inhibitory effect.[10,11] The polyphenols content of *Geranium sanguineum* has been studied previously, when apigenin, quercetin, myricetin, morin, kaempferol, hyperoside, caffeic acid, chlorogenic acid, and ellagic acid were found.[12] Our study confirms the presence of other polyphenols such as caftaric acid and quercitrin, which haven't been identified in *Geranium sanguineum* before, and the absence of chlorogenic acid. Based on the results obtained by extraction with and without acid hydrolysis, we suggested the presence of an unknown kaempferol glycoside in *Geranium sanguineum.* These findings are of much importance as the inhibitory effect of *Geranium sanguineum* extract on herpes simplex virus replication is related to the rich content of polyphenol compounds.[13,14]

REFERENCES

1. Yang, B.; Kotani, A.; Arai, K.; Kusu, F. Estimation of the antioxidant activities of flavonoids from their oxidation potentials. Anal. Sci. **2001**, *17*, 599–604.

2. Pratt, D.E. *Phenolic Compounds in Food and their EffEcts on Health II*; Amer. Chem. Soc.: Washington, DC, 1992; 54–72.
3. Husain, S.R.; Cillard, J.; Cillard, P. Hydroxyl radical scavenging activity of flavonoids. Phytochemistry **1987**, *26* (9), 2489–2491.
4. Hertog, M.G.L.; Kromhout, D.; Aravanis, C. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries. Study. Arch. Int. Med. **1995**, *155*, 381–386.
5. Shahidi, F.; Wanasundara, P.; Hong, C. *Phenolic Compounds in Food and their EffEcts on Health I*. Amer. Chem. Soc.: Washington, DC, 1992; 214–222.
6. Amrik, L.K. *Phenolic Compounds in Food and their Effects on Health I*. Amer. Chem. Soc.: Washington, DC, 1992; 77–84.
7. Careri, M.; Elviri, L.; Mangia, A.; Musci, M. Spectrophotometric and coulometric detection in the high performance liquid chromatography of flavonoids and optimization of sample treatment for the determination of quercetin in orange juice. J. Chromatogr. A **2000**, *881*, 449–460.
8. Schieber, A.; Keller, P.; Carle, R. Determination of phenolic acids and flavonoids of apple and pear by high performance liquid chromatography. J. Chromatogr. A **2001**, *910*, 265–273.
9. Cimpan, G.; Gocan, S. Analysis of medicinal plants by HPLC: recent approaches. J. Liq. Chromatogr. & Rel. Technol. **2002**, *25*, 2225–2292.
10. Serkedjieva, J. Antiinfective activity of a plant preparation from *Geranium sanguineum*. L. Pharmazie **1997**, *52* (10), 799–802.
11. Serkedjieva, J. Influenza virus variants with reduced susceptibility to inhibition by a polyphenol extract from *Geranium sanguineum*. L. Pharmazie **2003**, *58* (1), 53–57.
12. Ivancheva, S.; Manolova, N.; Serkedjieva, J.; Dimov, V.; Ivanovska, N. Polyphenols from Bulgarian medicinal plants with anti-infectious activity. Basic Life Sci, **1992**, *59*, 717–728.
13. Serkedjieva, J.; Ivancheva, S. Antiherpes virus activity of extracts from the medicinal plant *Geranium sanguineum*. L. J. Ethnopharmacol. **1999**, *64* (1), 59–68.
14. Zgorniak-Nowosielska, I.; Zawilinska, B.; Manolova, N.; Serkedjieva, J. A study on the antiviral action of a polyphenolic complex isolated from the medicinal plant *Geranium sanguineum* L. VIII. Inhibitory effect on the reproduction of herpes simplex virus type 1. Acta Microbiol. Bulg, **1989**, *24*, 3–8.

Received July 1, 2005
Accepted July 17, 2005
Manuscript 6675