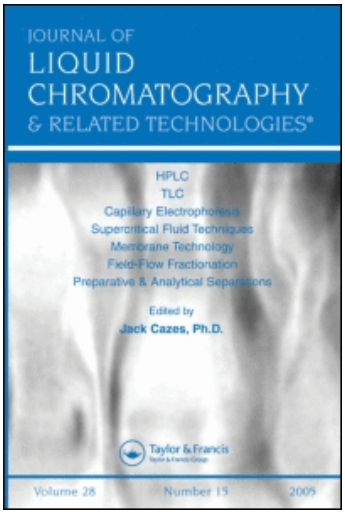

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IDENTIFICATION, ISOLATION, AND DETERMINATION OF FLAVONES IN *ORIGANUM VULGARE* FROM MACEDONIAN FLORA

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ABSTRACT

Assay of flavone aglycones in extracts of *Origanum vulgare* from Macedonia including identification and isolation is performed. Hydrolyzed extracts were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Three aglycones were identified and then isolated by column chromatography and TLC. Further TLC, HPLC, and UV/VIS spectrophotometric analysis showed that the isolated compounds are luteolin (the most abundant), apigenin, and diosmetin.

A new and rapid HPLC method was developed only for determination of the content of luteolin, which can serve as an appropriate criterion for evaluation of the flavonoid content in the drug. The accuracy of the method was proven by the method of standard additions, which gave satisfactory values for the recovery (98.2–100.3 %). The quantity of luteolin found in the drug ranged from 0.40–0.50 % (m/m).

INTRODUCTION

As a medicinal plant, oregano has been used for a long time. Certain biological effects of the *Origanum* species have been experimentally proven, such as spasmolytic (Van Den Broucke and Lemli[1]), antibacterial (Vokou et al.;[2] Abdel-Sattar et al.[3]), antifungal (Stiles et al.;[4] Adam et al.;[5] Markovic and Kosutic[6]), antioxidant (Lagouri et al.;[7] Nakatani;[8] Deighton et al.[9]), etc. In Macedonian folk medicine, dried overground parts of *Origanum vulgare* are widely used.

Homemade remedies are recommended for regulation of abdominal disorders. Usually a decoct is used in treatment of colds, bronchitis, etc. (Dervendzi[10]). These activities are due to the specific composition of the essential oils (Daouk et al.;[11] Paster et al.;[12] Shimoni et al.;[13] Panizi et al.[14]), flavonoids (Abdel-Sattar et al.;[3] Husain et al.[15]), phenolic acids (Kikuzaki and Nakatani[16]) and other chemical constituents of *Origanum*.

Much of the literature data about *Origanum* consider the composition of the essential oils of the *Origanum* species, as well as their biological activity. Less information is available concerning the flavonoids present in these plants. A very comprehensive work in this area is presented in the paper of Husain et al.,[15] where the distribution of flavone aglycones in a number of *Origanum* species is given.

Representatives of this species are divided into five groups according to the presence of luteolin, apigenin, chrysoeriol, and scutellarein, and their derivatives. *Origanum vulgare* is placed in the group characterized by the presence of luteolin in various glycoside forms (glycosides, diglycosides, glucuronides, and diglucuronides). There is, also, data about apigenin, eriodictiol, dihydrokaempferol, and dihydroquercetin found in *Origanum vulgare* (Olechnowicz-Stepien et al.[17]). Consulting the literature data, only information on identification and isolation of flavonoids could be found, whereas no data were found for quantitative analyses of these constituents of *Origani herba*.

Chemical composition of Macedonian *Origani herba* has been previously investigated and the content and composition of the essential oil was determined (Hristova et al.[18]). The aim of the present study is investigation of the flavonoids including identification, isolation, and quantitative determination.

EXPERIMENTAL

Materials

Plant Material

Aerial parts of the plant were collected in the flowering season during the summers of 1997 and 1998 in the region of Skopje (O1), the Belasica mountain

(O2), and in the Mavrovo region, western Macedonia (O3). The material was air dried, packed in paper bags, and kept in a dark and cool place until analysis. The plant identity was verified by Prof. V. Matevski from the Institute of Biology, Faculty of Science in Skopje. Voucher specimens were deposited at the Herbarium, at the Department of Botany, Institute of Biology, Faculty of Science, Skopje.

Reagents and Authentic Samples

The reagents used were of highest purity (>99.95 purity), methanol HPLC grade, and glacial acetic acid (Merck, Darmstadt, Germany), authentic samples of apigenin, luteolin, chrysoeriol, and diosmetin (Extrasinthese, Lyon, France).

Extraction Procedure

Plant material (10 g) was extracted with 100 mL acetone and 5 mL 25 % HCl in an Erlenmeyer flask (with condenser) on a water bath with continuous mixing. The extract was then cooled, filtered, and transferred to a separating funnel. 100 mL of water was added and extraction with ethylacetate was repeated until negative reaction on flavonoids (test with TLC in system S_1).

The ethylacetate fractions were collected and washed three times with 100 mL of water each, then dried with anhydrous Na_2SO_4 for 6 hours, filtered and evaporated to dryness under low pressure. The residue was dissolved in 2 mL of mixture of methanol and chloroform (1:1) and this solution was used for identification and isolation of flavone aglycones.

The solution for quantitative analysis of luteolin was prepared by an analogous procedure starting with 0.5 g of plant material. The dry residue after evaporation of ethylacetate was dissolved in methanol and filled to volume (10 mL). This solution was used for HPLC analysis of luteolin.

Isolation of Flavone Aglycones

Column chromatography on Silica G 60 (70–230 mesh), combined with preparative TLC, was used for isolation of flavone aglycones. The elution system consisted of chloroform and ethylacetate with growing fraction of ethylacetate, and then of ethylacetate and methanol with growing fraction of methanol. The eluates were tested by TLC and the identical ones were combined. The obtained six fractions were then subjected to preparative TLC in the systems S_1, S_2, and S_3. The final purification of the isolated compounds was also performed by preparative TLC using the system S_2.

Identification of Flavone Aglycones

Flavone aglycones were identified in the prepared extracts using TLC and HPLC compared to authentic samples. The isolated flavone aglycones were identified by TLC, as well as by UV/VIS spectral analysis.

TLC

Commercial TLC aluminium sheets with Silica HF 254 and homemade plates with Silica HF 254 were used. The following mobile phases were used:

S_1: Toluene : EtAcO : HCOOH = 58 : 33 : 9 (*V/V*);

S_2: $CHCl_3$: MeOH = 97 : 3 (*V/V*);

S_3: $CHCl_3$: n-Hexane : MeOH = 40 : 40 : 3 (*V/V*);

S_4: Toluene : MeCOEt : CH_3COOH = 18 : 5 : 1 (*V/V*).

The visualization of the spots was performed under UV light at 254 and 366 nm before, and after, spraying with $AlCl_3$ solution.

UV/VIS Spectral Analysis

UV/VIS spectra of the isolated flavone aglycones were recorded in methanol using Perkin-Elmer UV/VIS spectrometer model Lambda 16. The identification of the components was made according to changes observed in the spectra after adding the classical shift reagents (NaMeO, $AlCl_3$, $AlCl_3$/HCl, $NaCH_3COO$, and $NaCH_3COO/H_3BO_3$, (Mabry et al.[19])).

HPLC

Varian HPLC system equipped with a ternary pump Model 9012 and UV-Diode Array detector Model 9065, and a reverse phase column C18 (250 × 4.6 mm, 5 μm particles) were used. The mobile phase consisted of two solvents: 5 % CH_3COOH (A) and CH_3OH (B), and the elution program for screening the extracts was the following: 0–5 min 70 % A; 10–15 min 60 A; 20–25 min 50 % A; 30–35 min 40 % A, and 40–45 min 20 % A. The flow rate was 1.3 mL/min and the temperature was set to 30 ºC. The elution was monitored at 254 and 348 nm.

Quantification of Luteolin

A new and rapid HPLC method was developed for quantitative determination of luteolin in the examined plant material. The experimental conditions were similar to those described for the identification. The significant difference that enables rapid quantification of luteolin is in the elution program, starting with a stronger mobile phase (45 % A from 0–15 min and then 20 % from 20–30 min) and higher working temperature (40 ºC). The flow rate was 0.8 mL/min.

Calibration was done in the concentration range of 0.05–1 mg/mL, or expressed in terms of mass of luteolin injected in the column from 1-20 μg luteolin (sample loop 20 μl). The data acquisition was made at 254 and 348 nm and the results obtained for both wavelengths were compared. The accuracy of the method was checked by the method of standard additions.

RESULTS AND DISCUSSION

Identification of Flavones

The flavone aglycones were identified in the extracts obtained, after hydrolysis of flavone glycosides in acetone using HCl. Obtained aglycones were transferred into a mixture of chloroform and methanol (1:1, *V/V*) and tested by TLC and HPLC. The results are presented in Table 1.

Two or three dark violet spots were recognized under UV light (366 nm) which become clear yellow to orange-yellow after spraying with $AlCl_3$ solution. The R_f values of two of these spots in different solvent systems were very close to the ones obtained for authentic samples of luteolin and apigenin . As for the third one, the R_f value was close to both the ones of chrysoeriol and diosmetin and TLC could not distinguish between the two compounds.

The screening HPLC method confirmed the presence of significant quantity of luteolin and much smaller of apigenin, according to the retention times (25.2 min and 31.4 min, respectively) and UV spectra, compared to those for authentic samples. Likewise, in this case, the third component with a retention time (32.6 min) was close to both chrysoeriol and diosmetin (32.2 and 32.8, respectively, Fig. 1).

Both components have very similar UV spectra, as well. Here, the identification was made using spiking of the sample with these two compounds and studying the obtained chromatograms. The results showed that the third peak is due to flavone diosmetin (Fig. 1b).

Table 1. TLC and HPLC Identification of Flavones in Hydrolyzed Extracts of *O. vulgare*

Component	TLC*												HPLC
	S_1			S_2			S_3			S_4			(t_R/min)
	O1	O2	O3	O1	O2	O3	O1	O2	O3	O1	O2	O3	O1
F1	0.36	0.48	0.36	0.40	0.40	0.39	0.12	0.11	0.11	0.16	0.14	0.16	25.2
F2	0.60	0.60	0.58	0.70	0.69	0.68	0.20	0.18	0.19	0.18	—	—	31.4
F3	—	—	—	—	—	—	0.25	0.24	0.25	0.40	0.40	0.48	32.6
Luteolin		0.38			0.41			0.13			0.18		25.2
Apigenin		0.56			0.50			0.19			0.40		31.3
Chrysoeriol		0.60			0.72			0.25			0.35		32.2
Diosmetin		0.62			0.74			0.25			0.38		32.8

* R_f values of the spots in different solvent systems (S_1–S_4) for TLC; O1 – sample from Skopje region; O2 – sample from the Belasica mountain; O3 – sample from Mavrovo region; t_R – HPLC retention times.

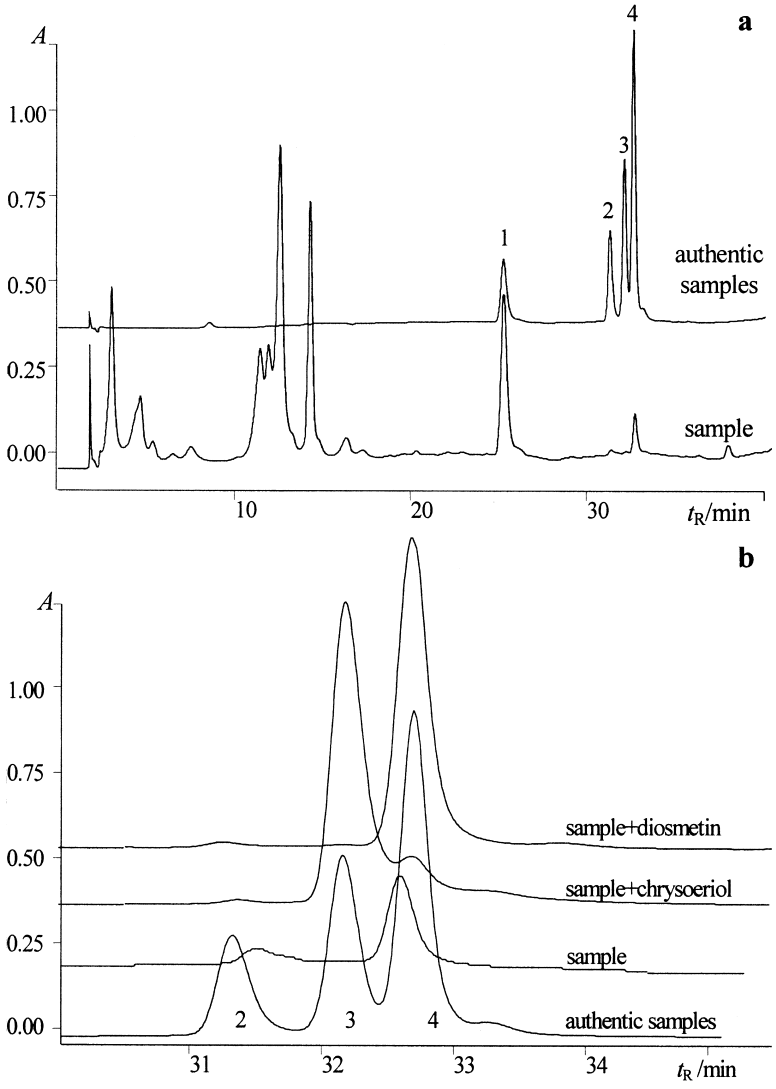

Figure 1. Chromatograms of: **a.** a sample and a mixture of authentic samples for screening of hydrolyzed extracts of *Origanum vulgare* (1-luteolin, 2-apigenin, 3-chrysoeriol, 4-diosmetin); **b.** spiked sample for identification of diosmetin.

Isolation of Flavones

Three flavone aglycones were isolated from chloroform-methanol solutions, after hydrolysis, using column chromatography and preparative TLC. The components marked as F1, F2, and F3 were subjected to identification using TLC, HPLC, and UV/VIS spectrometry compared to authentic samples of luteolin, apigenin, chrysoeriol, and diosmetin.

Results shown in Table 2 led us to the conclusion that F1 is luteolin, F2 apigenin, and F3 diosmetin, which proves our previously established identification of the components from the extracts.

Table 2. $R_{f\,\text{and}}$ t_R Values and Spectrophotometric Identification of the Flavone Aglycones Isolated from Macedonian *O. vulgare*

Spot	Relative Yield[a]	R_f Values S_3	R_f Values S_4	λ max/nm	t_R/min	Identification
F1	++++ ++++	0.12	0.16	MeOH: 254, 268, 347 NaOMe: 270, 406 $AlCl_3$: 274, 328, 4202 $AlCl_3$ + HCl: 275, 300, 355, 385 NaOAc: 270, 326, 384 NaOAc+H_3BO_3: 260, 375	5.2	5,7,3,4-Tetra-OH-flavone
F2	+	0.20	0.19	MeOH: 269, 333 NaOMe: 270, 323, 390 $AlCl_3$: 269, 300, 349, 384 $AlCl_3$+HCl: 276, 300, 343, 380 NaOAc: 270, 380 NaOAc+H_3BO_3: 270, 342	31.3	5,7,4'-Tri-OH-flavone
F3	++	0.25	0.42	MeOH: 240, 252, 267, 244 NaOMe: 270, 302, 386 $AlCl_3$: 273, 298, 360, 390 $AlCl_3$+HCl: 276, 295, 352, 385 NaOAc: 275, 322, 367 NaOAc+H_3BO_3: 253, 267 348	32.6	5,7,3'-TriOH-4'-Ome-Flavone

[a] Judged by the areas of the HPLC peaks and the areas and intensities of fluorescence of the TLC spots.

Quantitation of Luteolin

The screening HPLC analysis of extracts obtained after hydrolysis, showed that the main flavone component in samples of *Origanum vulgare* is luteolin (Fig. 1a). As for the other components having UV spectra of flavonoids, the peaks due to diosmetin and apigenin had significantly smaller area compared to the one obtained for luteolin. All this brought the idea that determination of the content of luteolin, only, can be an appropriate criterion for evaluation of the flavonoid content of *Origanum vulgare*, having in mind that the quantities of apigenin and diosmetin are very low and not significant.

Here, it is interesting to discuss the selection of the optimal wavelength for monitoring the elution of the components from the column. Our choice was 254 nm as the most conventional one and 348 nm where luteolin exhibits absorption maximum. Comparison of the chromatograms obtained at both wavelengths (Fig. 2), supports using the second (348 nm) primarily for the better resolution, which is 6.6 compared to 2.0 at 254 nm. This is due to the presence of an unidentified component eluting before luteolin, which absorbs significantly at 254 nm, but not at all at 348 nm.

The calibration curve was made in the concentration range of 0.05–1 mg/mL, or expressed in terms of mass of luteolin injected in the column

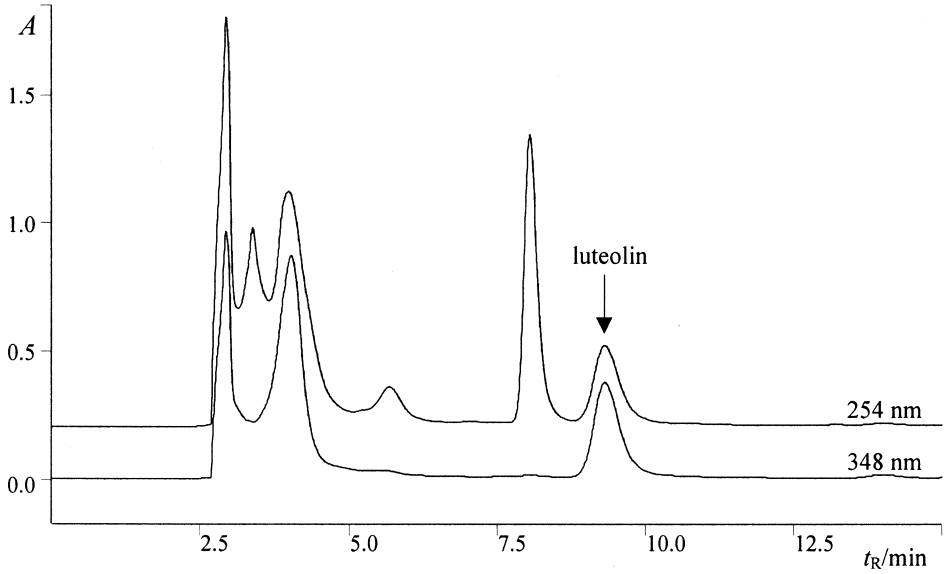

Figure 2. Chromatograms of a hydrolyzed extract of *Origanum vulgare* at 348 and 254 nm.

from 1–20 μg luteolin. The linear dependence of the mass of luteolin injected in the column was established in the whole range. The linear regression equations obtained for 254 nm and 348 nm with the corresponding *RSD* values and the coefficient of correlation are the following:

$$254 \text{ nm: area} = 3.6884 \cdot 10^5 \, m(\text{luteolin}), \; RSD = 3.51 \, \%, \; r = 0.9939$$

$$348 \text{ nm: area} = 3.0592 \cdot 10^5 \, m(\text{luteolin}), \; RSD = 3.67 \, \%, \; r = 0.9991$$

The higher value for the slope of the regression curve obtained for 348 nm, gives better sensitivity to this method, and together with the better resolution of the peak of luteolin, leads to the conclusion that 348 nm is the optimal wavelength for detection and determination of luteolin.

The accuracy of the method was checked by the method of standard additions (Table 3). High values for the recovery (98.2–103.0 %) show that the proposed HPLC method has a satisfactory accuracy.

The sensitivity of the method was determined by construction of a calibration curve in the low concentration region (5–50 μg/mL, i.e. 0.1–1.0 μg luteolin), which is approximated as a detection and quantification limit. The regression equation of this curve was: area = $4.1182 \cdot 10^5$ *m*(luteolin) with *SD* = 10802 and *r* = 0.9979. The limit of detection was calculated as three times the ratio between the *SD* and the slope of the low concentration curve (LOD = 3·*SD/slope*), and the limit of quantification as ten times the same ratio (LOQ = 10·*SD/slope*).[20] The LOD was found to be 0.08 μg and the LOQ 0.26 μg of luteolin.

The results from the determination of luteolin in three samples of *Origanum vulgare* from different regions in Macedonia, are presented in Table 3.

Table 3. Results from the Standard Additions Method for Checking the Accuracy of the Procedure and for the Content of Luteolin in Specimens of Macedonian *O. vulgare*

Standard Additions			
Measured	Luteolin Added (m/μg)	Calculated	Recovery (%)
4.05	—	—	—
5.02	1.0	5.05	99.4
6.43	1.5	6.55	98.2
9.32	5.0	9.05	103.0
Region	Skopje Region	Belasica Mountain	Mavrovo Region
Content of luteolin in herbal drug/% (m/m) (*n* = 3)	0.50 ± 0.03	0.40 ± 0.02	0.46 ± 0.02

The content of total luteolin in air-dried material (8.6 % moisture) is found to be 0.40–0.51 % (m/m). Results obtained are in good agreement with previously published data concerning the presence of luteolin in *Origanum vulgare* (Husain et al.[3]). According Asenov and Nikolov,[21] apigenin and diosmetin, besides luteolin, are also present in herbal parts of the plant. At the same time, no quantitative data are available in these references.

As was mentioned above, *Origani herba* is widely used in traditional medicine for treatment of abdominal disorders. Homemade extracts prepared by infusion of the drug with boiling water are the most important forms of use. Hence, luteolin and its water-soluble glycosides are probably the main components responsible for the spasmolytic action of the drug. This activity of luteolin was experimentally proved previously by investigation of different plant extracts containing luteolin (Van den Broucke and LemLi;[22] Van den Broucke;[23] Van den Broucke et al.[24]).

The present work offers a new and rapid HPLC method for determination of total luteolin content in *Origani herba*, which can be used in routine analysis of the drug. The proposed method is more important because of a lack of methods for quantification purposes present in available literature.

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