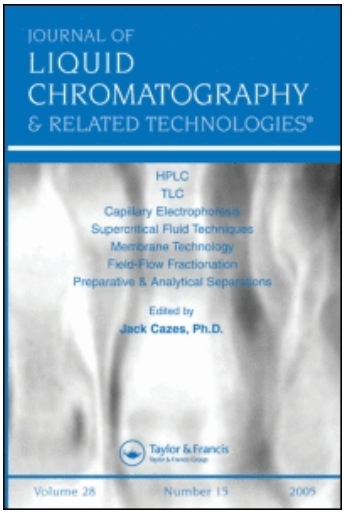

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ASSAY OF FLAVONOLS AND QUANTIFICATION OF QUERCETIN IN MEDICINAL PLANTS BY HPLC WITH UV-DIODE ARRAY DETECTION

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ABSTRACT

A new and rapid procedure for screening of flavonols (myricetin, quercetin and kaempferol) and for determination of quercetin by RP-HPLC with UV-diode array detection in 16 medicinal plants is presented. Screening of the extracts showed that quercetin is the most abundant flavonol, especially in *Hyperici herba*, *Uvae ursi folium* and *Pruni spinosae flos*. Kaempferol was the most abundant in *Robiniae pseudoacaciae flos* and *Pruni spinosae flos*, whereas myricetin was identified only in *Betulae folium*.

The method for quantification of quercetin was checked by the method of standard additions, and satisfactory values for the recovery (97.0-103.5 %) were obtained. The limit of detection was found to be 0.004 mg/mL and the limit of quantification

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0.013 mg/mL of quercetin. The content of quercetin in the plant material ranged from 0.026-0.552 % (m/m).

INTRODUCTION

In the last decade, polyphenols have gained much more attention, owing to their antioxidant capacity (free radical scavenging and metal chelating) and their possible beneficial implications in human health, such as in the treatment and prevention of cancer, cardiovascular disease, and other pathologies.[1] They have also been studied for their inhibitory activity against human immunodeficiency virus (HIV)-1 protease and flavonols, especially quercetin, and were found to be the most potent inhibitors of the target enzyme.[2]

The interest in identification and quantification of flavonols, especially quercetin, in different products, is growing owing to their antioxidant potential. In the majority of assays, high performance liquid chromatography with spectrophotometric or electrochemical detection is the method of choice. There is data from measurements of flavonols in fruits, vegetables, and beverages,[3-8] and also in wines, especially red wines, which are found to be very rich in flavonols.[9-11]

There is not much data about flavonols content in medicinal plants used in the official and traditional medicine. We have published our results from determination of total flavonoids and total and free quercetin in *Hypericum perforatum* using UV-VIS spectrophotometry and HPLC.[12] Continuing our research in the developing and validation of analytical methods for determination of flavonoids with potential antioxidant activities,[13] in this work we present an assay of flavonols and a method for determination of quercetin by RP-HPLC in 16 medicinal plants. A relatively rapid and simple procedure is proposed for identification of myricetin, quercetin, and kaempferol, and quantification of quercetin, as the most abundant, after acid hydrolysis using an RP-HPLC method with diode array detection.

EXPERIMENTAL

Materials

Plant Material

Certain parts of plants (leaves, herbs, flowers) were collected in the flowering season during summer 1999 and 2000 at different locations throughout the entire territory of Macedonia. The materials were air dried, milled, packed in paper bags, and kept in a dark and cool place until analysis. The following specimens were included in the investigation:

- *Hyperici herba*, herbs of *Hypericum perforatum* L.
- *Uvae ursi folim*, leaves of *Arctostaphylos uva-ursi* (L.) Spreng
- *Pruni spinosae flo*s, flowers of *Prunus spinosa* L.
- *Sambuci flos*, flowers of *Sambucus nigra* L.
- *Betulae folim*, leaves of *Betula pendula* Roth.
- *Primulae flos*, flowers of *Primula veris* L.
- *Herniariae herba*, herbs of *Herniaria glabra* L.
- *Centaurii herba*, herbs of *Erythrea centaurium* (L.) Pers.
- *Tiliae flos*, flowers of *Tilia platyphyllos* Scop.
- *Bursae pastoris herba*, herbs of *Capsela bursa pastoris* (L.) Med.
- *Robiniae pseudoacaciae flos*, flowers of *Robinia pseudoacacia* L.
- *Juniperi fructus*, berries of *Juniperus communis* L.
- *Lavanulae flos*, flowers of *Lavandula officinalis* Chaix., commercial sample
- *Melissae folium*, leaves of *Melissa officinalis* L.
- *Galii veri herba*, herbs of *Galium verum* L.
- *Maydis stigmata*, *Zea mays* L., commercial sample.

Reagents and Authentic Samples

The reagents used were of highest purity (>99.95 % purity), acetonitrile HPLC grade, glacial acetic acid (Merck, Darmstadt, Germany), and authentic samples of quercetin, myricetin, and kaempferol (Extrasynthese, Lyon, France).

Extraction Procedure

Milled plant material (2 g) was extracted twice with 50 mL of acetone, 2 mL of concentrated HCl and 1 mL of 1 % solution of urotropine in water, each time. The extraction was performed in an Erlenmeyer flask with reflux in a water bath for 30 min. The extract was then cooled, filtered, and filled to volume with acetone (100 mL). 25 mL of this extract were then transferred to a separating funnel, 50 mL of water was added and extraction with ethylacetate was repeated 3 times with 15 mL each. The ethylacetate fractions were collected and washed three times with 50 mL of water each, then dried with anhydrous Na_2SO_4, filtered, and evaporated to dryness under low pressure. The residue was dissolved in 10 mL of methanol and this solution was used for identification of flavonols and quantification of quercetin by HPLC.

HPLC Analysis

A Varian HPLC system equipped with a ternary pump Model 9012 and UV-Diode Array detector Model 9065 and a reverse phase column C18 (250 x 4.6

mm, 5 μm particle diameter) were used. The mobile phase consisted of two solvents: 5 % CH_3COOH (A) and CH_3CN (B), and the elution program was the following: 0-10 min 70 % A and then 20-30 min 40 % A. The flow rate was 1.0 mL/min and the temperature was set to 30 °C. The elution was monitored in the whole UV range.

Calibration was done in the concentration range of 0.05-1.00 mg/mL or expressed in terms of mass of quercetin injected in the column from 1-20 μg quercetin (sample loop 20 μL). The accuracy of the HPLC method was checked by the method of standard additions, as well as the accuracy of the whole extraction procedure. The limit of detection (LOD) and limit of quantification (LOQ) were established by construction of a calibration curve in the low concentration region (0.005-0.050 mg/mL).

RESULTS AND DISCUSSION

Assay of Flavonols

The assay of flavonols in the plant extracts was performed by comparing the chromatograms obtained from the samples to the one obtained for the mixture of the three authentic samples of myricetin, quercetin, and kaempferol. The retention times, together with the UV-spectra of the studied flavonols, were used for identification. The retention times obtained for myricetin, quercetin, and kaempferol are 5.4; 9.4, and 16.3 min, respectively. This elution sequence is due to their structural characteristics. Namely, they are all 3,5,7-trihydroxy substituted flavones differing in the number of hydroxyl groups substituted in the B-ring of flavone (kaempferol has one OH-group in position 4', quercetin two OH-groups at 3', 4', and myricetin three OH-groups at 3', 4', 5' in the B-ring). This substitution controls the order of elution from the reverse phase column: more polar groups—less retention on the column—shorter retention time.

Screening of the extracts showed that quercetin is the most abundant flavonol, especially in *Hyperici herba*, *Uvae ursi folium*, *Pruni spinosae folium*, *Sambuci flos,* and *Betulae folium*. Lesser amounts were found in *Primulae flos*, *Herniariae herba*, *Centaurii herba*, *Tiliae flos,* and *Bursae pastoris herba*, and only traces in *Robiniae pseudoacaciae flos*, *Juniperi fructus*, and *Lavandulae flos*. Kaempferol was found to be most abundant in *Robiniae pseudoacaciae flos*, *Pruni spinosae flos*, and *Tiliae flos*, whereas myricetin was identified only in *Betulae folium*. It is interesting to point out, that in the chromatograms obtained for extracts of *Primulae flos*, *Sambuci flos*, *Herniariae herba*, and *Pruni spinosae flos*, a component having UV-spectrum almost identical with the one of quercetin and a retention time (16.9 min) close to the one of kaempferol is eluted. It is probably a flavonol with a substitution pattern of quercetin, but with one methy-

lated OH-group. The results from the assay of flavonols in the 16 studied medicinal plants are presented in Table 1. The chromatograms obtained for extracts of *Hyperici herba*, *Robiniae pseudoacaciae flos*, *Pruni spinosae flos*, *Sambuci flos*, *Betulae folium*, and *Primulae flos*, together with the chromatogram of the mixture of authentic samples of myricetin, quercetin, and kaempferol are given in Fig. 1.

Quantification of Quercetin

Having in mind the results from the screening HPLC analysis of extracts obtained after hydrolysis, which showed that the main flavonol component in majority of samples is quercetin, we developed and validated a procedure for quantitative analysis of this flavonol. As explained in the experimental section, this method includes acid hydrolysis during extraction for releasing quercetin (and other flavonols) bonded to various sugars and then determination of total quercetin in the prepared extracts.

Table 1. Results from Assay of Flavonols, After Hydrolysis

Herbal Drug	Myricetin t_R-5.4	Quercetin t_R-9.4	Kaempferol t_R-16.3	Unidentified Flavonol t_R-16.9
1. *Hyperici herba*	−*	+++++**	−	−
2. *Uvae ursi folium*	−	+++++	+	−
3. *Pruni spinosae flos*	−	+++++	+++++	+
4. *Sambuci flos*	−	++++	+	++
5. *Betulae flos*	+	++++	−	−
6. *Primulae flos*	−	+++	++	++++
7. *Herniariae herba*	−	++	traces	++
8. *Centaurii herba*	−	++	++	−
9. *Tiliae flos*	−	+	+++	−
10. *Bursae pastoris herba*	−	+	+	−
11. *Robiniae pseudoacaciae flos*	−	traces	+++++	−
12. *Juniperi fructus*	−	traces	−	−
13. *Lavandulae flos*	−	traces	−	−
14. *Melissae folium*	−	−	traces	−
15. *Galii veri herba*	−	−	−	−
16. *Maidis stigmata*	−	−	−	−

*Not found in the extract.
**Relative quantity approximated in relation to peak area at 254 nm.
t_R - Retention time in min.

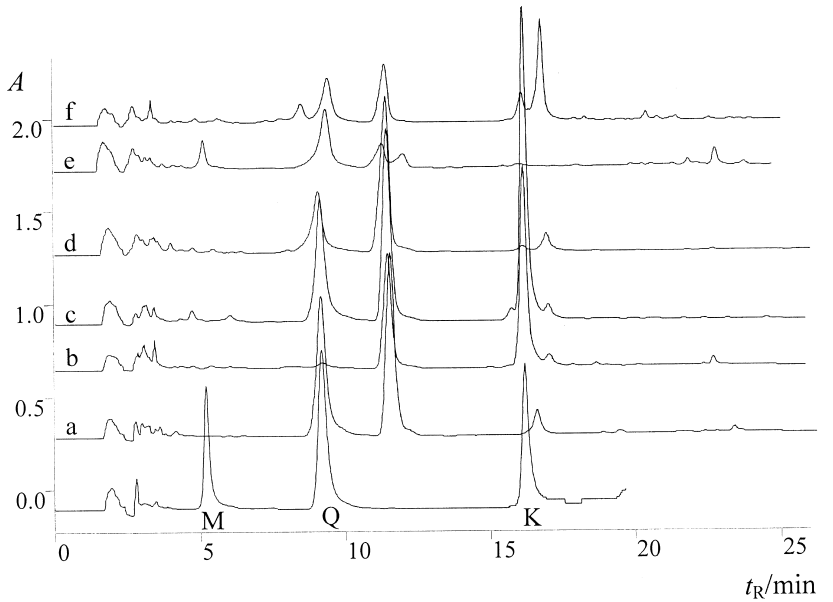

Figure 1. Chromatograms obtained for extracts of a. *Hyperici herba*; b. *Rabiniae flos*; c. *Pruni spinosae flos*; d. *Sambuci flos*; e. *Betulae folium*, and f. *Primulae flos*, and for mixture of authentic samples of myricetin (M), quercetin (Q), and kaempferol (K).

The selection of the optimal wavelength for monitoring the elution of the components from the column is, in this case, interesting to discuss. 254 nm was selected as the most conventional one and the other choice was 367 nm, where quercetin exhibits absorption comparable to the one at 254 nm, but much more selective for flavonols in relation to other components, which are not of our interest here. Comparison of the chromatograms obtained for *Hyperici herba* extract at both wavelengths (Fig. 2) supports using the second one (367 nm) primarily for the better resolution, which is higher than 10 compared to around 2.0 at 254 nm. This is due to the presence of an unidentified component eluting right after quercetin, which absorbs significantly at 254 nm, but not at all at 367 nm, which is obvious from the UV-spectra of the two compounds given in Fig. 2.

The calibration curve was made in the concentration range of 0.05-1.00 mg/mL, or expressed in terms of mass of quercetin injected in the column from 1-20 μg quercetin. The linear dependence of the mass of quercetin injected in the column was established in the whole range. The linear regression equations obtained for 254 nm and 367 nm with the corresponding *RSD* values and the coefficient of correlation are the following:

254 nm: area = $8.9568 \cdot 10^6 \gamma$(quercetin), *RSD* = 3.96 %, r = 0.9991

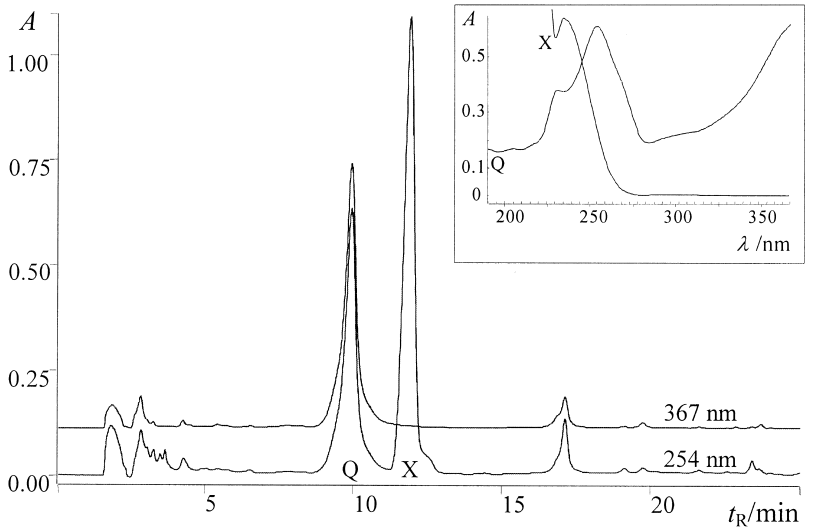

Figure 2. Chromatograms of an extract of *Hyperici herba* at 367 and 254 nm and UV-spectra of quercetin (Q) and interfering component (X).

367 nm: area = $8.7830 \cdot 10^6 \gamma$(quercetin), *RSD* = 3.87 %, $r = 0.9991$

The value of the slope for both regression curves obtained for 254 nm and 367 nm are comparable, but the better resolution of the peak of quercetin at 367 nm is favorable for using the latter wavelength as optimal for determination of quercetin.

The accuracy of the HPLC method was checked by the method of standard additions, as well as the accuracy of the whole procedure, which was tested by adding certain amounts of quercetin dihydrate (corresponding to 20; 10, and 5 mg of quercetin) in the plant material at the start of extraction. The results from these analyses are presented in Table 2. High values for the recovery (97.0-103.5 %) show that the proposed HPLC method, as well as the whole procedure, is acceptable for quantification of quercetin in plant material.

The sensitivity of the method was determined by construction of a calibration curve in the concentration region approximated as a detection and quantification limit (5-50 μg/mL). The regression equation of this curve was:

area = $8.7168 \cdot 10^6 \gamma$(quercetin), *SD* = 11278, $r = 0.9980$

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 this ratio (LOQ = 10·*SD/slope*).[14] The LOD was found to be 0.004 mg/mL and the LOQ 0.013 mg/mL of quercetin.

Table 2. Results from the Standard Additions Method for Checking the Accuracy of the HPLC Method and of the Whole Procedure for Analysis of Quercetin in Plant Material

HPLC Method			
	γ (quercetin)/ (mg/mL)		
Measured	Added	Calculated	Recovery/ %
0.276	-	-	-
0.387	0.100	0.376	103.0
0.493	0.200	0.476	103.5
0.590	0.300	0.576	102.4
Whole Procedure			
	γ (quercetin)/ (mg/mL)		
Measured	Added	Calculated	Recovery/ %
0.276	-	-	-
0.414	0.125	0.401	97.0
0.532	0.250	0.526	101.1
0.753	0.500	0.776	103.2

The results from the determination of quercetin in 10 samples of medicinal plants from different regions in Macedonia are presented in Table 3. The content of total quercetin ranged from 0.026 % in *Bursae pastoris herba* to 0.552 % in *Hyperici herba*.

It is well known that *Betullae folium*, *Sambuci flos*, and *Tiliae flos* are appreciated in phytotherapy because of their flavonoid content, and the main flavonol component in these herbal drugs is quercetin. Quality control of these herbal drugs, among other measurements, includes spectrophotometric determination of total flavonoids, using $AlCl_3$ as a complex forming reagent.[15] Other herbal drugs that contain predominantly flavonoids (quercetin and other flavonols and their glucosides) are *Primulae flos, Pruni spinosae flos, Robiniae pseudoacaciae flos, Galii veri herba*, and *Maidis stigmata,* according to literature data.[15,16] To the best of our knowledge, no method for quantitative determination of specific flavonoid components useful in routine analysis for such herbal drugs is suggested in literature data. In quality control of *Hyperici herba*, determination of hypericin was accepted as the quantitative standard. Among other investigations, bitterness index for *Centaurii herba* and hemolytic index for *Herniariae herba* were accepted as tests for evaluation of their quality, as well.

We suggest, here, a method which can be used in quality control, according to the content of quercetin. The assay of flavonoids in herbal drugs included in

Table 3. Results from the Determination of Quercetin in 10 Plant Samples

Plant Material	γ(Quercetin) in Extract/ (mg/mL)	*w* (Quercetin) in Drug/ %
1 *Hyperici herba*	0.276±0.009	0.552
2 *Uvae ursi folium*	0.241±0.002	0.482
3 *Pruni spinosae flos*	0.253±0.005	0.506
4 *Sambuci flos*	0.141±0.004	0.281
5 *Betulae flos*	0.156±0.007	0.312
6 *Primulae flos*	0.098±0.004	0.197
7 *Herniariae herba*	0.051±0.001	0.102
8 *Centaurium herba*	0.046±0.004	0.092
9 *Tiliae flos*	0.023±0.001	0.047
10 *Bursae pastoris herba*	0.013±0.001	0.026

n= 3.

our investigation, in most of the cases, confirmed the literature data, but the lack of flavonoids in *Juniperi fructus, Lavandulae flos, Melissae folium, Galii veri herba*, and *Maidis stigmata* was rather unexpected.

As already pointed out, flavonols, and especially quercetin, are nowadays very much studied for their antioxidant potential and our research is a contribution to qualitative and quantitative analysis of these compounds in medicinal plants used in traditional and official medicine. The present work offers a new and rapid HPLC method for assay of flavonols and determination of total quercetin content in medicinal plants, which can be used in routine analysis of various plant materials.

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