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HPLC DETERMINATION OF QUERCETIN AND RUTIN IN SUPERCRITICAL CARBONDIOXIDE EXTRACTS OF *HYPERICI HERBA*

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Introduction

With the fast development of modern chromatographic and spectroscopic techniques, the chemistry of natural products has made great progress during past decades.[1] Since pharmacologically active compounds in herbal plants usually are in low concentrations, a great deal of research has been done to develop more effective and selective extraction methods for effective recovery of these compounds from the raw materials. Analytical scale supercritical fluid extraction (SFE) is a well-recognized alternative to conventional solvent-based extraction procedures. Supercritical fluid extraction poses several advantages over traditional liquid-solvent-based extraction methods including improved selectivity, expeditiousness, automation and environmental safety.[2,3] The avoidance of organic solvents is a major goal in the isolation of natural products, which may be commercialized as food additives.

Hypericum perforatum L. (also known in Anglo-Saxon folk medicine as St. John's Wort) is a herbaceous perennial plant, belonging to Hypericaceae family. It is a well known medicinal plant since the antiquity, and was used to heal wounds, remedy kidney troubles, and alleviate nervous disorders, even insanity.[4] Today St. John's Wort is best known for its use in the treatment of mild-to-moderately severe depressive disorders.[5,6] It was one of the top-selling herbal products for 1997.[7]

Flavonoids are low-molecular-mass compounds found in all vascular plants. They act as antioxidants or as enzyme inhibitors, are involved in photosynthesis and cellular energy transfer processes, and may serve as precursors of toxic substances.[8,5,1] Specific flavonoids as quercetin and rutin are known to have pharmacological activity, particularly anti-allergic, anti-inflammatory, anti-viral, or anti-carcinogenic effects. Beside this, flavonoids are concerned to be free radical scavengers and metal chelators and inhibitors of lipid peroxidation.

Most of the methods reported for analysis of *Hypericum perforatum* flavonoids are based on HPLC,[9,10] and usually involve a previous fractionation by liquid-liquid extraction or solid phase extraction. In some cases it was found that SFE recovered higher levels of natural antioxidants, such flavonoids are, than sonication in liquid solvents[11]. There are few reported papers where supercritical fluid extraction was employed as a sample preparation technique prior to HPLC analysis.[12] Neither of them offered detailed study about the optimization of supercritical fluid extraction conditions.

Our research was focused on investigation of quercetin and rutin in supercritical fluid extracts of *Hyperici herba*. We have optimized the conditions for efficient SFE on pure compounds spiked on inert solid material,[13] and further more in this paper we will present the results of quantitative determination of investigated compounds in SFE extracts with RP-HPLC.

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Experimental

Chemicals and Reagents. HPLC grade solvents and water were used from Merck (Darmstadt, Germany). Standards of rutin and quercetin purchased from Sigma-Aldrich (USA) were used for identification and quantification purposes. Stock solutions of rutin and quercetin (1000 μg/ mL) were prepared in methanol and diluted to desired concentrations just prior to use. The solutions were kept in a 4 °C refrigerator and were stable for at least one month.

Plant Materials. Hyperici herba was supplied from commercial sources. Prior to analysis whole herba (grains, leaves and flowers) was grounded and passed through a sieves, so two types of samples were obtained: with particle diameter 0.150-0.300 mm and 0.300-0.750 mm. For extraction, different sample weights were taken: 0.10, 020, 030, 0,40 and 0,50 g.

Supercritical Fluid Extractions. Supercritical fluid extractions reported here were carried out on Hewlett Packard 7680 T instrument, and carbondioxide was used as supercritical fluid, and as a modificator 15 % methanol (v/v) was used in a static mode. Extractions were done in 7 mL extraction tubes sealed with caps. The extractions were performed at pressure of 380 bar and temperature of 50 °C. Static extraction time was 30 minutes, and dynamic extraction was 60 minutes. Trap temperature was 50 °C for extraction, and 30 °C for rinse step. The flow rate of a supercritical CO_2 was 1.0 mL/ min. As a trapping material ODS chromatographic packing was used. After the extraction step was completed, analytes were recovered in 1.0 mL methanol, which passed through the trap with rate of 0.5 ml/ min. Each extraction was done in three replicates

HPLC-DAD Analysis. Extracts were analyzed with HPLC-DAD on Perkin Elmer system equipped with quaternary LC pump series 200, autosampler series 200, Diode Array Detector 235C with wavelenght range from 190 to 365 nm. Supelcosil LC 18 (250mm×4.6 mm; 5 □m particle size) analytical column was used, thermostated on 30 °C. Chromatograms were recorded at 270 nm, while UV spectra were collected during the whole run. For the mobile phase acidified water with formic acid (pH 2.25-2.30) was used as eluent A, acetonitrile as eluent B, and methanol as eluent C. The pump program was four step gradient, whereas starting with 100 % of A, 0 % of B and C the total run was finished in 30 minutes with 10 % of A, 70 % of B and 20 % of C. Injection volume was 25 μL, and the flow velocity was 0.7 mL/ min. Analytes were identified by comparing their retention times, and UV-Vis spectra with standard solutions of examined compounds.

Results and Discussion

Calibration Procedure. For both quercetin and rutin quantification was done with establishing calibration curves by plotting the area of peaks obtained at 270 vs. different concentrations of examined compounds, within the concentration range from 1 μg/ mL to 100 μg/ mL (expressed in mass of rutin and quercetin injected into HPLC system from 25 to 2500 ng). Limits of detection and quantification were calculated as 3SD/slope and 10SD/slope[14] respectively, where SD is the standard deviation of the calibration curve constructed for concentration range from 1 to 40 μg/ mL (expressed in mass of rutin and quercetin injected into HPLC system from 25 to 1000 ng). The obtained calibration curves, regression coeficients, LOD's and LOQ's are given in Table 1.

Table1. Quantification parameters for rutin and quercetin

270 nm	LOD/μg mL^{-1}	LOQ/ μg mL^{-1}	calibration curve	r
rutin	3.4	11.5	$A = -0.97774 \cdot 10^5 + 0.57132\ 10^5 \gamma$	0.9943
quercetin	2.9	9.8	$A = -1.19567 \cdot 10^5 + 0.78590\ 10^5 \gamma$	0.9991

Quantification of Rutin and Quercetin. Obtained extracts by previously described SFE procedure were further analysed by HPLC, and obtained chromatograms from standard solutions of pure components of rutin and quercetin, and SF extract from *Hyperici Herba* are shown on Figure1. The results from quantitative determinations are shown in Table 2 and 3.

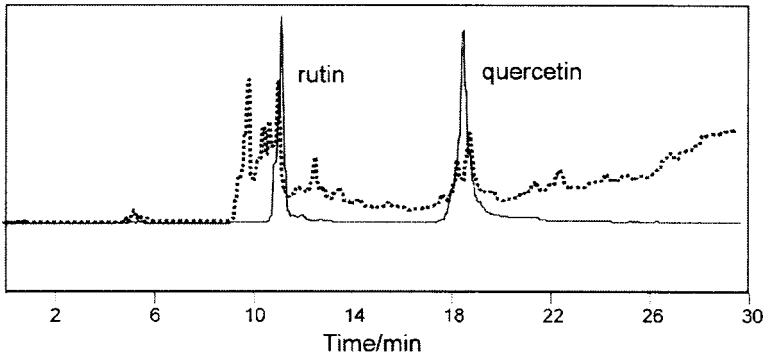

Figure 1. Chromatograms of standard solutions of rutin and quercetin (solid line) and SFE extract from *Hyperici Herba* (dashed line).

Table 2. Results from quantitative determination of quercetin in *Hyperici herba* extract.

sample weight/ mg	particle diameter 0.150-0.300 mm			particle diameter 0.300-0.750 mm		
	γ/μg mL^{-1}	RSD/%	w / %	γ/μg mL^{-1}	RSD/%	w / %
100	29.018	14.52	0.029 ± 0.003	17.865	18.63	0.018 ± 0.003
200	64.019	8.50	0.032 ± 0.003	33.900	6.20	0.017 ± 0.001
300	84.717	4.19	0.028 ± 0.001	44.655	7.58	0.015 ± 0.001
400	109.512	5.33	0.027 ± 0.001	60.382	3.76	0.015 ± 0.001
500	121.762	5.36	0.024 ± 0.001	71.604	7.86	0.014 ± 0.001

γ and w – mean values from three replications

As it can be seen from the results listed in Table 2 and 3, the sample weight has some influence on the SFE process, even since there is no irrefutable explanation for this phenomena.[2] Actually the parameter which influences the expeditiousness is sample size to cell volume ratio, and which must be determined experimentally. In our case the best extractions in all cases were performed when sample size vas 0.2 g, and naturally the cell volume always was 7 mL.

Table 3. Results from quantitative determination of rutin in *Hyperici herba* extract.

sample weight/ mg	particle diameter 0.150-0.300 mm			particle diameter 0.300-0.750 mm		
	γ/μg mL^{-1}	RSD/%	w/ %	γ/μg mL^{-1}	RSD/%	w / %
100	19.737	6.36	0.020 ± 0.001	14.382	5.25	0.014 ± 0.000
200	43.251	7.88	0.022 ± 0.002	35.079	4.35	0.018 ± 0.001
300	67.555	6.73	0.023 ± 0.002	54.399	9.00	0.018 ± 0.002
400	79.109	4.39	0.020 ± 0.001	64.538	5.80	0.016 ± 0.001
500	95.069	7.44	0.019 ± 0.001	76.618	4.59	0.015 ± 0.001

γ and w – mean values from three replications

Concerning the dependence of the SFE efficiency from the particle size of the sample, it can be notified that bigger concentrations of rutin and quercetin were determined when the particles were with diameter 0.150-0.300 mm. With decreasing the particle size, sample surface area to weight ratio increases. Reduction of the particle sizes solves the problems derived from sample heterogeneity.

Conclusions

It is important to amplify that when performing an SFE on natural products, it should be customary to pre-treat samples by grinding, sieving and adding a modifier prior to SFE analysis. Sample size and particle diameter influence on the efficiency of the extraction procedure.

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