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## THE INFLUENCE OF THE OPERATION CONDITIONS AND THE EXTRACTION TECHNIQUES ON THE YIELD, KINETICS AND THE COMPOSITION OF ETHANOL EXTRACTS OF *Hieracium pilosella* L.

*The optimal operational extraction conditions (50 % v/v ethanol, solvomodule 1:15 m/v, temperature: the solvent boiling temperature) were determined by investigating the influence of the ethanol concentration (30 – 95 % v/v), solvomodule (1:10 – 1:25 m/v) and the temperature of the maceration extraction on the yield and kinetics of total extraction matter, chlorogenic acid, umbelliferone and apigenin-7-O-glucoside from Hieracium pilosella L. Based on the results of Soxhlet and Tillepape extraction kinetics, the investigations of the total extraction matter and the components under the optimal maceration operation conditions it was found that the highest yield of the extraction matter from the dry plant material (43.0 g/100 g of the dry plant material, i.e. 97.7 % of the extractive matter content in the plant material), chlorogenic acid (20.30 g/100 g of dry plant material, i.e. 94 % of the chlorogenic acid content in the plant material), umbelliferone (0.30 g/100 g of the dry plant material, i.e. 96.7 % of umbelliferone content in the plant material, and apigenin-7-O-glucoside (0.24 g/100 g of the dry plant material, i.e. 96 % of the apigenin-7-O-glucoside content in the plant material) was obtained by using a Soxhlet extraction method. The contents of chlorogenic acid, umbelliferone and apigenin-7-O-glucoside in the extracts were determined by HPLC method. Chlorogenic acid is the component with the highest share in all the extracts. (It was found) The high content of chlorogenic acid in the investigated plant material (21.60 %) and the high level of its extraction from the plant material (75–94 %) were found under the defined optimal maceration conditions, Soxhlet and Tillepape extraction.*

*Key words: Hieracium pilosella L., extraction techniques, HPLC analysis, chlorogenic acid, Umbelliferone, Apigenin-7-O-glucoside.*

*Hieracium pilosella* Linne (Family Asteraceae) is a perennial herb. It is widespread in mountainside and hill pastures, in the areas of oak woods and shrubbery. It is used in the traditional medicine for the treatment of bronchitis, bronchial asthma, dropsy and edema, and as an ointment for wound healing. It is especially recommended for intensifying urination and eliminating slime, sand and small stones from the urinary tract [1]. In the traditional European medicine it is used for its diuretic and anti-inflammatory effects [2]. The components most commonly found in all *Hieracium* species are phenol and flavonoids [3–5]. The chemical composition and the quantitative content of individual components depend on the species and the locality where the investigated species thrive [6]. A phytochemical screening of the diethyl ether extract revealed that *Hieracium pilosella* L. leaves contained coumarins, flavonoids and terpenes [7]. The common phenol components found in all *Hieracium* species methanol extracts are: chlorogenic acid, 3,5-dicaffeoylquinic acid, and flavonoid luteolin

7-O-glucoside [8]. The chlorogenic and caffeic acids are found in green leaves and the water extracts from *Hieracium pilosella* L. dry leaves, and the root contains umbelliferone [9]. *Hieracium pilosella* L. flowers contain flavonoids and phenolic acids [5]. Phenolic acids and flavonoids are natural antioxidants [10–12] with anti-mutagenic and anti-carcinogenic [13,14], cardio-protective [15] and antimicrobial properties [11,16,17]. For the extraction of phenolic compounds from the plant material, methanol, ethanol and acetone are most often used as extragents [18, 19].

In the available reference works there is no data on the composition of the ethanol extracts from the leaves and roots of this herb from the locality of Southeast Serbia, or data on the effects of the operational conditions and extraction techniques on the yield and composition of extractive matter and the kinetics of the extractions.

Based on the comparative investigation of the extract composition and the kinetics of the macerations extraction, Soxhlet and Tillepape extraction, the aim of this work is to define the optimal operational conditions and the extraction techniques for obtaining the maximum yield of the extraction matters (chlorogenic acid, umbelliferone and apigenin-7-O-glucoside) and to determine the parameters in the extraction kinetics equations.

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## EXPERIMENTAL

### Plant material

*Hieracium pilosella* L. (a whole plant, without flowers). The plant was picked in the area of southeast Serbia in June, 2005. The plant material was dried in the shade, in a drafty place, and after drying it was stored in paper bags and kept at room temperature (water content in the plant material being 14.87 %). All the experiments were carried out with the same plant material.

### Standards

Chlorogenic acid was obtained from Sigma-Aldrich (Steinheim, Germany), apigenine-7-O-glucoside and umbelliferone were purchased from Extrasynthese (Genay, France).

### Solvents and reagents

Acetonitrile was of HPLC-grade from Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade (Sigma).

### Extractive matter content in the plant material

The measured quantity (10 g) of the crushed and homogenized plant material was placed in a Soxhlet extraction apparatus. 150 ml of solvent (50% v/v ethanol) was added into a receiving flask. The extraction was being carried out at boiling temperature lasted for 6 hours. The solvent was evaporated on a rotary vacuum evaporator at 50°C. The extract obtained was dried in a vacuum dryer at 50°C till constant mass and the content of the extraction matter in the plant material was calculated.

### Water content in the plant material

The content of the water was determined on a SCALTEC SMO 01 apparatus (Scaltec Instruments, Germany) at 105°C.

### HPLC analysis

HPLC analysis was used to determine the qualitative and quantitative composition of chlorogenic acid, umbelliferone and apigenine-7-O-glucoside in the extracts based on calibration curves of the investigated components standards. The identification and determination of the content of bioactive components in the extracts was carried out by HPLC analysis under the following conditions: Agilent 1100 Series, Waldborn, Germany; Column: Zorbax-Eclipse XDB-CN; 4.6x250 mm, 5 µm. Eluent: acetonitrile: water, 30:70 v/v. Flow rate: 1 ml/min. Injection volume: 20 µl. Detection: 205 nm UV detector. The calibration curves for quantitative determination were constructed with seven different concentrations of standard components solution, under

the same conditions of HPLC analysis of the extract composition. The concentration ranges were: 1–500 µg/ml, 0.15–15 µg/ml and 4–670 µg/ml of chlorogenic acid, umbelliferone and apigenine-7-O-glucoside, respectively.

### Maceration

The measured quantity of crushed and homogenized plant material (3 g) was poured over with 1:15 m/v ethanol (30–95% v/v) and the maceration extraction with reflux was being carried out for 2 hours at 25°C. The extract was separated by filtering under a weak vacuum. The content of the extractive matter (dry extract) was determined on a SCALTEC SMO 01 apparatus (Scaltec Instruments, Germany) at 105°C. The yield of the extractive matter was calculated on the basis of the dry residue content. Based on the extractive matter yield, the yield and the content of chlorogenic acid, umbelliferone and apigenine-7-O-glucoside in the dry extracts and the optimal concentration of ethanol were determined. With the optimal concentration of ethanol and with other operational conditions unchanged, the effect of solvmodule (ratio of plant material: solvent, m/v) (1:10, 1:20 and 1:25 m/v) on the yield of the matter extracted was investigated, as well as the yield and the content of the investigated components in the dry extracts. Based on the results obtained, the optimal solvmodule was determined. Similarly, employing the optimal ethanol concentration and the optimal solvmodule, the effect of the temperature on the extraction kinetics and the bioactive components was investigated to select the optimal extraction temperature.

### Soxhlet extraction

Under the optimal maceration conditions the kinetics of Soxhlet extraction, the extractive matter, chlorogenic acid, umbelliferone and apigenine-7-O-glucoside were investigated.

### Tillepape extraction

Under the optimal maceration conditions the kinetics of Tillepape extraction, the extractive matter, chlorogenic acid, umbelliferone and apigenine-7-O-glucoside were investigated.

## RESULTS AND DISCUSSION

Phenolics and flavonoids are the components most commonly found in all *Hieracium species* [3–5]. Phenolic acids and flavonoids are natural antioxidants [10–12] with anti-mutagenic, anti-carcinogenic [13,14], cardio-protective [15] and antimicrobial properties [11,16,17]. According to HPLC analysis all the obtained extracts of *Hieracium pilosella* L. contain three phenolic components: chlorogenic acid, umbelliferone and apigenine-7-O-glucoside.

Maximum contents of the matter extracted, chlorogenic acid, umbelliferone and apigenine-7-O-glucoside in the initial plant material determined by Soxhlet extraction with 50 % v/v ethanol were 44.0, 21.60, 0.31 and 0.25 g /100 g dry plant material, respectively. Maximum contents of chlorogenic acid, umbelliferone and apigenine-7-O-glucoside in (the) dry extracts, determined by Soxhlet extraction with 50 % v/v ethanol, were 49.61, 0.72 and 0.58 g /100 g dry extract, respectively.

Retention times of the components chlorogenic acid, umbelliferone and apigenine-7-O-glucoside obtained by HPLC method were 2.10, 4.60 and 5.30 min, respectively. The equation obtained from the calibration graph used for determining the concentrations of the components: the chlorogenic acid, umbelliferone and apigenine-7-O-glucoside, in the extracts through the dependence of the peak area upon the concentration of the standards obtained by HPLC method is:

$$P = q + r \cdot c \quad (1)$$

where: P is peak surface (mAU), c – standard concentration (mg/ml), and q and r – constants. The values of q for chlorogenic acid, umbelliferone and apigenine-7-O-glucoside were 75.84, 60.08 and 235.61, respectively. The values of r for chlorogenic acid, umbelliferone and apigenine-7-O-glucoside were 30891, 79939 and 153296, respectively. The linear correlation coefficient was 1,000.

The influence of ethanol concentration on the yield of the extractive substances and the bioactive

components can be seen in Table 1. The highest yields of the extractive substances (29.50 g/100 g of dry plant material), chlorogenic acid (13.80 g/100 g of dry plant material), umbelliferone (0.23 g/100 g of dry plant material), and apigenine-7-O-glucoside were obtained by the extraction with 50 % v/v ethanol. When the ethanol concentration is increased, the yield of the matter extracted is reduced due to the reduction of the solvent polarity, i.e. the decreased solubility of the hydrophilic compounds. The content of chlorogenic acid in the dry extract is not significantly affected by the increase of the ethanol concentration above 50 % v/v. The content of umbelliferone and apigenine-7-O-glucoside in dry extracts is significantly increased (9.09 and 155.84 % in the extraction with 70 % ethanol, and 37.5 and 110 % in the 95 % ethanol extraction, respectively), which is the consequence of better solubility of these components in ethanol. Further investigations were carried out with the extractions with 50 % ethanol, this being the optimal solvent.

Table 2 shows the effect of solvomodule on the yield of extractive substances, the yield and the content of the bioactive components in dry extracts obtained by the maceration.

With the increase of solvomodule to above 1:15 m/v, the total extractive matter content was decreased (2.27 and 5.18 % for solvomodule 1:20 and 1:25 m/v, respectively). With the increase of solvomodule (1:20 and 1:25 m/v) the content of chlorogenic acid (5.05 and 16.33 %, respectively), umbelliferone (21.73 and 30.43 %, respectively), and apigenine-7-O-glucoside (8.33 %)

Table 1. The effect of ethanol concentration on the yield of the matter extracted and the bioactive components (extraction time: 120 minutes, solvomodule: 1:15 m/v, temperature: 25°C)

Solvent, Ethanol % (v/v)	Matter extracted, g/100g d.p.m.	Chlorogenic acid		Umbelliferone		Apigenin-7-O-glucoside	
		g/100g d.e.	g/100g d.p.m.	g/100g d.e.	g/100g d.p.m.	g/100g d.e.	g/100g d.p.m.
30	28.91	43.40	12.55	0.72	0.21	0.29	0.08
50	29.50	46.71	13.80	0.77	0.23	0.40	0.12
70	24.61	47.19	11.61	0.84	0.21	0.55	0.11
95 %	6.24	47.40	2.96	1.97	0.12	0.84	0.05

d.e.– dry extract, d.p.m. – dry plant material

Table 2. The effect of solvomodule on the yield of the matter extracted and the bioactive components (extraction time: 120 min, solvent: 50% v/v ethanol, temperature: 25°C)

Solvomodule, m/v	Matter extracted, g/100g d.p.m.	Chlorogenic acid		Umbelliferone		Apigenin-7-O-glucoside	
		g/100g d.e.	g/100g d.p.m.	g/100g d.e.	g/100g d.p.m.	g/100g d.e.	g/100g d.p.m.
1:10	23.44	45.60	10.68	0.72	0.17	0.49	0.11
1:15	29.50	46.71	13.80	0.77	0.23	0.40	0.12
1:20	28.83	44.35	12.78	0.63	0.18	0.39	0.11
1:25	27.97	39.08	10.93	0.58	0.16	0.40	0.11

d.e.– dry extract, d.p.m. – dry plant material

in the dry extracts was decreased. Solvmodule 1:15 m/v was accepted as the optimal one.

The influence of the temperature on the matter extracted yield, the yield and the content of bioactive components in the macerated dry extracts with 50 % v/v ethanol and solvmodule 1:15 m/v was monitored by investigating the kinetics of the extraction of the matter extracted, chlorogenic acid, umbelliferone and apigenine-7-O-glucoside (a-d, respectively) at different extraction temperatures (Figure 1).

The yield of the matter extracted, chlorogenic acid, umbelliferone and apigenine-7-O-glucoside is increased with the increase of temperature. The content of chlorogenic acid, umbelliferone and apigenine-7-O-glucoside in dry extracts is not significantly changed; therefore, the solvent boiling point temperature was accepted as the optimal extraction temperature.

The figure shows that, in the maceration extraction process, there are two characteristic periods: the period of the fast extraction and the period of the slow extraction, in accordance with Ponomarjev's empirical equation [20]. Based on the results obtained, the optimal extraction conditions were defined: 50 % v/v

ethanol, solvmodule 1:15 m/v and the solvent boiling point temperature.

Under the optimal maceration extraction conditions, the kinetics of the matter extracted and bioactive components were monitored by the circulation extraction techniques. A comparative survey of the kinetics of the maceration extraction with reflux, Tillepape and Soxhlet extraction of the extractive matter, chlorogenic acid, umbelliferone and apigenine-7-O-glucoside (a-d, respectively) under the optimal maceration extraction conditions is shown in Figure 2.

The figure shows that a greater extraction level of matter extracted ( $(q_0 - q_i)/q_0$ ) was achieved by the circulation extraction techniques, when compared to the maceration technique with reflux under the same extraction operation conditions. The highest level of the matter extracted was achieved with Soxhlet extraction for a period of 240 minutes (97.73 %).

The extraction kinetics curves (Figure 2) are the curves typical for the extraction of the cellular material [20-22] with two extraction periods. In the first period, the fast extraction occurs when the matter extracted is washed out from the surface of the destructed cells by water. In the second period, a slow molecule diffusion of

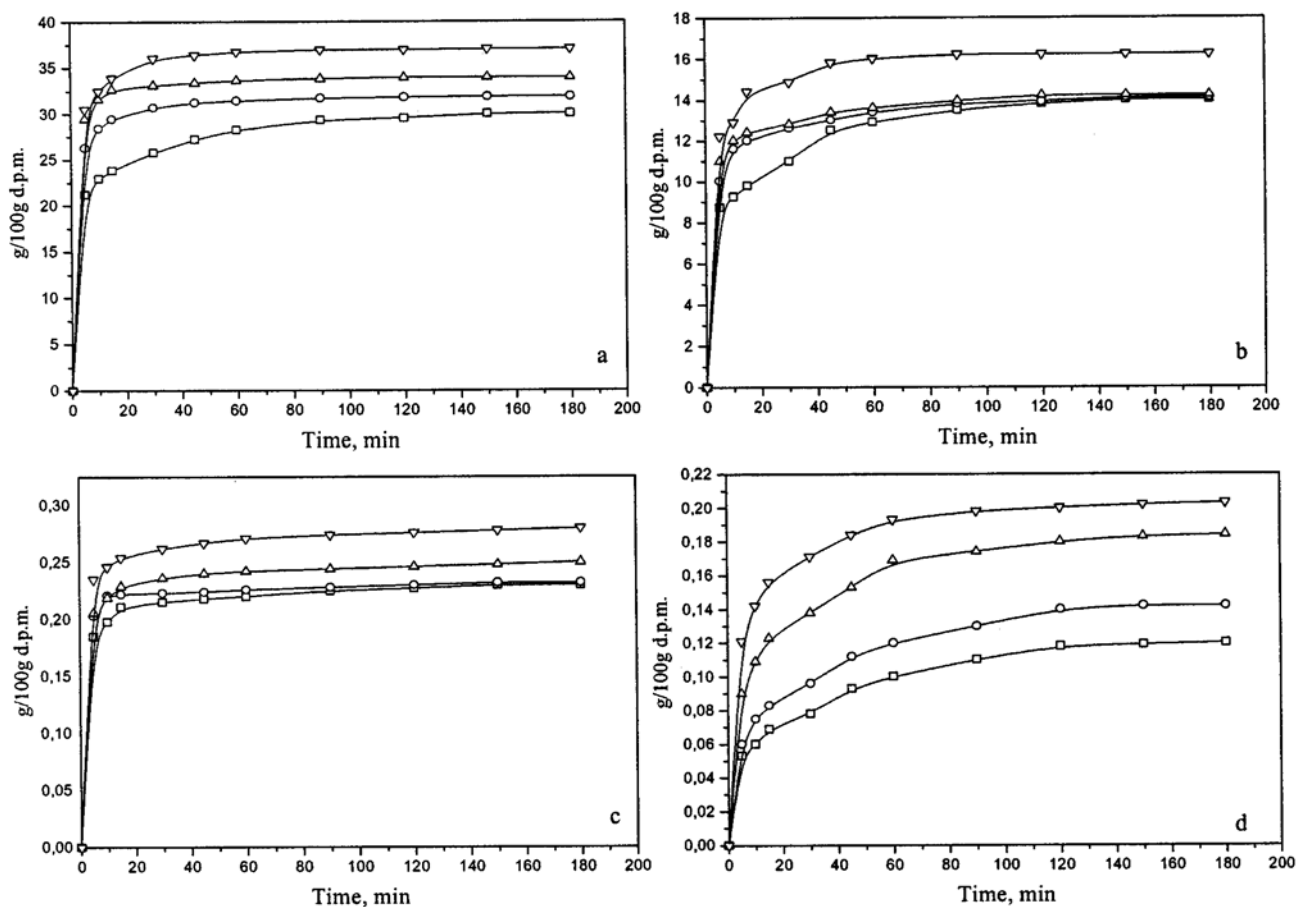


Figure 1. The extraction kinetics of the matter extracted, (a) chlorogenic acid; (b) umbelliferone; (c) and apigenine-7-O-glucoside; (d) maceration at various temperatures (solvent: 50% v/v ethanol, solvmodule: 1:15 m/v (□) - 25°C, (O) - 40°C, (Δ) - 60°C, (▽) - solvent boiling point temperature)

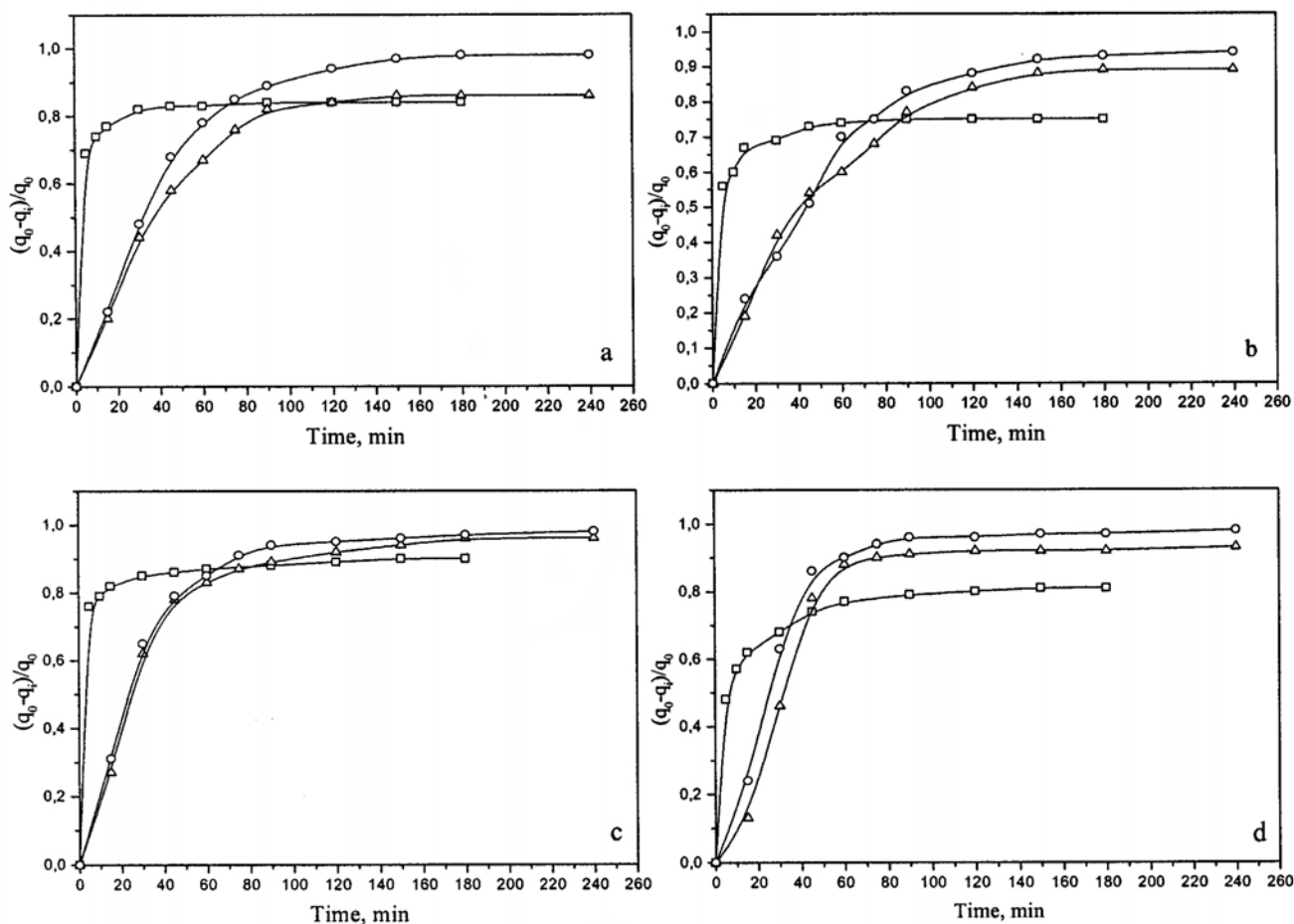


Figure 2. Tillepape and Soxhlet extraction kinetics and reflux maceration of the matter extracted (a) chlorogenic acid; (b) umbelliferone; (c) and apigenine-7-O-glucoside; (d) under the optimal maceration conditions ( $\square$  – reflux,  $\Delta$  – Tillepape, O – Soxhlet)

the matter extracted from the internal part of the non-destroyed cells occurs (the slow extraction). The fast extraction (the curvilinear part of the extraction curve) is characterized by the washing coefficient  $b$ , and the slow extraction by the slow extraction coefficient,  $k$ , in the following equation [20]:

$$\frac{q_0 - q_i}{q_0} = b + k \cdot t \quad (2)$$

where  $q_0$  is the extractive matter content in the initial plant material;  $q_i$  – the content of the extractive matter in the plant material after the period  $t$ ;  $b$  – the coefficient of the fast extraction period; and  $k$  – the coefficient of the

slow extraction period. The values of  $b$  and  $k$  for the extraction of the extractive matter, chlorogenic acid, umbelliferone and apigenine-7-O-glucoside, by using various techniques under the optimal conditions, are given in Table 3. The duration times of the fast extraction and the extraction levels for the total extractive matter, and the investigated components are given in Table 4. During the fast extraction period, 83–94 % of the extractive matter, 74–92% of chlorogenic acid, 87–94 % of umbelliferone and 77–96 % of apigenine-7-O-glucoside were extracted by dissolution of the extractive matter from surfaces of the plant particles (Table 4), indicating that the crushing of the plant material had been relatively high.

Table 3. The values of  $b$  and  $k$  coefficients in the equations of the extraction kinetics of the matter extracted, chlorogenic acid, umbelliferone and apigenin-7-O-glucoside using various techniques under the optimal maceration conditions (Ponomarjev's empirical equation [20])

Coefficients	Reflux maceration		Tillepape extraction		Soxhlet extraction	
	$b$	$k \times 10^4, \text{min}^{-1}$	$b$	$k \times 10^4, \text{min}^{-1}$	$b$	$k \times 10^4, \text{min}^{-1}$
Matter extracted	0.830	0.67	0.832	1.33	0.916	2.95
Chlorogenic acid	0.740	0.67	0.868	0.95	0.889	2.14
Umbelliferone	0.856	2.67	0.917	1.90	0.918	2.70
Apigenin-7-O-glucoside	0.756	3.33	0.902	1.13	0.946	1.40

Table 4. The fast extraction time and the extraction level in the equations of the extraction kinetics of the matter extracted, chlorogenic acid, umbelliferone and apigenin-7-O-glucoside using various techniques under the optimal extraction conditions (Ponomarev's empirical equation [20])

	Reflux maceration		Tillepape extraction		Soxhlet extraction	
	PBE, min	SE, %	PBE, min	SE, %	PBE, min	SE, %
Matter extracted	60	83	120	84	120	94
Chlorogenic acid	60	74	150	88	150	92
Umbelliferone	60	77	90	91	90	96
Apigenin-7-O-glucoside	60	87	150	94	90	94

PBE – period of fast extraction

SE – extraction level

In all the extracts the chlorogenic acid was represented by the highest share. The content of this acid in dry extracts obtained by maceration with reflux, Soxhlet and Tillepape extractions under the optimal conditions, was 43.92, 47.17 and 50.74 g/100g of the dry extract, respectively. The preparative isolation of chlorogenic acid confirmed that the yield of chlorogenic acid in the isolated preparation corresponds with this component content established in liquid extracts by HPLC analysis. The high content of phenolic acid as a natural antioxidant [4] is an indication of the possible use of the ethanol extract in food and pharmaceutical industries.

## CONCLUSION

Based on the results obtained, it can be concluded that the operation conditions and the extraction methods used have a considerable influence on the yield of the matter extracted, the extraction kinetics and the composition of the extracts obtained from *Hieracium pilosella* L. The optimal extraction conditions are: the solvent: 50 % v/v ethanol; solvmodule 1:15 m/v; the extraction temperature: the solvent boiling point temperature. The highest yield of the matter extracted and the individual bioactive components was achieved by using Soxhlet extraction. The components identified through HPLC analysis in all of the extracts are: chlorogenic acid, and umbelliferone, apigenine-7-O-glucoside. Chlorogenic acid is the component with the highest share in all the extracts. A relatively high content of chlorogenic acid in the investigated plant material (21.60 %) and the high level of its extraction from the plant material (75–94 %) under the defined optimal maceration conditions, Soxhlet and Tillepape extraction, facilitate its high level of the extraction as a pure substance that has an important application in the pharmaceutical and food industries.

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