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The GC/MS and HPLC/DAD analysis of phenolic acids from Winter Savory (Satureja montana)

Analiza kwasów fenolowych w cząbrze górskim (Satureja montana) metodami GC/MS i HPLC/DAD

INTRODUCTION

Phenolic acids (cinnamic and benzoic acids derivatives) are the aromatic secondary plant metabolites (a subclass of phenolics) widespread throughout the plant kingdom. Cinnamic and benzoic acid derivatives are physically dispersed throughout the plants and appear in seeds, leaves, roots and stems. The majority of phenolic acids are linked through the glycoside, ester, ether, or acetal bonds either to the structural components of plants (e.g., to lignin and cellulose), or to the larger polyphenols (e.g., to flavonoids and tannins), smaller molecules (e.g., glucose and tartaric acid), and the other natural products (e.g. terpenes). Depsides are intermolecular esters which are formed by condensation of two or more molecules of the same or different phenolic acids. In plants, the most common depside is chlorogenic acid. All forms of phenolic acids are well soluble in organic solvents [1].

Phenolic acids exert various biological and pharmacological effects. They have been connected with the diverse functions such, as synthesis of proteins, enzymatic activity, photosynthesis of structural components, and alleopathy. They play an important role in the natural host defense mechanism of plants against the infectious diseases and inhibit multiplication of the plants' pathogenic bacteria, viruses, and fungi. Stress conditions (such, as UV light, wounding, or infection) induce biosynthesis of phenolic compounds [2].

Phenolic acids can be found in many traditional herbal medicines. Most of them have shown an excellent scavenging activity toward the free radicals. Certain phenolic acids have anti-inflammatory and antirheumatic properties. Recent investigations have linked a series of phenolic acids (e.g., caffeic and ferulic acid) with anticancer activity. In recent years, a lot of clinical studies

were focused on phenolic acids as a group of potential immunostimulating compounds. Several phenolic acids exert antimicrobial and antifungal properties. Moreover, certain phenolic acids (4-hydroxybenzoic and chlorogenic acid) show a hypoglycemic effect and additionally increase the serum insulin levels and the liver glycogen content. Ferulic acid exhibits cholesterol-lowering activity and it is considered as a potential drug against coronary heart disease. Other biological activities of phenolic acids are also known such, as adstringent (rosmarinic acid), sedative exerted on the central nervous system (rosmarinic acid, chlorogenic acid), choleretic and cholekinetic (most phenolic acids), hepatoprotective, analgetic, antipyretic, and keratolytic (salicylic acid) [1].

Because of the aforementioned curative properties of phenolic acid, fractions thereof are often investigated in various different plant materials. However, only a small number of plants have been systematically examined for the biologically active phenolic compounds. Thus, the data on phenolics contained in plants, fruits and vegetables are incomplete [2]. For this reason, there is a continuous need for the investigation of the phenolic acid fraction originating from various different plant materials.

The principal goal of this study on winter savory (Satureja montana) was to compare the GC/MS and HPLC/DAD results on identification of phenolic acids contained in this plant. The GC/MS analysis was carried out on the steam distillation residue extracted by means of the SPE technique, and ultimately analyzed in the derivatized form. Additionally, the GC/MS analysis was carried out on the extracts of the winter savory sample obtained with use of ASE and then derivatized. The non-derivatized ASE extract was analyzed by means of HPLC/DAD. Partial identification of phenolic acids derived from the plant material was performed, a comparison of the GC and HPLC results was made, and the conclusions were drawn as to the identified compounds, depending on the applied analytical approach.

MATERIAL AND METHODS

H e r b a l m a t e r i a l. Winter savory (*Satureja montana*) investigated in this study was harvested in Pharmacognosy Garden of the Medical University, Lublin, Poland. The plant material comprised all parts of the plant (i.e. roots and the aerial parts) and it was dried for 40 h in an oven with a forced air flow at 35 to 40°C. Then the obtained dry material was stored in the deep-freeze compartment of refrigerator until the commencement of the analysis. Finally, plant material was weighed and ground with a mechanical blender.

REAGENTS AND ACCESSORIES

In this study, the following reagents were used: 36% aqueous HCl and solid NaOH (both analytical purity grade, P.O.CH, Gliwice, Poland); methanol (HPLC purity grade, P.O.CH); N,O-bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (BSTFA+TMCS, 99:1; Supelco, Bellefonte, USA); diethyl ether and pyridine (both analytical purity grade, Baker, Deventer, Holland). The phenolic acid standards used for direct identification by means of HPLC/DAD were manufactured by the firm Roth, Karlsruhe, Germany.

Besides, the following apparatus and accessories were used:

- The Dionex model ASE 200 accelerated solvent extraction unit (Sunnyvale, CA, USA);
- The ultrathermostat type U2 (VEB Medingen, Germany);

- The ultrasonication bath (Banderin, Berlin, Germany); and
- The set for the vacuum solid phase extraction, SPE (Supelco) and the SPE cartridges filled with octadecyl silica (DSC-18) and polyamide (DPA-6S) packings (Supelco).

SOLID-PHASE EXTRACTION (SPE) AND DERIVATIZATION OF PHENOLIC ACIDS

Liquid left from the steam distillation of the *Satureja montana* sample with use of the Deryng apparatus (as recommended by Polish Pharmacopoeia VI applied to the 50-g plant samples [3]) underwent the acidic and basic hydrolysis in parallel, in order to liberate phenolic acids most probably present in the ester and the glycoside form. The acidic hydrolysis was performed in the following way: Liquid was adjusted with 36% HCl to pH ≈ 2 and the entity was kept boiling for 2 h under the reflux. In the case of basic hydrolysis, the second portion of the liquid was adjusted with 4M NaOH to pH ≈ 10 and the entity was also kept boiling for 2 h under the reflux. The obtained hydrolysates were neutralized and filtered to separate the particulate matter and finally they underwent the solid phase extraction.

The SPE tubes were first conditioned with 5 mL methanol and 10 mL redistilled water. After passing the hydrolyzed and filtered samples through the SPE cartridges, elution of the adsorbed compounds was carried out with 6 mL methanol. The eluates were condensed to dryness in the air stream and then underwent the derivatization procedure.

Derivatization was carried out by adding 100 µL BSTFA+TMCS and 1 mL pyridine to the dry residues and heating the obtained solutions at 80°C for 2 h on the oil bath. After cooling, samples underwent the GC/MS analysis.

Identification of individual compounds was based on the Selected Ion Monitoring (SIM) procedure, which depends on the search of the matching ions in the whole spectral range with aid of the software library (NIST, Gaithersburg, MD, USA).

GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC/MS) OF THE DERIVATIZED PHENOLIC ACIDS

Samples were analyzed with use of a TRACE 2000 model GC with an MS TRACE model mass detector (ThermoQuest), equipped with an autosampler (Combi PAL), and the TR-35MS capillary column (30 m ↔ 0.25 mm i.d., 0.5-µm film thickness; Thermo Scientific, Northumberland, UK). Helium (p = 100 kPa) was used as carrier gas. Gradient analysis was run using the following temperature program: 80ºC (3 min); 80–160ºC (20ºC/min); 160–280ºC (20ºC/min); and 280ºC (20 min). The temperature of the injector was kept constant at 280ºC. Mass spectrometer was fitted with an EI source operated at 70 eV.

ACCELERATED SOLVENT EXTRACTION (ASE) AND DERIVATIZATION OF PHENOLIC ACIDS

The 2.5-g aliquots of the mechanically ground winter savory sample were placed in the extraction cell. At the first step, the samples were twice extracted with petroleum ether at 120°C under the pressure of 100 atm. This procedure was in principle meant to remove chlorophyll from plant material, yet the joint petroleum ether extract (denoted as E0) was checked for the possible phenolic acid losses at this initial step of the procedure.

The same plant material was then extracted with methanol at 100°C under the pressure of 80 atm, and the methanol extract was condensed to dryness in the air stream. The dry residue was washed with four 2.5-mL portions of warm redistilled water and the joint water solution was stored in a cool place for 24 h.

After filtering off the particulate matter, water solution was extracted with five 2-mL portions of diethyl ether. The joint diethyl ether extract was denoted as E1. Water residue from the diethyl ether extraction underwent the acidic hydrolysis (in order to liberate phenolic acids present as glycosides or esters in the plant material), as described in sub-section 2.3. The hydrolyzate was extracted with five 2-mL portions of diethyl ether and the joint extract was denoted as E2. Each of the three extracts (E0, E1, and E3) was condensed to dryness and then derivatized, as described in sub-section 2.3, in order to facilitate the GC/MS analysis (which was carried out, as described in sub-section 2.4). The described procedure (which was an in-home modification of the original procedure introduced in [4]) is summarized in scheme 1.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE ARRAY DETECTION (HPLC/DAD) OF THE NON-DERIVATIZED PHENOLIC ACIDS

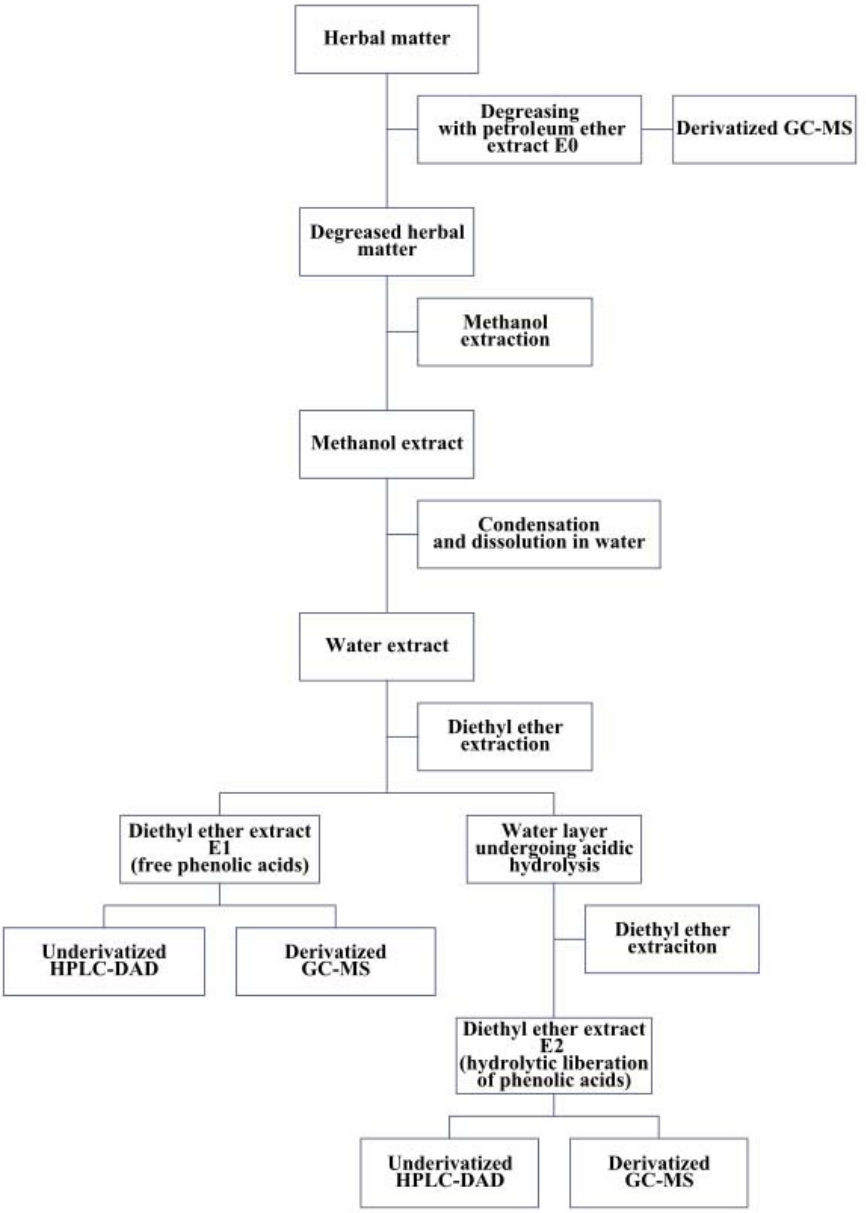
High performance liquid chromatographic analysis was carried out on the underivatized ASE extracts using a Gyncotek liquid chromatograph (Gyncotek, Macclesfield, UK) equipped with a Gyncotek Gina 50 model autosampler, Gyncotek P 580A LPG model pump, Gyncotek DAD UVD 340U model diode array detector, and Chromeleon Dionex v. 6.4 software for data acquisition and processing. The analyses were carried out with the acetonitrile (A) – water modified with 1% glacial acetic acid (B) mobile phase at a flow rate of 0.7 mL min^{-1} in the gradient mode, using an RP-18 Hypersil GOLD (5 μm particle size) column (250 mm x 4.6 mm i.d.; Thermo Scientific, Waltham, MA, USA; cat. no. 0694830N). The following gradient was applied (v/v): 0-7 min, A + B, 20 + 80; 8-13 min, A + B, 40 + 60; 14-19 min, A + B, 60 + 40; 20-36 min, A + B, 100 + 0; and 37-40 min, A + B, 20 + 80. The chromatographic column was thermostated at 35°C with use of the Varian Pro Star 510 model column oven.

RESULTS AND DISCUSSION

ANALYSIS BY MEANS OF GC/MS

An attempt was made to analyze phenolic acids contained in the residue water from the steam distillation in the Deryng apparatus. Steam distillation was earlier used to derive the volatile fraction from *Satureja montana*, which was then analyzed by means of the GC/MS technique [5]. In general, it can be stated that the SPE technique applied to the samples after both, the acidic and the basic hydrolysis, resulted in the low phenolic acids yields, which might probably be the natural characteristics of winter savory (*Satureja montana*).

In the extract originating from basic hydrolysis, then condensed by means of SPE, and finally derivatized by means of the trimethylsilylation reagent, four phenolic acids were identified (as the respective trimethylsilyl derivatives), i.e., 2,3-dihydroxyphenylacetic acid, syringic acid, caffeic acid, and α-hydroxydihydrocaffeic acid (Fig. 1A). In the extract originating from the acidic hydrolysis and then analogically preprocessed, two phenolic acids were identified (also in the form of the respective trimethylsilyl derivatives), i.e., caffeic acid and o-hydroxydihydrocaffeic acid (Fig. 1B).



Scheme 1. Presentation of the ASE extraction and acidic hydrolysis of bonded phenolic acids

The results of the accelerated solvent extraction (ASE) of the winter savory samples largely confirmed the above findings. In the trimethylsilylated extract E0, methyl esters of caffeic acid and 3,4-dihydroxyphenylacetic acid were identified. In the trimethylsilylated extract E1**,** methyl esters of 2-hydroxyphenylpropionic acid and 3,4-dihydroxyphenylacetic acid were found. In the trimethylsilylated extract E2, no phenolic acids were detected.

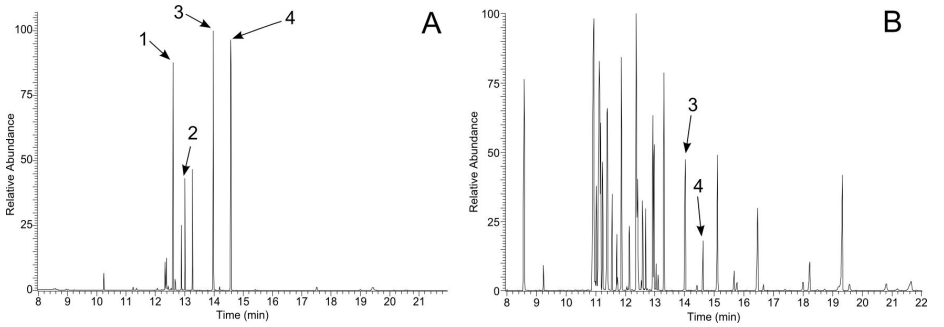


Figure 1. Fingerprint GC/MS chromatograms of the non-volatile *Satureja montana* fraction obtained from the hydrolyzed, SPE-condensed, and finally trimethylsilylated water residue steam distilled in the Deryng apparatus. A: basic hydrolysis; B: acidic hydrolysis. 1: 2,3-dihydroxyphenylacetic acid; 2: syringic acid; 3: α-hydroxydihydrocaffeic acid; and 4: caffeic acid (see Table 1).

ANALYSIS BY MEANS OF HPLC/DAD

The HPLC/DAD analysis was performed for the underivatized (i.e., the non-trimethylsilylated) extracts E1 and E2, obtained with use of the ASE procedure described in sub-section 2.5, and extract E0 was not analyzed by this method. In extract E1, cinnamic acid and p-hydroxybenzoic acid were identified, and in extract E2, chlorogenic acid and elagic acid were found (see Tab. 2 and Fig. 3).

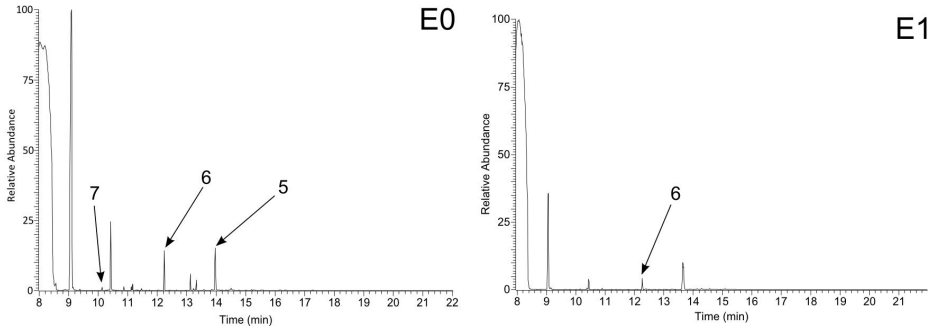


Figure 2. Fingerprint GC/MS chromatograms of the non-volatile *Satureja montana* fraction obtained from the trimethylsilylated ASE extracts (samples E0 and E1). 5: caffeic acid methyl ester; 6: 3,4-dihydroxyphenylacetic acid methyl ester; and 7: 2-hydroxyphenylpropionic acid (see Table 1).

Table 1. Seven derivatized phenolics detected in the water residue from the extraction of the volatile *Satureja montana* fraction with use of the Deryng apparatus and ASE, their respective chemical structures, and peak numbers, as in the GC/MS chromatograms shown in Figs. 1 and 2

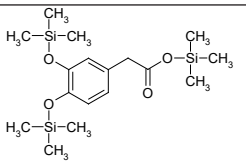
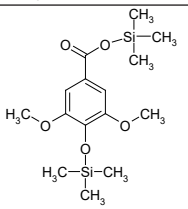
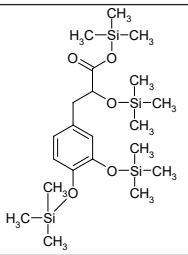
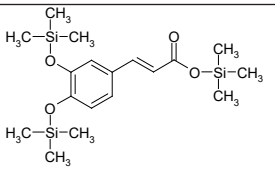
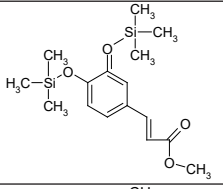
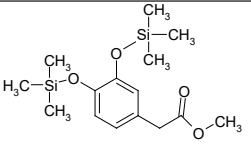
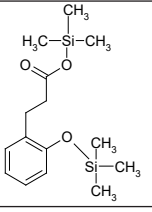
Original compound	Chemical structure of derivative	Peak no.
2,3-Dihydroxyphenylacetic acid		1
Syringic acid		2
α-Hydroxydihydrocaffeic acid		3
Caffeic acid		4
Caffeic acid methyl ester		5
3,4-Dihydroxyphenylacetic acid methyl ester		6
2-Hydroxyphenylpropionic acid		7

Table 2. Four non-derivatized phenolic acids detected by means of HPLC/DAD in extracts E1 and E2 of *Satureja montana* obtained with use of ASE, their respective retention times (tR), and semi-quantitative evaluation of their contents in plant material (mg per 1 gram dry herbal matter)

Extract	Phenolic acid	Retention time, t_R [min]	Contents [mg g^{-1} dry herbal matter]
B	Cinnamic acid	3.38	0.30928
	p-Hydroxybenzoic acid	3.98	0.00004
C	Elagic acid	2.70	0.00004
	Chlorogenic acid	2.89	0.00050

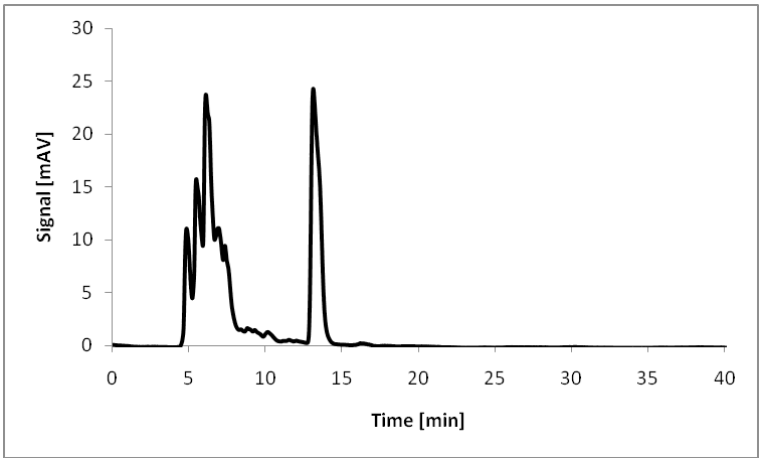


Figure 3. Fingerprint HPLC/DAD chromatogram of the non-volatile *Satureja montana* fraction contained in the diethyl ether extract E2 after the acidic hydrolysis.

In the paper published by Ćetković et al. [6], the folowing phenolic acids were found by means of the RP-HPLC analysis: gallic, protocatechuic, vanillic, caffeic, syringic, p-coumaric, and ferulic. Some of these acids i.e., syringic and caffeic, were also found in our experiments. There are, however, numerous differences among the phenolic acids found in *Satureja montana* in the compared experimental data. They can be due to the differences in the plant habitat (Serbia vs. Poland), but also due to the different methodologies of sample preparation and/or analysis. These observations confirm the necessity of using various different analytical techniques in the investigations of plant metabolites.

CONCLUSION

The methodical aspect of the analysis of the phenolic acids fraction contained in winter savory (*Satureja montana*) was considered in this study. Usefulness of the complementary approach was shown, i.e., of combining the GC/MS and the HPLC/DAD technique. A convincing proof of this usefulness was that each of these two techniques allowed detection of the different phenolic acids.

REFERENCES

1. Wójciak-Kosior M., Oniszczuk A., Sample Preparation and TLC Analysis of Phenolic Acids. in Thin Layer Chromatography in Phytochemistry (Waksmundzka-Hajnos M., Sherma J., Kowalska T., Eds), CRC Press, Taylor & Francis Group, Boca Raton, Fl. pp. 331-364, 2008.
2. Hyötyläinen T., Kivilompolo M., Application of HPLC in the Analysis of Phenols, Phenolic Acids and Tannins. in High Performance Liquid Chromatography in Phytochemical Analysis, (M. Waksmundzka-Hajnos, J. Sherma, Eds), CRC Press, Taylor & Francis Group, Boca Raton, Fl. pp. 477-512, 2011.
3. Polish Pharmacopoeia VI, Polish Pharmaceutical Society, Warsaw, 2002
4. Ibrahim R.K., Towers G.H., Identification by chromatography of plant phenolic acids. Arch. Biochem. Biophys. 87 125-127, 1960.
5. Rzepa J., Sajewicz M., Baj T., Gorczyca P., Włodarek M., Głowniak K., Waksmundzka-Hajnos M., Kowalska T., A comparison of methodical approaches to fingerprinting of the volatile fraction from winter savory (*Satureja montana*). Chromatography Research International, doi:10.1155/2012/596807. (8 pages).
6. Ćetković G.S., Mandić A.I., Čanadanović-Brunet J.M., Djilas S. M., Tumbas V.T., HPLC screening of phenolic compounds in winter savory (*Satureja montana* L.) extracts. J. Liq. Chromatogr. Relat. Technol., 30 293-306, 2007.

SUMMARY

An effort was undertaken to identify phenolic acids contained in the water sample remaining from steam distillation of the volatile fraction contained in winter savory (*Satureja montana*). Solid phase extraction (SPE) was employed to isolate the phenolics from water. Additionally, the accelerated solvent extraction (ASE) was used to isolate the phenolics from the dried winter savory material. The phenolics derived both from the water residue and those obtained with use of ASE were first derivatized and then analyzed by means of GC/MS. The non-derivatized phenolics originating from ASE were fingerprinted and semi-quantitatively assessed by means of HPLC/DAD. A comparison was made of the GC and HPLC results, and the conclusions were drawn as to the identified compounds, depending on the applied analytical approach. Usefulness of the complementary approach was shown, as each of these two techniques allowed detection of the different phenolic acids.

Keywords: savory, *Satureja montana*, phenolic acids, SPE, ASE, HPLC/DAD

STRESZCZENIE

Podjęto próbę identyfikacji kwasów fenolowych zawartych w wodnej pozostałości po destylacji z parą wodną lotnej frakcji zawartej w cząbrze górskim (*Satureja montana*). Zastosowano ekstrakcję do fazy stałej (SPE) do izolacji związków fenolowych z wody. Dodatkowo użyto ciśnieniowej ekstrakcji z wymuszonym przepływem rozpuszczalnika (ASE) do izolacji związków fenolowych z suchego materiału roślinnego. Związki fenolowe pochodzące z wodnej pozostałości i te pochodzące z ASE były derywatyzowane (przeprowadzane w lotne pochodne) a następnie analizowane za pomocą GC/MS. Nie-derywatyzowane związki fenolowe pochodzące z ASE były identyfikowane i szacowne pół-ilościowo za pomocą HPLC/DAD. Przeprowadzono porównanie wyników uzyskanych za pomocą obu technik (GC/MS i HPLC/DAD) i wyciągnięto wniosek, że identyfikowane związki zależą od użytej metody analitycznej. Ponieważ każda z tych technik pozwala na detekcję innych kwasów fenolowych wykazano użyteczność obu komplementarnych metod.

Słowa kluczowes: cząber górski, *Satureja montana*, kwasy fenolowe, SPE, ASE, HPLC/DAD