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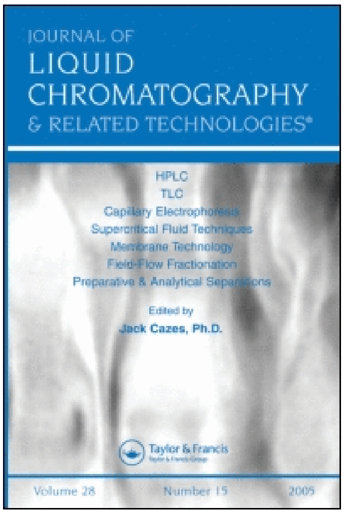

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TLC-Based Start-to-End Method of Analysis of Selected Biologically Active Compounds Contained in Common Sage (*Salvia officinalis* L.)

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Abstract: Common sage (*Salvia officinalis* L.) is an herb native to the Mediterranean region and nowadays is cultivated in many parts of the world. This plant has been known and utilized for hundreds of years in natural medicine, due to its curative properties and good performance in combating various diseases. In spite of its well recognized curative potential, the chemical composition of common sage is far from being sufficiently explored, and research is needed in the areas of phytochemistry and pharmacognosy to better scrutinize its constituents and therapeutic properties. It was the aim of this study to elaborate a start-to-end approach to extraction and chromatographic separation of the compounds contained in common sage, based on thin layer chromatography with densitometric detection and preparative layer chromatography, additionally supported by high performance liquid chromatography with diode-array detection and head-space gas chromatography with mass spectrometry detection. This approach was meant to enable an effective fingerprint analysis, focused mainly on flavonoids, phenolic acids, and terpenes, of the common sage samples, and its performance was demonstrated by analysis of a commercially available common sage tea preparation.

Keywords: Common sage, Flavonoids, HPLC/DAD, HS-GC/MS, Phenolic acids, *Salvia officinalis* L., Terpenes, TLC/densitometry

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INTRODUCTION

Common sage (*Salvia officinalis L.*) originates from the Mediterranean region, where it grows wild and is gathered from natural stands. It is also cultivated in Central and East European countries. Pharmacopoeial material consists of leaves and leaf tops of the sage sprouts with a determined content of essential oil (1.0–2.5% w/w). Essential oil of sage contains thujone, cyneol, camphor, borneol, pinene, and other components.[1] Moreover, plant material contains diterpenes (carnosol, carnosolic acid, and rosmanol); triterpenes (oleanolic acid, ursolic acid, and germanicole); and catechine tannins and flavonoids, mainly the apigenine and luteoline derivatives.[1]

Common sage has a long history of effective medicinal use, and it is an important domestic herbal remedy for disorders of the digestive system. Its antiseptic qualities make it an effective gargle for the mouth and gums, with an additional effect of pain relief.[1] *S. officinalis* is reported to have a wide range of biological activities, i.e., anti-bacterial,[2] fungistatic, virustatic,[1,3] astringent, eupeptic, and anti-hydrotic,[1,4] and anti-inflammatory.[5] Water soluble polysaccharides from *S. officinalis* possess immunomodulatory activity,[6] and there are also reports available on the hypoglycaemic effects of sage.[7] Sage leaves are well known for their anti-oxidative properties[8–11] and hence, they are used in the food processing industry. Moreover, sage has been recognized over the centuries as a restorative for lost or declining mental function,[12–14] and for the antimutagenic activity of its extracts.[15,16]

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Essential oil from the sage leaves is used in perfumery, in hair shampoos, and as a food flavoring. It is a very effective perfume 'fixer', and it is used to flavor toothpastes and as an additive in bio-activating cosmetics.

Chromatographic methods are often used for examination and standardization of plant material derived from sage. In recent years, however, the principal attention of scientists has been focused on *S. miltiorrhiza* and on its active compounds, tanshinones. Published papers dealing with the liquid chromatography of *S. miltiorrhiza* are abundant, and applications of thin layer chromatography (TLC) are given in other articles.[17,18] The volatile fraction of *S. officinalis* has often been examined by means of gas chromatography[19–21] and TLC.[22,23] Application of TLC to the separation and identification of diterpenes,[24–26] flavonoids, and/or phenolic acids[11,27] contained in sage has seldom been reported.

The aim of this research was to elaborate a start-to-end approach to extraction and chromatographic separation of the selected compounds contained in common sage (i.e., phenolic acids, flavonoids, and terpenes), based on thin layer chromatography with densitometric detection (TLC/densitometry) and preparative layer chromatography (PLC), additionally supported by high performance liquid chromatography with diode-array detection (HPLC/DAD), and head-space gas chromatography with mass spectrometry detection (HS-GC/MS).

EXPERIMENTAL

Materials and Reagents

We used dried common sage leaves commercially available from drugstores and suppliers of botanical nutritional supplements. An average sample of dried and ground common sage leaves (5 and 10 g) was obtained from a package of 30 sage teabags (each containing ca. 1.2 g of dried leaves and making a total amount of ca. 36 g). Firstly, all teabags were emptied into a glass dish. Then the leaves were carefully mixed, and portions of 5 and 10 g were weighed.

For extraction and liquid chromatography, chloroform, methanol, *n*-hexane, toluene, and 1,4-dioxane were used (all solvents were of analytical purity grade and manufactured by POCh, Gliwice, Poland). For identification purposes, we utilized solutions of standard phenolic acids and flavonoids listed in Table 1 (manufactured by the firm Roth, Karlsruhe, Germany).

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Extraction

Traditional Extraction

Ten g of ground common sage leaves and 200 mL of *n*-hexane were placed in a round bottom reaction flask equipped with a reflux cooler, and the contents were vortexed for 24 h at ambient temperature (22 + 2°C). The leaves were filtered, a fresh amount of 100 mL *n*-hexane was added, and, again, the contents were vortexed for 24 h at ambient temperature. *n*-Hexane extracts were discarded, as the aim of this extraction step was to remove chlorophyll from the sage sample (this natural pigment negatively affects further analysis due to its abundance and intensive color). The sage leaves were filtered, dried, treated with 100 mL of chloroform, and vortexed for 24 h at ambient temperature. The chloroform extract was evaporated to dryness in a stream of air. The obtained dry residue was dissolved in 10 mL of methanol and centrifuged.[28] The decanted methanol solution was passed through an Anotop syringe filter, filled with aluminium oxide adsorbent (barrel diameter 25 mm, average particle diameter 0.02 μm; Merck KGaA, Darmstadt, Germany, cat. no. 11320). The decanted and additionally filtered solution was ready for TLC analysis.

Table 1. The retardation factor (R_F) values for standard compounds used in TLC [stationary phase, silica gel; mobile phase, toluene – dioxane, 7:2 (v/v)]

Standard compound	R_F
Vanillic acid	0.48
Caffeic acid	0.21
trans-Cinnamic acid	0.54
Syringic acid	0.37
Rosmarinic acid	0.04
m-Coumaric acid	0.39
o-Coumaric acid	0.38
p-Coumaric acid	0.36
Chlorogenic acid	0
Gallic acid	0.24
m-Hydroxybenzoic acid	0.16
p-Hydroxybenzoic acid	0.19
Ellagic acid	0.10
Protocatechuic acid	0.23
Isovanillic acid	0.35
Ferulic acid	0.40
Apigenine	0.29
Luteoline	0.16
Myricetine	0.03
Quercetine	0
Quercitrine	0
Isoquercitrine	0
Rutine	0
Kaempferol	0.37
Hyperoside	0
Apigenine glucoside	0.27
Kaempferol glucoside	0
Luteoline glucoside	0.17
Hesperetine glucoside	0

Accelerated Solvent Extraction (ASE)

Extraction was carried out according to the three different working procedures in an ASE 200 model accelerated solvent extraction unit (Dionex, Sunnyvale, CA, USA). The operating parameters valid for each individual procedure are given in Table 2.

Each extract obtained with use of the ASE apparatus was evaporated to dryness in a stream of air, and then 5 mL of methanol was added to the dry residue. Vials with dry residue and 5 mL of methanol were ultrasonicated for 15 min in a model RK 255H Sonorex Super ultrasonic bath

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Table 2. The employed extracting solvents and working parameters of accelerated solvent extraction

No.	Extracting solvent	Sample weight (g)	Preheat (min)	Heat (min)	Static (min)	Pressure (atm)	Temperature (°C)	No. of cycles	Total time (min)	Total solvent volume (mL)
1	*n*-Hexane* (one cycle)	5	10	5	5	65	40	2	32	40 + 40
	MeOH** (one cycle)		2	5	5		100			
2	MeOH	5	2	5	5	68	100	1	12	40
3	MeOH-H_2O, 8:2 (*v*/*v*)	5	10	5	5	65	40	1	20	40

**n*-Hexane extract was discarded, as the first extraction step was meant to eliminate chlorophyll from the sage samples.

**MeOH = methanol.

(Bandelin, Berlin, Germany). Finally, the contents of each vial were condensed by evaporation of the excess methanol to 1 mL. Extracts were filtered through an Anotop syringe filter with aluminium oxide adsorbent, and the samples were ready for liquid chromatographic analysis.

TLC/Densitometry

Thin layer separation of the sage extracts (traditional extract and the ASE extracts Nos. 1–3, listed in Table 2) was performed on commercial glass plates (20 cm × 20 cm) precoated with 0.25 mm layers of silica gel 60 F_{254} (Merck, cat. No. 1.05715). Each extract was spotted onto the thin layer in aliquots of 5 µL spot^{-1} using an AS 30 model autosampler (Desaga, Heidelberg, Germany). Development of the chromatograms was carried out at 22 ± 2°C for a distance of 15 cm in the one-dimensional development mode, using the binary mobile phase toluene – dioxane (7:2, *v*/*v*). This mobile phase was our own modification of the ternary mobile phase proposed by Glensk et al.[29] This modification consisted of removing formic acid from the mobile phase composition, which was the cause of an unfavorable solvent demixing. The chromatograms were dried for 3 h at ambient air and then evaluated by means of densitometry.

Acquisition of densitograms was carried out with a Desaga CD 60 model densitometer equipped with Windows-compatible ProQuant software (Desaga). Concentration profiles of the development lanes for the sage samples were recorded in reflected ultraviolet (UV) light from a deuterium lamp at 340 nm. The dimensions of the rectangular light beam were 2.0 mm ×0.1 mm. The obtained densitograms were primarily assessed for providing the fingerprint response. The chromatograms were additionally scanned in 254 nm UV light using a Chromimage flatbed scanner (AR2i, Le Plessis Robinson, France).

PLC

A 40 µL aliquot of the ASE extract No. 1 (for details see Table 2) was applied to a preparative layer by means of the AS 30 model Desaga autosampler. Application was band-wise with a band width of 16 cm.

Preparative layer chromatographic separation of the investigated sage extracts was performed on commercial glass plates (20 cm × 20 cm) precoated with 2 mm layers of silica gel 60 F_{254} (Merck, cat. No. 1.05745). These separations were carried out using a procedure analogous to that employed in TLC, i.e., using the binary mixture toluene – dioxane (7:2, *v*/*v*) as the mobile phase. The plates were developed at 22 ± 2°C for a distance of 15 cm. Based on visual inspection, the area of the chromatogram was divided into five sections. Each section of the silica gel layer

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was carefully scraped from the plate, mixed with 20 mL of methanol, and placed in an ultrasonic bath for 30 min. The ultrasonicated solution was passed through an Anotop syringe filter with aluminium oxide adsorbent in order to separate silica gel particles from the liquid, which was then evaporated to dryness in a stream of air. Dry residue from each fraction was dissolved in 1 mL of methanol, the obtained solution was again Anotop filtered, and 40 µL aliquots were analyzed by means of HPLC/DAD.

HPLC/DAD

The HPLC/DAD analysis was carried out for the traditional extract, ASE extracts Nos. 1–3, and for the five individual fractions of the ASE extract No. 1, separated by means of PLC (in each case, a 40 µL aliquot of the sample was used).

High performance liquid chromatographic analysis was carried out 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 in the isocratic mode, using an RP-18 (5 µm particle size) column (250 mm × 4.6 mm i.d.; Varian, Harbor City, CA, USA; cat. No. 1215–9307), and acetonitrile – water (55:45, v/v) mobile phase with a trace amount of glacial acetic acid (quantitative composition specially developed for the purpose of this analysis) at a flow rate of 0.6 mL min^{-1}.

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HS-GC/MS

Gas chromatographic analysis additionally complemented our TLC-based strategy and was carried out for the 5 g samples of the dried sage leaves (without any preprocessing). The HS-GC/MS analysis was performed with use of a Trace 2000 model gas chromatograph with an MS Trace Finnigan model mass detector (Thermo Finnigan, San Jose, CA, USA); CTC Analytics model autosampler (Combi PAL, Basel, Switzerland); and DB-5, 30 m × 0.25 mm i.d. column with film thickness 0.25 µm (Agilent Technologies, Palo Alto, CA, USA). The working parameters of the gas chromatograph were carrier gas, helium, $p = 100$ kPa; injector temperature, 150°C; ionization energy, 70 eV; and temperature program, 40°C (3 min), 40 to 150°C (8°/min), and isothermal conditions for 15 min at 150°C.

The autosampler was operated in the head space sampling mode. Working parameters of the autosampler were temperature of desorption, 70°C; time of desorption, 15 min; and amount of the head space phase introduced onto the gas chromatographic column, 0.5 mL.

For identification purposes, we additionally chromatographed ether oils of known composition, namely pine oil, peppermint oil, eucalyptus oil, and juniper oil, as sets of volatile standards (Apotheca Pacis, Rybnik, Poland), which enabled attribution of the retention times to the respective volatile compounds contained in sage. Identification of these compounds was based on agreement between retention times of the known components of the ether oil standards with those obtained from the sage, and on the comparison of mass spectra of the sage components with those from the NIST Mass Spectral Library with the Search Program in the HS-GC/MS system software.

RESULTS AND DISCUSSION

Extraction

At the first stage of our investigation, we focused on elaboration of the best performing extraction method to enable efficient analysis of the common sage samples by means of TLC/densitometry, PLC, and HPLC/DAD. On the basis of the obtained thin-layer chromatograms (see Figure 1), it became evident that the most efficient extraction technique was the two-step ASE procedure employing *n*-hexane and methanol as the extracting solvents, denoted as ASE extraction No. 1 in Table 2. Additional confirmation of this finding was derived from a comparison of the respective densitograms obtained for the four extracts considered, i.e., traditional extract and ASE extracts Nos. 1–3 (see Figure 2).

General advantages of the ASE extraction methods compared to the traditional one are the short time of running the extractions and relatively low consumption of solvents used (compare total extraction times and total consumption of solvents for the traditional approach and for those when using ASE, summarized in Table 2).

ASE extract No. 1, obtained according to the best performing procedure (see Table 2), was further analyzed by means of the liquid chromatographic techniques (TLC, PLC, and HPLC).

TLC/Densitometry

The main goal of the thin-layer chromatographic analysis with densitometric detection (apart from a comparison of the performance of the employed extraction procedures) was elaboration of the optimum working conditions for separation of the relatively high number of biologically

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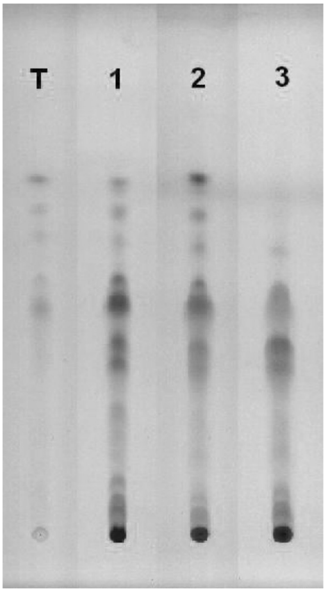

Figure 1. Chromimage scans of thin layer chromatograms of the analyzed common sage extracts, recorded at 254 nm. Stationary phase, silica gel; mobile phase, toluene – dioxane, 7:2 (*v*/*v*); sample aliquot, 5 μL. T, extract obtained by traditional extraction; 1, ASE extract No. 1; 2, ASE extract No. 2; 3, ASE extract No. 3.

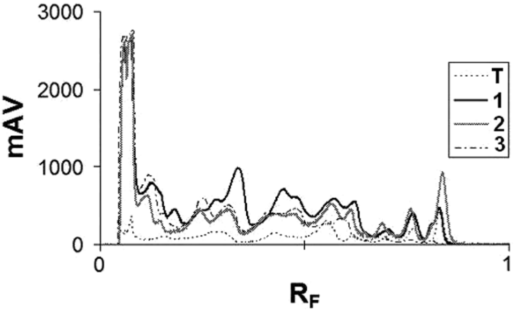

Figure 2. Densitograms obtained from the thin layer chromatograms of the common sage extracts at 340 nm, shown in Figure 1. Stationary phase, silica gel; mobile phase, toluene – dioxane, 7:2 (v/v); sample aliquot, 5 μL. T, extract obtained by traditional extraction; 1, ASE extract No. 1; 2, ASE extract No. 2; 3, ASE extract No. 3.

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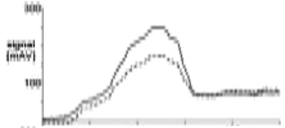
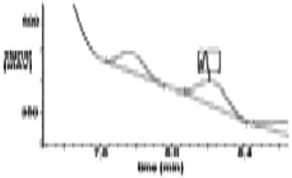
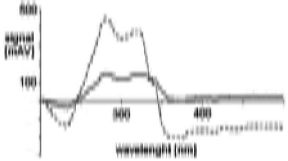
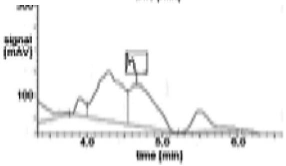
Table 3. Summary of identified active compounds present in common sage as the respective HPLC chromatogram fragments with confirmatory UV spectra (HPLC/DAD) and in situ recorded UV spectra (TLC/densitometry). In densitometrically recorded UV spectra, a solid line denotes a spectrum collected from the sage chromatogram and a dashed line denotes a spectrum collected from standard sample

	Instrumental evidence	
Compound	HPLC/DAD	TLC/densitometry
Ferulic acid	not identified	
o-Coumaric acid		
Caffeic acid		not identified
t-Cinnamic acid		not identified

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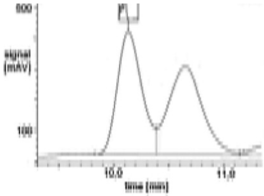
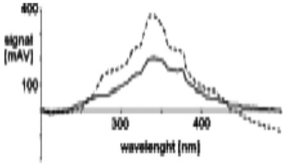
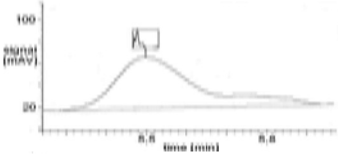
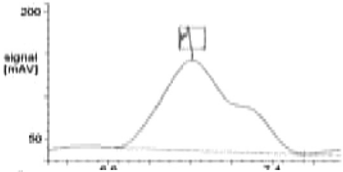
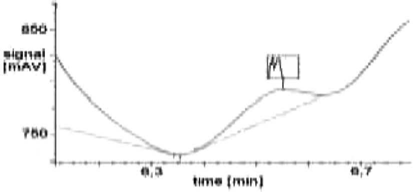
Apigenine		
Apigenine glucoside	not identified	
Rutine		not identified
Kempherol		not identified
Myricetine		not identified

active compounds present in the sage samples for the purpose of fingerprint analysis.

In elaborating the most suitable thin-layer chromatographic conditions, we considered working systems recommended in the literature[30–34] and applied them to the separation and identification of flavonoids and phenolic acids retrieved from plant material. Based on a considerable amount of our own test trials, we chose silica gel and toluene – dioxane (7:2, *v*/*v*) as the best system. With use of this system, we were able to get a fingerprint image showing at least 12 different compounds contained in the ASE extract No. 1 (see chromatogram 1 and densitogram 1, respectively, in Figures 1 and 2). In order to identify at least some of these compounds, we chromatographed selected flavonoids and phenolic acids as external standards. Comparing the retardation factor (R_F) values and densitometrically recorded UV spectra for the separated extract components and standard samples, we managed to identify the following compounds: ferulic acid, *o*-coumaric acid, apigenine, and apigenine glucoside. The R_F values of the standards of interest are given in Table 1, and the respective UV spectra are shown in Table 3.

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HPLC/DAD

In Figure 3, we show the chromatogram obtained from the ASE No. 1 extract of the investigated common sage sample. As can be seen from Figure 3, due to an abundance of chemical compounds present in this extract and revealed by means of HPLC, the obtained chromatogram bears importance as a fingerprint. By comparison of the retention time (t_R) values and UV spectra of the extract components with those of the external standards listed in Table 1, we managed to identify several

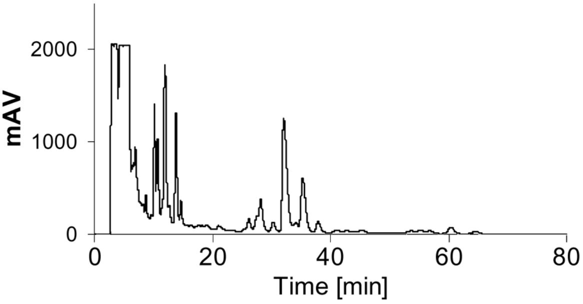

Figure 3. High performance liquid chromatogram of the ASE extract No. 1 of common sage recorded at 225 nm. Column, RP-18 (250 mm × 4.6 mm i.d., 5 µm); mobile phase, acetonitrile – water, 55:45 (*v*/*v*), with the trace amount of glacial acetic acid.

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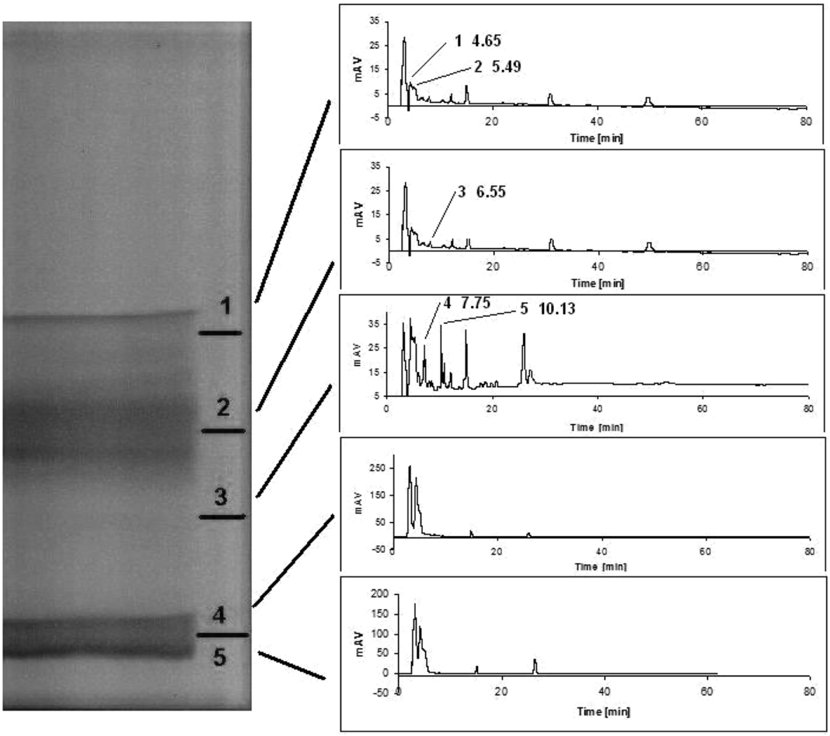
phenolic acids: *o*-coumaric acid, caffeic acid, and *t*-cinnamic acid, and flavonoids: apigenine, rutine, kempherol, and myricetine. In Table 3, we show the enlarged fragments of the HPLC chromatogram together with the confirmatory UV spectra of the identified compounds.

PLC

As shown in the Chromimage scan of the preparative layer chromatogram (Figure 4a), upon visual inspection we divided the investigated sage



(a) (b)

Figure 4. (a) Chromimage scan of the preparative layer chromatogram of the analyzed common sage extract (recorded at 254 nm) with the indicated five fractions; (b) Fingerprint comparison of the five high performance liquid chromatograms obtained from individual sections separated by PLC. Column, RP-18 (250 mm × 4.6 mm i.d., 5 μm); mobile phase, acetonitrile – water, 55:45 (*v*/*v*), with the trace amount of glacial acetic acid. For the sake of example, peaks of the following identified compounds (and their respective retention times, t_R) are marked: 1, caffeic acid; 2, rutine; 3, myricetine; 4, *t*-cinnamic acid; and 5, apigenine.

extract into five fractions, which were retrieved from the five scraped out sections of the adsorbent layer. For the next step, 40 μL aliquots of the methanol solutions of fractions 1–5 underwent examination by means of HPLC/DAD. Respective high performance liquid chromatograms of individual fractions are shown in Figure 4b. For the sake of an example, selected identified compounds (along with their respective retention times) are marked in this figure (i.e., caffeic acid, rutine, myricetine, *t*-cinnamic acid, and apigenine).

From a comparison of the chromatograms shown in Figure 4b as fingerprints, it is evident that individual fractions significantly differ both in qualitative and quantitative terms. Hence, it can be concluded that PLC is an important intermediary step, making further HPLC separation and identification of the sage extract composition a considerably easier and more efficient analytical task than analyzing the total extract.

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HS-GC/MS

HS-GC/MS was a complementary analytical step employed in our study (and combined with the TLC-based strategy), basically due to the wealth of essential oils contained in common sage and to their recognized curative potential. Volatile organic compounds were retrieved from the plant sample and analyzed by means of HS-GC/MS. The aim of this experiment was identification of the volatile compounds, based on their retention times (Table 4) and mass spectra contained in the NIST Mass Spectral Library. In that way, 10 volatile organic compounds were identified in the sage sample: α-pinene, β-pinene, camphor, eucalyptol,

Table 4. Retention times (t_R) of the volatile organic compounds contained in common sage, determined by means of HS-GC/MS

Volatile compound	t_R (min)
α-Pinene	11.0
Camphene	11.5
β-Pinene	12.2
Eucalyptol	13.5
Thujonone	15.3
Camphor	16.3
Borneol	16.7
Bornyl acetate	18.8
Isocariophylene	21.7
Cariophylene	22.5

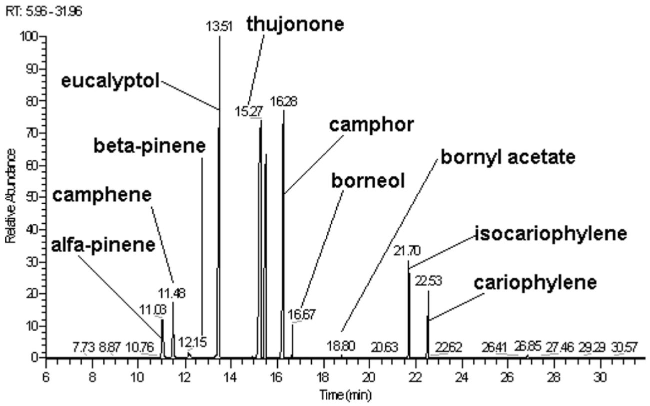

Figure 5. Head-space gas chromatogram of the common sage sample recorded by means of the HS-GC/MS system, with the volatile compounds identified with use of the NIST Mass Spectral Library.

borneol, bornyl acetate, camphene, thujonone, cariophylene, and isocariophylene (see Figure 5).

CONCLUSIONS

The following conclusions can be derived from our experiments:

- Two-step accelerated solvent extraction of plant material with *n*-hexane and methanol was more effective than traditional heating with a solvent under reflux.
- The thin-layer chromatographic system of choice (silica/toluene:dioxane, 7:2, *v*/*v*) could be applied to obtain a fingerprint profile of the common sage extract with 12 separated components and with ferulic acid, *o*-coumaric acid, apigenine, and apigenine glucoside identified by the retention parameters and UV spectra.
- A complementary HPLC profile enabled identification of *o*-coumaric acid, caffeic acid, *t*-cinnamic acid, apigenine, rutine, kempherol, and myricetine in the common sage extracts.
- PLC enabled isolation of five partly separated fractions, in which caffeic acid and rutine (fraction 1), myricetine (fraction 2), and *t*-cinnamic acid and apigenine (fraction 3) were identified. PLC was an important intermediary step, making further HPLC separation of the sage extract and identification of its components a much easier and more efficient analytical task than analyzing the total extract.

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- A complementary method for fingerprint analysis of the volatile fraction derived from plant material was HS-GC/MS, which enabled identification of numerous terpenoids.

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