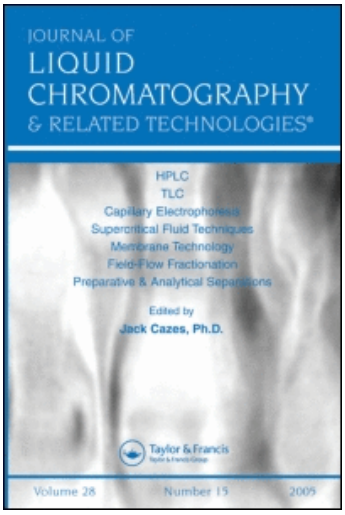

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Thin Layer Chromatographic Techniques (TLC, OP TLC) for Determination of Biological Activated Compounds from Herb Extracts

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Abstract: In the current contribution the comparison of various chromatographic techniques (TLC, OP TLC) for the determination of volatile organic compounds including monoterpenes from peppermint (*Folium Mentha piperita*) is presented. Flavonoids from hawthorn (*Crategus oxyacantha*), *Passiflora incarnata*, hop (*Humulus lupulus*), cacao (*Theobroma cacao*), as well as tea (*Thea sinensis*) extracts were also determined by TLC and OP TLC. Planar chromatographic techniques, thin-layer chromatography (TLC) and over pressure thin-layer chromatography (OP TLC) have been developed for the quantitative determination of constituent menthol and menthone in extracts of peppermint leaves. The optimisation of separation of monoterpenes from peppermint based on their principles of polarity including the selection of solvents, stationary phases, and chromatographic conditions, was performed. For separation of determined compounds from plant material, different extraction methods such as supercritical fluid extraction (SFE) and solvent extraction were used.

Keywords: Sample preparation, TLC, OP TLC, Herbs analysis

INTRODUCTION

Peppermint (*Folium Mentha piperita*) is widely used in food, cosmetic industries, and medicine. Generally only leaves of peppermint are used in herbal medicine. Peppermint leaves contain about 0.5–4% volatile oil that

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is composed of 50–78% free menthol and 5–20% other volatile constituents. Menthol is used in confectionery, perfumery as well as liqueurs, cigarettes, nasal inhalers, and cough drops production. It is also used as a component of anaesthetic, antiseptic, and gastric sedative drugs. Menthone can be found in various volatile oils, such as pennyroyal, peppermint, and geranium. It is used in perfume and flavour compositions. Because of the wide range of applications of menthol and menthone in food and drug production, the determination of these compounds is important not only for consumers but also for analytical chemists. Peppermint oil is classified as a carminative, meaning it helps ease intestinal cramping and tones the digestive system. Peppermint oil, or peppermint tea, is often used to treat flatus and indigestion. It may also increase the flow of bile from the gallbladder. Peppermint tea is a traditional therapy for colic in infants and a double blind study has confirmed its effectiveness.[1–4]

During the growth of peppermint leaves, various volatile organic compounds including monoterpenes are produced.[5] During the peppermint growth the concentration of essential oil and monoterpenes is quite different. Young plants are characterised by the high concentration of essential oil and the low concentration of monoterpenes. Therefore, only young peppermint herbs are taken for the industrial production of peppermint oil. However, during the peppermint growth the concentration of menthone is gradually dwindling, but the concentration of menthol explicitly goes up.

The liquid chromatography techniques such as column chromatography and planar chromatography (includes paper chromatography and thin-layer chromatography (TLC)) give satisfactory results for analysis of herbs and spices.[6,7] The thin layer stationary phase in TLC requires smaller sample size and shorter development distances to reveal their separation potential and to provide faster separation, better resolution, and because spots are more compact and the optical properties of the layer more favourable for *in situ* detection, much better detection limits.[8] Many advantages only become apparent during application, development and scanning densitometry. TLC still finds many applications for the qualitative analysis of simple mixtures, for which little in the way of instrumentation is needed.[8] For this reason, TLC remains popular for isolation and determination of components from natural products, cosmetics, pharmaceutical products, therapeutic monitoring of drugs in biological fluids, a detection of aflatoxins in agricultural products, etc.[7–10]

Menthol (5-methyl-2-[1-methylethyl] cyclohexanol) and menthone (5-methyl-2-[1-methyl-ethyl] cyclohexanone) belong to the group of terpenes. Usually, thin layer chromatography (TLC) is applied for the determination of these compounds, isolated from real samples by liquid–liquid and/or liquid – solid extraction. At the present time extraction methods such as solid phase microextraction (SPME), head space (HS), supercritical fluid extraction (SFE) coupled with gas chromatography (GC), and/or high performance liquid chromatography (HPLC) is successfully applied for

isolation, enrichment, and determination of individual analytes in herbs, food, drugs, cosmetics, sewage, etc.[11–14]

The second group of examined compounds were flavonoids which belong to the polyphenols group. These compounds play an important role in biological and chemical activities of plant material. Flavonoids are a large group of occurring phenolic compounds widespread in plants. In the 1960s, these compounds were widely viewed as metabolic waste products that were stored in plant vacuoles. However, some flavonoids are found in plants (leaves and fruits), herbs, and other products such as wines, beers, juices, etc., but also the presence of them in mosses and liverworts, and even their occurrence in an alga, is reported.[15] Over 4000 flavonoids have been identified, many of which occur in fruits, vegetables, and beverages (tea, coffee, beer, wine). They are categorized into classes according to their chemical structure.[15,16]

For many years, flavonoids have been known to display different pharmacological and biological activities; the most important are capillary protective effect and antioxidant activity. Recently, increasing attention is focused on flavonoids as natural antioxidants.[15,16]

Reactive oxygen species, such as $^1O_2$ and radicals: $O_2^{-\bullet}$, $HO_2^{\bullet}$, damage lipids, proteins, and DNA and participate in pathogenesis and aging. Organisms possess a wide array of antioxidant physiological defenses. However, in situations of oxidative stress additional exogenous antioxidants are needed to limit damage in biological systems. The natural antioxidants such as ascorbic acid, tocopherols, carotenoids, and flavonoids contribute to these defenses. The antioxidant activity of flavonoids is determined by chemical structure. The positions and degree of hydroxylation is of primary importance for their antioxidant activity.

They have some physiological functions and pharmacological properties. Also, flavonoids influence the taste of drinks, wines, and juices. Flavonoids influence colours, UV-absorbents, phytoalexins, and antimicrobial agents. They have become increasingly recognised as being important for long term health and a reduction in the risk of chronic disease. Flavonoids are strongly implicated as active contributors to the health benefits of wine, tea, fruit, and vegetables, cacao as well as chocolate. Other functions of flavonoids to be performed by plants are modulation of enzymatic activity, insect attraction or repulsion, nectar guides, viral, fungal, and bacterial protection, etc.[15]

Flavonoids are most successfully analyzed by LC (Liquid Chromatography) techniques. In most cases, we can identify peaks directly on-line by comparison with literature data or with standard compounds.[17–19] Researches of flavonoids need a variety of analytical techniques including TLC and OP TLC. Modern analytical chemistry of flavonoids which employs mass spectrometry techniques, such as FAB, MALDI-TOF, and electrospray have the ability to elucidate complex flavonoid glycosidicstructures through the ready determination of accurate molecular weights and limited fragmentation patterns. The development of advanced methods of separation,

e.g., capillary electrophoresis (CE) and HPLC, as well as HPLC/MS has been observed, especially in qualitative and quantitative analyses of flavonoids mixtures.[15]

In the current study the comparison of various chromatographic techniques, such as TLC and OP TLC is presented. Components of peppermint extract, especially monoterpenes, and were determined. Planar chromatographic methods, TLC and OP TLC, have been developed for the quantitative determination of constituent menthol, isomenthone, and menthone in plant leaves. The optimisation of separation of monoterpenes from peppermint based on their principles of polarity includes the selection of solvents, stationary phases, and chromatographic conditions was performed. For separation of determined compounds from plant material different extraction methods, such as supercritical fluid extraction (SFE) and solvent extraction were used.

Flavonoids were the second examined group of compounds. The hawthorn, *Passiflora incarnata*, hop, cacao, as well as tea extracts were analysed by TLC and OP TLC.

EXPERIMENTAL

Preparation of Peppermint Samples

Samples of peppermint leaves were bought in the local pharmacy. Dry peppermint leaves were ground in a mortar with pestle. Monoterpenes were extracted from the leaves by grinding with toluene (20 mL of toluene to 2 g of peppermint leaves). The extraction was performed during 24 hours. After that, the extract was filtered to remove solid plant debris. The plant extract was kept in a refrigerator (temp. 4°C). Chromatographic analysis was performed on 1 μL of the extracts.

For SFE, 2.9115 g dry peppermint leaves were ground in a mortar with pestle and loaded into stainless steel extraction vessels. The extraction of the sample was carried out in stainless steel cells using carbon dioxide at a pressure 12 MPa and at a temperature 50°C, during 60 min. The SFE system employs a linear, fused silica (20 cm, 100 μm i.d.) restrictor. Analytes were collected in 2 mL of dichloromethane, placed and cooled (2°C) in glass vials.

TLC Conditions for Peppermint Samples

Apparatus

For the TLC analysis of constituent monoterpenes from plant extracts, the horizontal chamber DS–M (obtained from Chromedes, Lublin Poland) introduced by Dzido and Soczewiński was used.

Reagents and Materials

All solvents were analytical grade; toluene and ethyl acetate were obtained from POCh (Gliwice, Poland).

Plates for TLC (DC-Alufolien, Kieselgel 60 F_{254}) were obtained from Merck (Darmstadt, Germany). For the radial developing these plates were cut with scissors to have dimensions 1×10 cm.

Preparation of Sample Solution

The mobile phase for the chromatography was a mixture of toluene and ethyl acetate. These mixtures had compositions, such as (1) 30:70, (2) 40:60, (3) 50:50, (4) 60:40, (5) 70:30, (6) 80:20, (7) 90:10, (8) 100:0 (v/v). Samples of dried leaves of *Mentha Piperita* were ground in a mortar with pestle. Monoterpenes were extracted from leaves by grinding in toluene. Toluene (20 mL) was added to 2 g of peppermint leaves. The resultant extract was centrifuged for several minutes and filtered to remove solid plant debris. The plant extracts were spotted onto plates with microcapillary tubes (2 μL). The chromatograms were developed for 25 minutes, which allows enough time for migration of monoterpenes in the mobile phase using each solution mixture.

Derivatization Reagent

All chromatograms were developed by use of a vanillin-sulphuric acid-methanol mixture.

OP TLC Conditions for Peppermint Samples

Apparatus

The OP TLC analysis of plant extracts was performed using OP TLC apparatus obtained from COBRAiD (Warsaw, Poland).

Reagents and Materials

All solvents were analytical grade; toluene and ethyl acetate were obtained from POCh (Gliwice, Poland). Plates for HP TLC (HP TLC-Alufolien, Kieselgel 60 F_{254}) were obtained from Merck (Darmstadt, Germany). For the radial developing, these plates were cut with scissors to have dimensions 10×10 cm.

Preparation of Sample Solution

The same solutions for OP TLC as for TLC were used.

Chromatographic Conditions

For OP TLC analysis, the plant extracts were spotted onto plates with micro-capillary tubes (2 μL). The radial chromatograms were developed for 5 minutes. The pressure of nitrogen was 0.4 MPa and the mobile phase was pumped with velocity 1.0 mL/min. The analysis of plant extracts was performed using all eight solutions (toluene and ethyl acetate mixtures).

Preparation of Herb Samples

The dry plant material, 0.2 g, was extracted by 1 mL of methanol. The extraction process was performed during 24 h. The extracts were centrifuged and filtered.

TLC and OP TLC Analysis of Plant Material Extracts

Chromatographic analyses were performed in the DS–L horizontal chamber (Chromdes). Additional OP TLC analyses of plant extracts were performed with over pressure TLC apparatus type 5121 (COBRABiD).

Glass backed silica gel 60 F_{254} plates, dimensions 10 cm × 20 cm (Merck, Darmstadt, Germany) and aluminium foil backed silica gel 60 F_{254} plates for HPTLC, HPTLC–Alufolien, Kieselgel 60 F_{254} (Merck, Darmstadt, Germany), were used.

All solvents were analytical grade; acetone and trichloromethane were purchased from POCh (Gliwice, Poland). Deionised water was prepared in the laboratory by use of a Milli-Q system (Millipore, El Paso, TX, USA).

The mobile phase used for chromatography was acetone-chloroform-water mixture in the volume proportions (80:20:10 v/v). The volume of each analysed extract was 20 μL. The distance of chromatograms were 8.5 cm (for TLC) and 3.5 cm (for OP TLC).

OP TLC conditions were as follows: gas pressure 0.6 MPa; flow velocity of the mobile phase 0.5 mL/min. Obtained chromatograms were developed by UV detection ($\lambda = 254$ nm).

RESULTS AND DISCUSSION

The aim of the presented research was the qualitative and quantitative analyses of menthol and menthone from peppermint extracts. Two extraction methods, such as supercritical fluid extraction (SFE) and solvent extraction (LE) were used in these experiments. For the final analysis, planar

chromatographic techniques, thin-layer chromatography (TLC) and over pressure thin-layer chromatography (OP TLC) were developed.

For quantitative analyses of menthol and menthone the external standard method was performed. The detection limits for determined menthol and menthone at the ppm level are observed. These values are 0.50 mg/L for menthol and 1.82 mg/L for menthone. The detection limit values are similar for the planar chromatographic techniques used. All calibration data, such as: calibration equations using intensity of spot colour for TLC and OP TLC, correlation coefficient of the calibration curve, planar chromatography parameters, particularly hR_f, R_M, k, and detection limits are presented in Table 1.

A typical TLC chromatogram obtained from the SFE extract of peppermint is presented in Figure 1.

From the analytical point of view, the important part of analysis is the assortment of analysis conditions. Therefore, the optimisation of the mobile phase composition for the separation of menthol and menthone from peppermint extracts was performed. Results of the optimisation of monoterpenes standards solution separated by TLC are presented in Figure 2.

The elution force of the mobile phase, which is the mixture of toluene and ethyl acetate increases with the high concentration of ethyl acetate. Therefore, when the concentration of ethyl acetate increases from 0 to 100% (v/v) the hR_F values of menthol increase from 9.4 to 96.2 and hR_F values of menthone increase from 53.0 to 94.7. However, for TLC and OP TLC of peppermint extracts analysis silica gel plates were used. This material is characterised by high polarity and it is dedicated for polar compounds analysis. While the concentration of ethyl acetate increases, the interactions between the compounds and stationary phase decrease and hR_F values for determined compounds approach 100.

For the final qualitative analysis of monoterpenes, ethyl acetate-toluene, 20 + 80 (v/v) as a mobile phase was used.

These studies were also performed for the comparison of TLC and OP TLC. The OP TLC method gave better resolution than TLC. The total analysis time of monoterpenes separation was much shorter (five times). The layers of adsorbent for OP TLC require smaller sample size and shorter development distances to reveal their separation potential and to provide faster separation. For methods comparison, the hR_F parameters of monoterpenes extracted from peppermint leaves were determined. Results obtained by using these two methods are good correlated ($R^2 = 0.9749$), as presented in Figure 3.

The results of qualitative and quantitative analyses of menthol and menthone in SFE and LE peppermint extracts utilising various chromatographic techniques are presented in Table 2.

The better efficiency extraction method for isolation of monoterpenes from peppermint extracts was SFE rather than LE. The obtained results for

Table 1. Calibration and retention data for determined monoterpenes

Technique	Compounds	Calibration curve	R^2	Retention	SD for retention parameters	Limit of detection (mg/L)
TLC	Menthone	$Y = 0.0005X + 0.0057$	0.9627	$hR_F = 62$ $R_M = -0.218$ $k = 0.605$	1	1.82
	Menthol	$Y = 0.0014X + 0.0085$	0.9790	$hR_F = 37$ $R_M = 0.236$ $k = 1.721$	1	0.50
OP TLC	Menthone	$Y = 0.0003X + 0.0157$	0.9655	$hR_F = 64$ $R_M = -0.243$ $k = 0.571$	2	1.82
	Menthol	$Y = 0.0013X + 0.0193$	0.9734	$hR_F = 38$ $R_M = 0.260$ $k = 1.605$	2	0.50

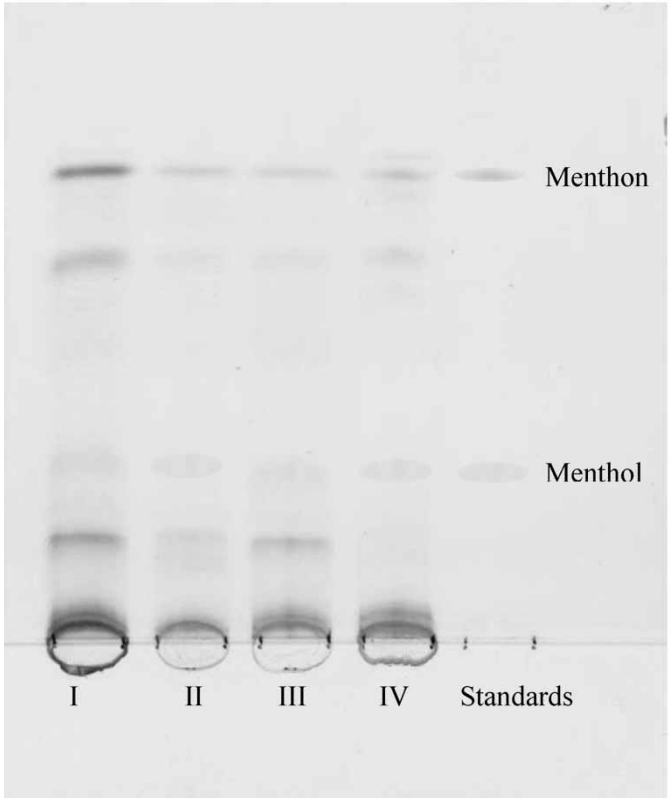

Figure 1. TLC chromatograms of monoterpenes obtained from SFE extracts of various samples of peppermint, separated with ethyl acetate-toluene, 20 + 80 (v/v) as a mobile phase.

L-menthone analysis are in the range from 1.43 $\pm$ 0.25 to 0.39 $\pm$ 0.07% w/w and for menthol are in the range from 0.13 $\pm$ 0.02 to 0.11 $\pm$ 0.06% w/w.

The peppermint plant material is conditioned by contents of monoterpenes. The high concentration of L-menthone in peppermint has the disadvantageous of a bitter flavour. However, a high concentration of menthol gives the refreshing and agreeable flavour of the peppermint infusion. During the peppermint growth the concentration of essential oil and monoterpenes is quite different. Young plants are characterised by the high concentration of essential oil and the low concentration of monoterpenes. Therefore, only young peppermint herbs are used for the industrial production of peppermint oil. However, during the peppermint growth, the concentration of menthone is gradually dwindling, but the concentration of menthol explicitly grows up. The final results of determination of menthol and menthone in plant

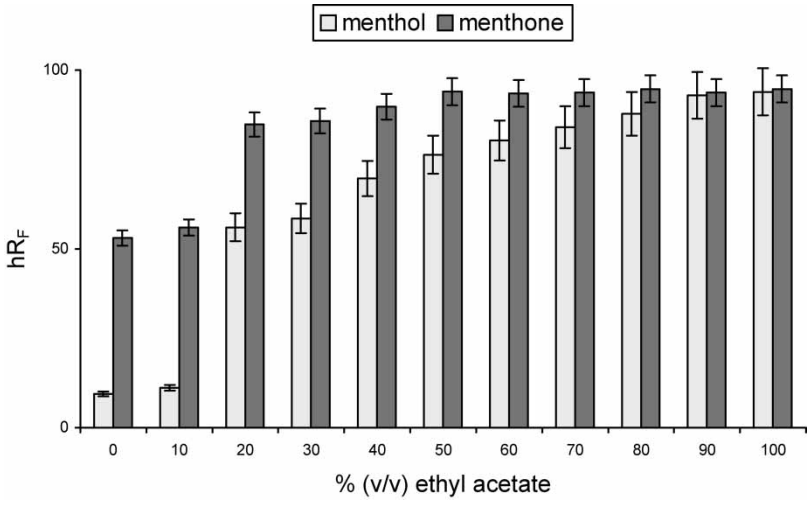

Figure 2. Dependence of the hR_F value of menthol and menthone from a peppermint extract on the ethyl acetate contents in mobile phase composition obtained by using of TLC.

materials suggest the possibility of application of extraction methods, such as SFE and LE coupled with thin layer chromatographic techniques. Particularly, the OP TLC in food and pharmaceutical products laboratory tests allows for about five times faster analysis performance.

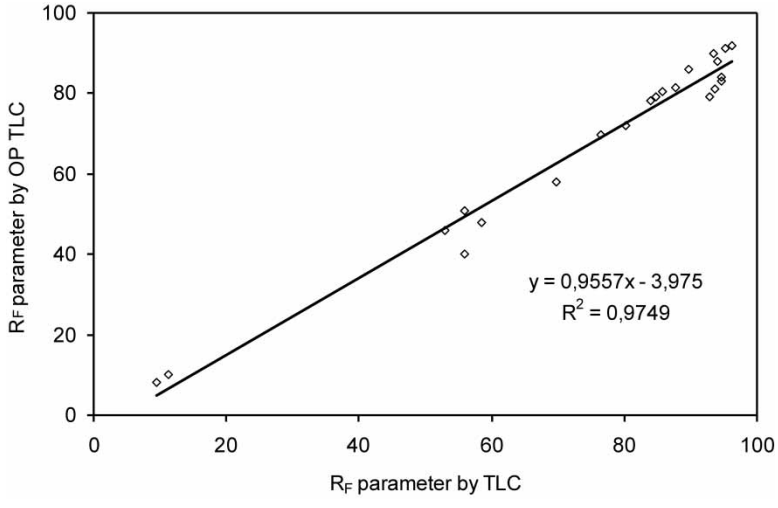

Figure 3. Linear correlation of hR_F values obtained for monoterpenes from peppermint extracts, determined by TLC and OP TLC technique.

Table 2. The comparison of menthone and menthol contents in peppermint extracts after using of different extraction methods and various thin layer chromatographic analyses

	Compounds	
Methods	Menthone (% w/w)	Menthol (% w/w)
SFE-TLC	0.39 ± 0.07	0.11 ± 0.06
LE-TLC	1.43 ± 0.25	Below detection limit
SFE-OP TLC	0.98 ± 0.04	0.13 ± 0.02
LE-OP TLC	Below detection limit	Below detection limit

TLC chromatogram obtained from herb extracts is presented in Figure 4. Obtained data after TLC and OP TLC analyses of herb extracts show the present of flavonols compounds. Particular results are as follows: hawthorn extracts containing rutin (ca. 1% w/w), chlorogenic acid (ca. 0.1% w/w), kaempferol (ca. 0.1% w/w); *Passiflora incarnata* containing rutin (ca. 1% w/w), catechin (ca. 2 w/w); hop containing rutin (ca. 1% w/w), catechin (ca. 0.5% w/w); tea containing rutin (ca. 0.1% w/w), quercetin (ca. 0.2% w/w), kaempferol (ca. 0.1% w/w), catechin and epicatechin (ca. 11% w/w); cacao containing catechin and epicatechin (ca. 5% w/w).

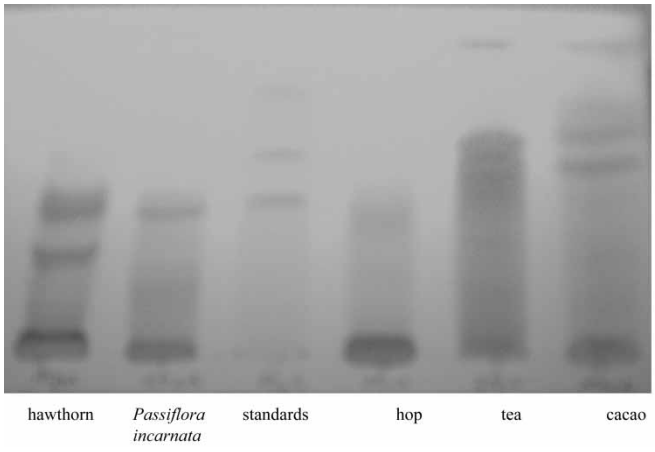

Figure 4. Example TLC chromatogram of flavonoids from a plant material extracts, separated with acetone-chloroform-water, 80 + 20 + 10 (v/v) as a mobile phase (standards: rutin, chlorogenic acid, quercetin, kaempferol).

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