This is the final version (unedited) of the paper published in Chromatographia
September 2012, Volume 75, Issue 17-18, pp 991-999
DOI: 10.1007/s10337-012-2233-5
http://www.scopus.com/record/display.url?eid=2-s2.0-84867021477&origin=inward&txGid=84BEE74147E8F102C075E2B7ED5C4E05.kqQeWtawXauCyC8ghhRGJg%3a11

Bioassay-guided isolation and identification of antimicrobial compounds from thyme essential oil by means of overpressured layer chromatography, bioautography and GC-MS

Ágnes M. Móricz[1], Péter G. Ott[1], Andrea Böszörményi[2], Éva Lemberkovics[2], Emil Mincsovics[3], Ernő Tyihák[1]

[1] *Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman O. Str. 15, H-1022 Budapest, Hungary;*
E-mail: moricz.agnes@agrar.mta.hu
[2] *Institute of Pharmacognosy, Faculty of Pharmacy, Semmelweis University, H-1085 Budapest, Üllői út 26, Hungary*
[3] *OPLC-NIT Engineering Co., Ltd, Budapest Andor Str. 60., H-1119 Budapest, Hungary,*

Corresponding author:
Ágnes M. Móricz, *Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences,* Herman O. Str. 15, H-1022 Budapest, Hungary; Tel.: 0036-48-77-515; Fax.: 0036-48-77-555; *E-mail: moricz.agnes@agrar.mta.hu*

Keywords
antimicrobial compounds, overpressured layer chromatography, direct bioautography, *Thymus vulgaris*, essential oil

Abstract
A simple method is described for efficient isolation of compounds having an antibacterial effect. Two thyme (*Thymus vulgaris*) essential oils, obtained from the market, were chosen as prospective materials likely to feature several bioactive components when examined by thin layer chromatography coupled with direct bioautography as a screening method. The newly developed infusion overpressured layer chromatographic separation method coupled with direct bioautography assured that only the active components were isolated by means of overrun overpressured layer chromatography with online detection and fractionation. Each of the 5 collected fractions represented one of the five antimicrobial essential oil components designated at the screening. The purity and the activity of the fractions were confirmed with chromatography coupled various detection methods (UV, vanillin-sulphuric acid reagent, direct bioautography). The antibacterial components were identified with GC-MS as thymol, carvacrol, linalool, diethyl-phthalate, and alpha-terpineol. The oil component diethyl-phthalate is an artificial compound, used as plasticizer or detergent bases in the industry. Our results support that exploiting its flexibility and the possible hyphenations, overpressured layer chromatography is especially attractive for isolation of antimicrobial components from various matrixes.

Introduction

In the last three decades the increased awareness of resistance to antibiotics, especially in multidrug resistant pathogenic microorganisms, as well as the emergence of undesirable side effects caused by inappropriate antibiotic use, have excited great interest in looking for new sources of useful agrochemicals and pharmaceuticals [1, 2]. Plants have been the main source of new structural types of compounds with potential antimicrobial properties to develop novel chemical prototypes [3].

Searching for bioactive natural products requires appropriate bioassays, focused on the desired activity (e.g. antifungal, antibacterial), as well as various techniques for isolation and identification.

Often the bioassay-guided isolation of bioactive natural substances goes through several fractionation and purification steps, each requiring bio-monitoring, so it can be a time-consuming process. Reducing the number of the steps may also lead to reduced loss of the interesting components.

The aim of this paper is to describe here a simple method with thyme (*Thymus vulgaris*) essential oil as an example, based on the use of thin layer chromatography (TLC), overpressured layer chromatography (OPLC) [4-6] and direct bioautography (DB) [7-9], for an efficient isolation of antibacterial compounds.

Numerous studies with essential oils of different *Thymus* species have shown their antimicrobial activity [10-12], and several of them have focused on the search for the responsible components [13]. The antibacterial effect of thyme essential oil has already been investigated by using TLC-DB [12, 14] as well as of OPLC-DB [14] in our laboratory.

Experimental

Materials

Aluminum foil-backed normal particle silica gel $60F_{254}$ chromatoplates (TLC, #5554 from Merck, Darmstadt, Germany) were used for TLC separation, as well as for OPLC but in this last case the adsorbent layers were sealed on all four edges (OPLC-NIT, Budapest, Hungary).
Analytical grade chloroform, 98% sulphuric acid, and vanillin were from Reanal Co. (Budapest, Hungary). Thymol, carvacrol, and (-)-linalool, as well as the analytical grade dye reagent MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), were from Sigma Aldrich Ltd. (Budapest, Hungary).
The investigated two essential oils of thyme (*Thymus vulgaris* L.) were obtained from the market: Naturol Ltd. (Budapest, Hungary; oil 1) and Gladiolus Ltd. (Kecskemét, Hungary; oil2). The quality of the essential oil 1 met the standards described in the *European Pharmacopoeia*, 4th edition [15].

Layer chromatography

Preparation of applied solutions: For standard solution 4 mg of each thymol, carvacrol and (-)-linalool were taken into 4 mL toluene. The essential oils were dissolved in toluene at the concentration of 5 mg mL^{-1}.

Thin layer chromatography: TLC separation of components of thyme essential oils dissolved in toluene was performed with pure dichloromethane as a mobile phase in an unsaturated TLC chamber, on 4 cm x 10 cm preconditioned (130 ^{o}C, 3 h) normal particle size silica gel layers. The samples were applied at 12 mm height with Linomat IV sample applicator (CAMAG, Muttenz, Switzerland).

Overpressured layer chromatography: Pre-heated (130 ^{o}C, 3 h) 20 cm x 20 cm analytical layer sealed at all four edges as the stationary phase and chloroform as the mobile phase were used for OPLC development using an automated OPLC system (OPLC BS50) (OPLC-NIT Co., Ltd, Budapest, Hungary) [4-6]. Off-line application of the samples at 3 cm from the edge of the layer was carried out by means of Linomat IV sample applicator (CAMAG, Muttenz, Switzerland).
The infusion OPLC development (closed mobile phase outlet) [6] was performed with the following parameters: sample application in a 4 mm wide band, external pressure: 50 bar, flow rate: 400 μL/min, initial flash volume: 450 μL, separation volume: 4380 μL and total development time: 668 s.
Antibacterial components of thyme essential oil were isolated by the use of OPLC with on-line detection operating mode [4, 5]. Conditions of the separation were as follows: 0.2 mL solution of thyme essential oil was applied in a 16 cm wide band: 50 bar external pressure: 400 μL rapid mobile-phase flush (15s, 1.6 mL min^{-1}): 1 mL min^{-1} mobile phase flow rate. The first part of the development was carried out in infusion conditions, that is, the mobile phase outlet was opened after 4 min and the mobile phase was introduced into the detector. The detection was achieved using an on-line coupled flow-through UV detector at the wavelength of 260 nm. The fractions were collected according to the UV detector signal and the R_F value of antibacterial non UV active components obtained from the OPLC-DB study. The fractions, concentrated by cold air stream, were chromatographed by infusion OPLC (conditions described above) and chemically and biologically evaluated.

Chromatoplates with samples, developed by TLC or infusion OPLC, were dried with a cold air stream from a hair-dryer (5 min). The chromatographic spots were visualised using a UV lamp (λ=254) (CAMAG), the vanillin-sulphuric acid reagent (dipping solution: 50 mg vanillin + 12 mL ethanol + 200 μL 98% sulphuric acid, after dipping the plate was heated to 110 ^{o}C for 5 min), as well as in two DB systems, and evaluated with a Shimadzu CS-930 dual-wavelength TLC scanner (Shimadzu Co., Kyoto, Japan) before and after the development with vanillin-sulphuric acid reagent at 270 and 600 nm, respectively.

Direct bioautography

The antibacterial effect of thyme essential oil components was evaluated *in vitro* with DB [7-9, 12, 14] against a Gram-positive soil bacterium *Bacillus subtilis* (Bs) (strain F1276 [16]) and a Gram-negative luminescence gene-tagged plant pathogenic bacterium *Pseudomonas syringae* pv. *maculicola* (Psmlux) [17]. Bs was grown in liquid Spizizen minimal salts medium [18] and Psmlux in King's B broth [19] at 28.5 ^{o}C and both cultures were well aerated by shaking to reach late exponential phase, exhibiting an optical density of 1.2 at 600 nm. The dried chromatoplates, developed with infusion mode, were immersed for 10 s into these bacterial cell suspensions and the excess liquid was drained down but the chromatoplates were not let dry.

The chromatoplates, developed by TLC or infusion OPLC and dipped into Bs suspension, were incubated for 1 h in a humidity chamber at 28 ^{o}C, then immersed for 5 s in an aqueous solution of MTT (80 mg MTT in 100 mL distilled water). The incubation of the treated chromatoplates was continued for 1 h until inhibition zones appeared as clear (off white) regions against a darker background where the viable, metabolically active cells could reduce the yellow MTT to the bluish MTT-formazan. The image of the bioautogram was recorded by use of a scanner (Mustek 1200 ED Plus), areas of the inhibition zones were evaluated by TLC scanner (Shimadzu Co., Kyoto, Japan) at λ=600 nm.

For detection of the bioluminescent light emission, the chromatoplates immersed into Psmlux cell suspension were put into a transparent glass cage ensuring an air space above the adsorbent layer and protecting against drying. Photography of the bioautograms started immediately after inoculation by use of a computer-controlled cooled CCD camera (IS-4000, Alpha Innotech, San Leandro, USA). The exposition time was continuously reduced from 15 to 1-2 min as background luminescence became stronger. The computer program gives a possibility to scan the intensity of the emitted light. The light emitted by the bacterial cells is closely dependent on their reductive metabolic activity (which in turn depends on viability), so the darker areas on the images indicate lack of metabolic activity.

GC-FID and GC-MS analyses

Analysis of chemical composition of the thyme essential oils was performed by a Fisons GC 8000 gas chromatograph (Carlo Erba, Milan, Italy) coupled with a flame ionization detector (FID). An Rt-β-DEXm (Restek) capillary column, 30 m long, 0.25 mm id., 0.25 μm film thickness, was used. Nitrogen was the carrier gas at 6.86 mL/min constant flow rate. The injection volume was 0.2 mL of a 0.1% solution (1 μl essential oil in 1 mL chloroform). Splitless injection was made. The temperatures of the injector and detector were 210°C and 240°C, respectively. The oven temperature was programmed from 60°C to 230°C at 8°C/min and finally held for 5 min. Identification of peaks was made by comparing retention time and standard addition; percentage evaluation was carried out by area normalization.

The identification of the isolated components was carried out by means of an Agilent 6890N/ 5973N GC-MSD, with Chrom Card Server ver. 1.2. Separations were performed using an A HP-5MS capillary column (30 m × 250 μm × 0.25 μm). Carrier gas was helium (p_{He} was 0.20 MPa), at 1 mL/min flow rate; 1 μL (10 μL/mL essential oil in ethanol) was injected at 0.7 mg/mL velocity, splitless type with an Agilent 7683 autosampler. The temperature of the injector was 280 °C, and the temperature of the transfer line was 275 °C. The oven temperature was increased at a rate of 8°C/min from 60°C to 230°C, with a final isotherm at 230°C for 5 min. MS conditions: ionization energy, 70 eV; mass range, m/z 40-500; 1 analysis/min. Peaks were identified based on the NIST spectral library and literary data.

Results and Discussion

In bioassay-directed isolation the steps of the procedure (extraction, separation, fractionation, and purification) are systematically followed by bioassay; that is the next step of the process is based on a bioassay result. For this reason, a fast, reliable, and relatively simple biological detection is needed in the system. DB, providing a measure of the biological response of a living microorganism to the presence of compounds which have cell proliferation inhibiting or promoting properties, meets these demands.

The isolation process, described in this paper, starts with the screening of the components of the samples using TLC-DB [7-9, 12, 14]. It is better than testing the active crude extracts, because in the crude extract the components can affect to each other, can alter the activity of each other. The TLC-DB study helps to choose the prospective samples containing effective components for further investigation. Developing infusion OPLC [6] separation method coupled with DB assures that we isolate only the active components by means of overrun OPLC with online detection and fractionation. Later on the purity of the fraction and the activity of the isolated components are confirmed after chromatography with OPLC-DB [14]; the antibacterial components are identified with GC-MS.

Screening with the use of TLC-DB

DB can be divided into two main steps, the separation and the biological detection. The separation has to be done using planar layer liquid chromatography, like TLC, high performance thin layer chromatography (HPTLC) or OPLC. *Nota bene*, the column chromatographic system is unusable for DB.

During biological detection, the developed chromatoplates containing separated components are dipped into an indicator cell suspension or sprayed with it, so the evaluation of the activity is performed directly on the adsorbent layer at the chromatographic spots. After a required incubation time, the inhibition zones of the bioautogram are usually visualized by the use of a vital dye. However, there is another way for visualisation of the bioautogram: just the light emitting phenomenon of some organisms has to be exploited; that is, a suitable luminescent indicator cells (e.g. natural or transgenic luminescent bacteria) can be applied.

TLC-DB, the widely used method for the detection of antifungal or antibacterial agents, potentiates the fast and easy sorting of effective components in matrixes, therefore, DB can be used in the selection of plant species, organs, development phases, cultivar varieties, and extraction conditions in the search for a prospective sample with the highest antibacterial properties.

The thyme essential oils were submitted to a standard TLC-DB using Bs and Psmlux for a rapid evaluation of their antibacterial activity. The TLC-DB revealed the presence of three active compounds of both essential oils (1 with the same development distance) (Fig. 1). The zones of

bacterial growth inhibition appeared as whitish, clear spots in the case of Bs (yellow MTT dye reagent can be reduced to bluish MTT-formazan only by metabolically active cells) and as black spots with Psmlux (indicating the lack of emitted light of alive bacteria cells).
According to these results, both essential oils are worth investigating further.

OPLC-DB

OPLC [4-6] is a forced flow planar layer chromatographic technique. The used OPLC BS 50 system is divided into two parts, the liquid delivery system and the separation chamber. The sealed layer with the applied samples is put into a cassette, covered by a flexible membrane, eliminating the vapour phase, and intrudes into a chamber under pressure. The mobile phase is driven through the closed layer by a pump. The linear eluent front line results from a micro groove in the covering foil, which enables a free flow of the mobile phase towards the sides.
The forced flow leads to a faster separation, and makes longer separation distances possible, increasing the zone capacity. The constant mobile phase velocity generates almost constant average theoretical plate height on the whole separation distance. For these reasons OPLC results in more compact chromatographic spots and better separation efficiency than conventional TLC/HPTLC.
Because of high-throughput, low cost, easy maintenance and selectivity of detection reagents, OPLC is an ideal technical solution for the screening of natural extracts' ingredients. OPLC coupling with DB bioassay enables the easy biological evaluation of separated components of numerous samples in parallel against a target microoganism.
Based on the preliminary TLC-DB biological screening we found some thyme essential oil components that display interesting antibacterial properties against Bs and Psmlux. To proceed, infusion OPLC-DB investigation was planned. The infusion operating mode means that we don't let the mobile phase flow out of the layer, therefore, the separated components remain on it, which can be achieved by closing of the mobile phase outlet.
The five antibacterial components of thyme essential oils were separated more efficiently by infusion OPLC than conventional TLC (Fig. 2 and 3). The separation of the antimicrobial compounds was confirmed after different visualisation methods (UV lamp, vanillin-sulphuric acid reagent, Bs and Psmlux), by densitometric evaluation or measurement of intensity of luminescent light (Fig. 4). Comparing the development distance of components visualised by different ways, it can be concluded that the antibacterial components can be detected by UV or vanillin-sulphuric acid reagent.
We have to note that for screening the TLC-DB gives enough information, but for further investigations, e.g. identification and isolation, the use of OPLC, providing better separation, is better.

OPLC isolation

The flexibility of OPLC means that the principal separation steps can be combined [4]. The sample application can be off-line using a spotter/streaker or on-line using an injector. The separation can be carried out on a dry or conditioned layer; the detection can be performed by use of on-line flow-cell detection or off-line (*in-situ*) by densitometry. The fully off-line mode is similar to conventional TLC/HPTLC, while the fully on-line mode is similar to HPLC.
OPLC with *on-line* detection and subsequent fraction collection is well suited for efficient isolation of various substance types in general, including potential antimicrobial components from different matrixes, like essential oils or plant extracts.

The developed off-line infusion OPLC separation method can be adopted for the isolation process, just the development has to be continued, that is, the mobile phase has to be overrun and led into a flow-cell detector. Later on, the detected overrun components can be collected e.g. by a fraction collector.
The antibacterial thyme essential oil components, found previously active in TLC- and OPLC-DB studies, were isolated by mixed infusion-transfusion mode of OPLC [6] including off-line sample application and on-line detection. This method seemed promising for the efficient separation and isolation of antibacterial ingredients of thyme essential oils (Fig. 5). The fractions 1, 2 and 4 were collected according to the UV detector signal, however, the retention time of fractions 3 and 5 (non-UV-active antibacterial components) was calculated based on the R_F values obtained from the OPLC-DB study (Fig. 4).

Evaluation of the fractions using chemical and biological detection
The collected fractions were evaluated by chemical and biological detection (Fig. 2 and 3). An aliquot of each fraction was applied onto a chromatosheet and chromatographed by infusion OPLC. The developed plates were observed under UV light. Later on the layers were sprayed with vanillin-sulphuric reagent or were evaluated by DB using Bs or Psmlux as test microorganisms. Development distance of the spots obtained from different visualisation methods were compared to each other and with standards.
The results of the four detection methods show that each fraction represents one of the five designated antimicrobial essential oil components. Also the purity of the fractions seems quite good: according to the chemical, biological and densitometric evaluations, all fractions contain only one oil component. Fraction 4 contained another compound (with a low R_F value) that has an antibacterial effect against Bs, but it did not originate from the oil, probably from the layer (e.g. binding material).

Identification of the isolated antibacterial components
The isolation of bioactive compounds can be followed by the characterization of their chemical structure that can be performed by various spectroscopic and spectrometric techniques.
The composition of thyme essential oils was determined by GC-FID technique. Identification of GC-FID peaks was made by comparison of retention times of standards and co-addition of standards; the percentage evaluation of compounds (Table 1) was carried out by area normalization. The main components of the oils were thymol and p-cymol. The second oil sample contained also high amount of diethyl-phthalate, which is used as plasticizer or detergent bases in the industry.
GC-MS analysis (Fig. 6 and 7) identified and determined the following compounds in the fractions (numbered): **1**- thymol; **2**- carvacrol; **3**- linalool; **4**- diethyl-phthalate; **5**- alpha terpineol (Table 2). GC-MS peaks were identified by comparison with MS and retention data of standards and spectra from the NIST library. All fractions contained silanols; it is especially observable in the fraction 3, because of the relative low concentration of linalool. The origin of the silanols was the silica gel layer.

The economical aspect of the method
This method gives a relatively cheap possibility for the isolation of antimicrobial components from various matrices. The use of layer chromatographic technique provides the selection, separation and collection of active compounds. The forced flow technique and the high-throughput direct bioautography assure that the process is time consuming and requires less

mobile phase than column chromatographic techniques. The parallel separation of 12-16 samples with infusion OPLC, and the fractionation of 1 mg of sample demand only less than 15 and 60 mL of mobile phase, respectively. After sample application and infusion OPLC development of the layer, it can be used immediately for direct bioautography, which gives result within 1 h about all separated components.

Conclusions

The forced-flow OPLC technique, a real layer chromatographic version of HPLC, achieves better separation e.g. with more compact spots than conventional TLC/HPTLC. As a flexible tool, it provides off-line and on-line separation modes, making possible the further chemical, physical, or biological *in situ* detections within the chromatographic spots in the adsorbent bed, or *ex situ* after fraction collection and isolation. OPLC-DB is a useful tool for study plant species with antimicrobial potential and to follow the bioactivity of fractionated components and pure compounds, which can be identified by the use of different spectroscopic and spectrometric techniques.

Acknowledgement

The authors thank Jun Fan (John Innes Center, Department of Disease and Stress Biology, Norwich, UK) for luminescent *Pseudomonas syringae* pv. *maculicola*, and József Farkas (Central Food Research Institute, Budapest, Hungary) for the *Bacillus subtilis* strain.
This work was supported by OTKA grant No. PD83487

References

1. Lorber B (2006) Ann Intern Med 145:354-360
2. Wedge E, Camper ND (2000) In: Cutler HG, Cutler SJ (eds) Biologically Active Natural Products: Agrochemicals and Pharmaceuticals, CRC Press, Taylor & Francis Group, Boca Raton
3. Koduru S, Grierson DS, Afolayan AJ (2006) Pharm Biol 44:283-286
4. Mincsovics E, Garami M, Kecskés L, Tapa B, Végh Z, Kátay Gy, Tyihák E (1999) J AOAC Int 82:587–598
5. Mincsovics E, Kátay Gy, Ott PG, Király-Véghely Zs, Móricz ÁM, Tyihák E (2005) Chromatographia 62:S51-S56
6. Mincsovics E, Sárdi É, Velich I, Kátay Gy, Tyihák E (2002) J Planar Chromatogr 15:280–285
7. Botz L, Nagy S, Kocsis B (2001) In: Nyiredy Sz (ed) Planar Chromatography, Springer, Budapest
8. Móricz ÁM, Tyihák E, Ott PG (2010) J Planar Chromatogr 23:180-183
9. Choma IM, Grzelak EM (2011) J Chromatogr A 1218:2684-2691
10. Rota C, Carraminana JJ, Burillo J, Herrera A (2004) J Food Protect 67:1252-1256
11. Nelson RR (1997) J Antimicrob Chemother 40: 305-306
12. Horváth Gy, Jámbor N, Végh A, Böszörményi A, Lemberkovics É, Héthelyi É, Kovács K, Kocsis B (2010) Flavour Fragr J 25:178-182
13. Juven BJ, Kanner J, Schued F, Weisslowicz H (1994) J Appl Bacteriol 76:626-631
14. Móricz ÁM, Horváth Gy, Molnár P, Kocsis B, Böszörményi A, Lemberkovics É, Ott PG (2010) J Planar Chromatogr 23:406-410
15. European Pharmacopoeia, 4th edition, Druckerei C.H. Beck, Noördlingen, 2002, p. 183.
16. Wang PZ, Doi RH (1984) J Biol Chem 259:8619-8625

17. Fan J, Crooks C, Lamb C (2008) Plant J 53:393–399
18. Anagnostopoulos C, Spizizen J (1961) J Bacteriol 81:741-746
19. King EO, Ward MK, Raney DE (1954) J Lab Clin Med 44:301-317

Figures:

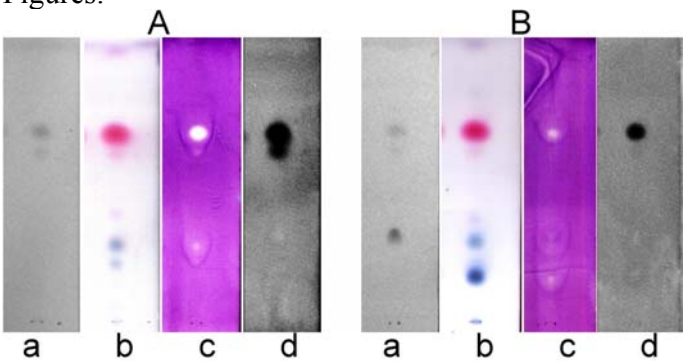


Fig. 1

Detection of components of thyme essential oils (20 µg of each oil 1 (A) and oil 2 (B)) separated by TLC

a – the developed layers under UV light (254 nm)

b – visualisation with vanillin-sulphuric acid reagent

c – bioautogram using *Bacillus subtilis* (light spot = inhibition zone)

d – bioautogram using luminescent *Pseudomonas maculicola* (dark spot = inhibition zone)

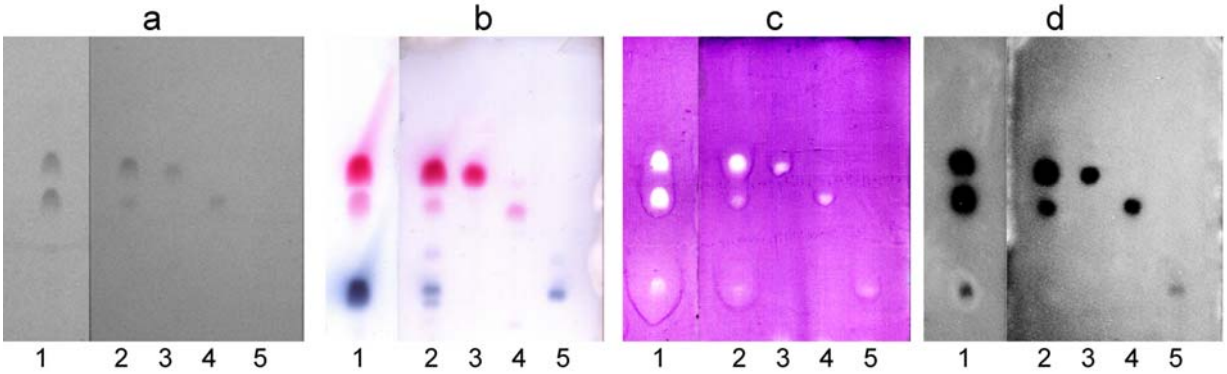


Fig. 2

Detection of thyme essential oil (oil 1) components and the collected fractions (1-3) separated by infusion OPLC

a – the developed layers under UV light (254 nm)

b – visualisation with vanillin-sulphuric acid reagent

c – bioautogram using *Bacillus subtilis* (light spot = inhibition zone)

d – bioautogram using luminescent *Pseudomonas maculicola* (dark spot = inhibition zone)

1 – thymol, carvacrol and linalool test substances (R_f=0.48; 0.41 and 0.25, respectively) (2µg of each); 2 – 20 µg of oil 1; 3 – fraction 1; 4 – fraction 2; 5 – fraction 3

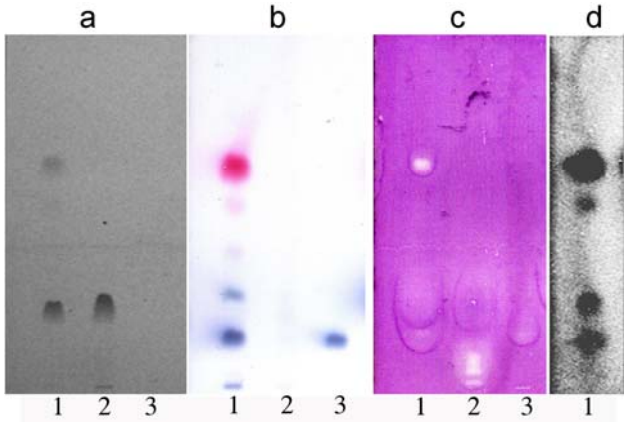


Fig. 3

Detection of thyme essential oil (oil 2) components and the collected fractions (4-5) separated by infusion OPLC

a – the developed layers under UV light (254 nm)

b – visualisation with vanillin-sulphuric acid reagent

c – bioautogram using *Bacillus subtilis* (light spot = inhibition zone)

d – bioautogram using luminescent *Pseudomonas maculicola* (dark spot = inhibition zone).

1 – 30 μg of oil 2; 2 – fraction 4; 3 – fraction 5

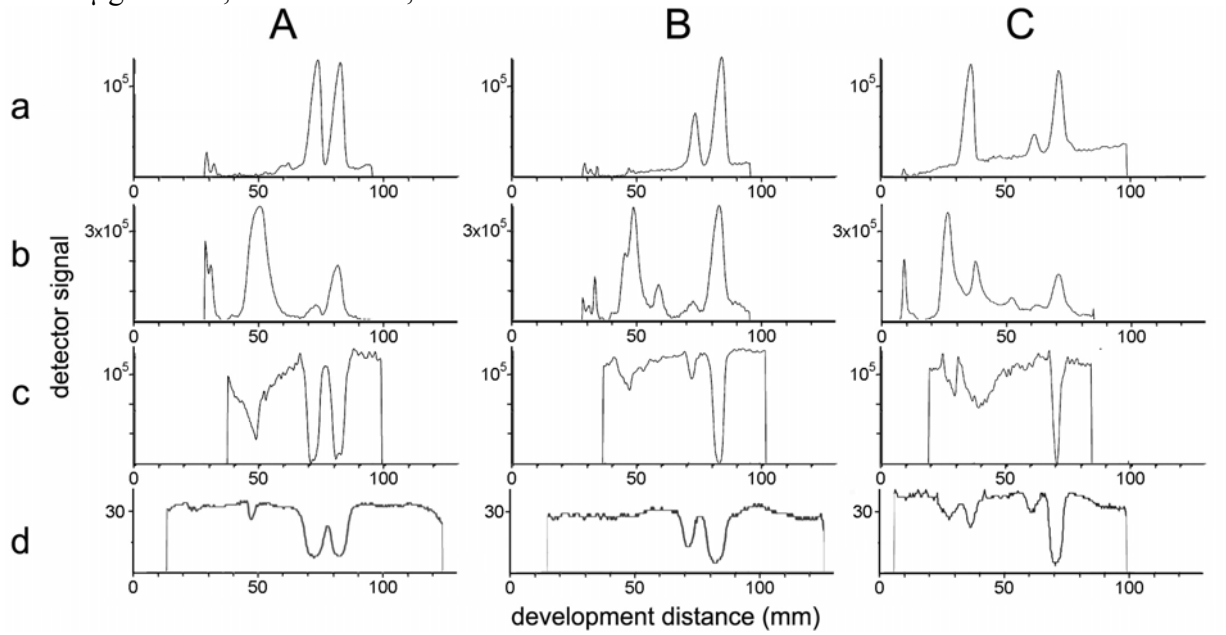


Fig. 4

Detection of thyme essential oil components separated by infusion OPLC after various visualisation methods. A – 2 μg of each thymol, carvacrol and linalool test substances; B – thyme essential oil 1; C – thyme essential oil 2

a – densitometric evaluation right after the separation at 270 nm

b – densitometric evaluation after the use of vanillin-sulphuric reagent at 600 nm

c – densitometric evaluation after biological detection with *Bacillus subtilis* at 600 nm

d – the measurement of the intensity of the luminescent light after biological detection with *Pseudomonas maculicola*

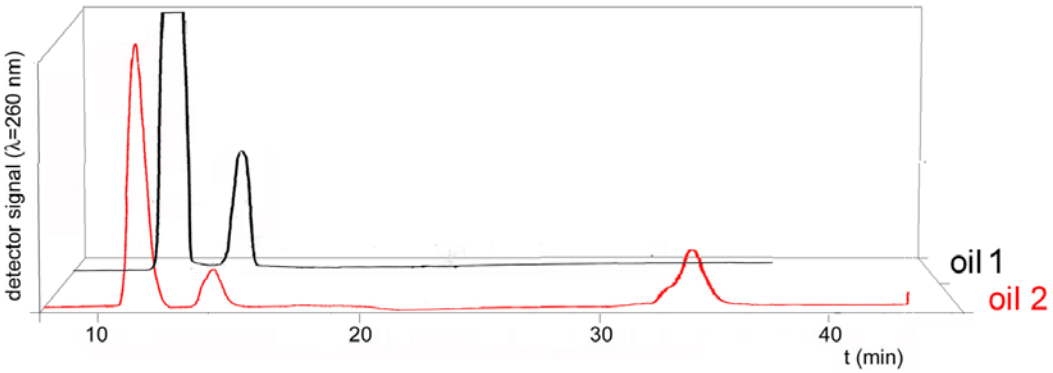


Fig. 5
OPLC separation of 1 mg of thyme essential oils; UV detection was achieved at 260 nm with flow-cell detector; the fractions 1-5 were collected at t_R 11-12.5; 14-15; 25-29; 31.5-35 and 37-42 min, respectively

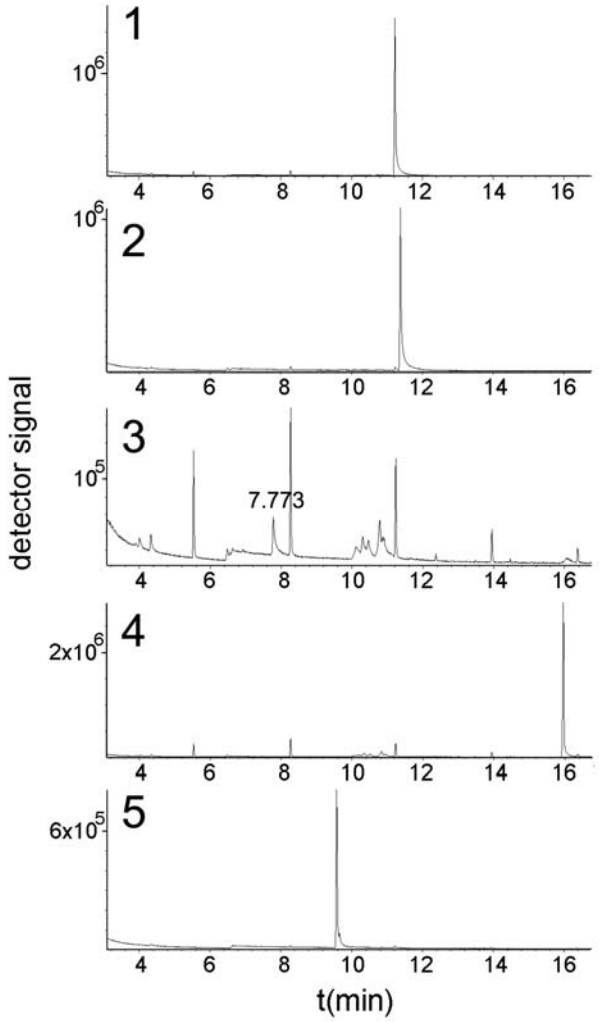


Fig. 6
TIC chromatograms of the 5 thyme fractions obtained by GC-MS analysis

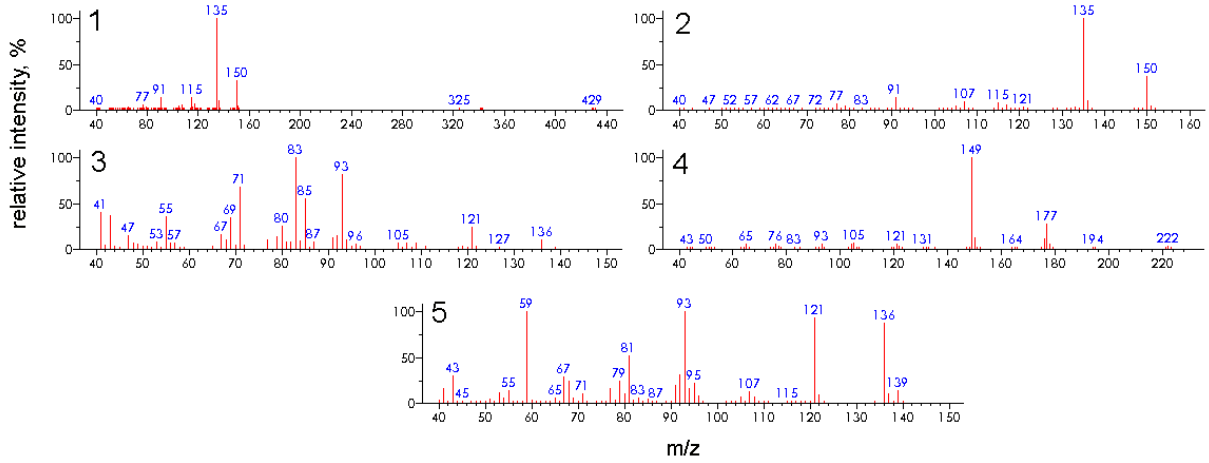


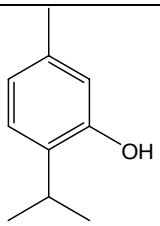
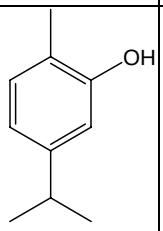
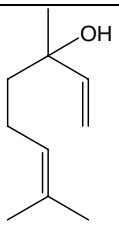
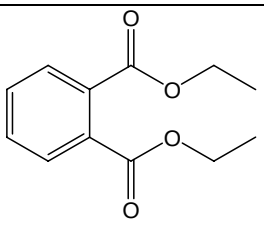
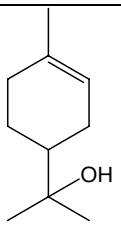
Fig. 7
The MS spectra of the main components of the thyme essential oil fractions
1 – peak in fraction 1 at t_R 11.23 min, identified as thymol
2 – peak in fraction 2 at t_R 11.38 min, identified as carvacrol
3 – peak in fraction 3 at t_R 7.80 min, identified as linalool
4 – peak in fraction 4 at t_R 15.97 min, identified as diethyl phthalate
5 – peak in fraction 5 at t_R 9.58 min, identified as α-terpineol

Table 1
Chemical constituents of the essential oils of *Thymus vulgaris* L. measured by GC–FID.

Compound	t_R (min)	Composition (%)	
		oil 1 (Naturol)	oil 2 (Gladiolus)
α-pinene	5.78	2.32	2.23
camphene	6.22	0.31	0.25
β- myrcene	6.61	2.69	-
β- pinene	6.76	-	0.91
α-terpinene	7.43	0.50	1.29
p-cymene	7.65	25.15	31.51
eucalyptol + γ-terpinene	7,95	7.62	0.44
linalool	10.15	5.97	4.92
α-terpineol	12.30	-	12.80
borneol	12.35	2.46	-
β-caryophyllene	14.82	1.67	0.65
thymol	15.51	38.11	22.13
carvacrol	15.86	10.58	3.62
diethyl-phthalate	19.90	-	14.84

t_R: retention time

Table 2
Isolated oil components identified by GC-MS

fractions	**1**	**2**	**3**	**4**	**5**
components detected by GC-MS	thymol	carvacrol	linalool	diethyl phthalate	α-terpineol
t_R (min)	11.23	11.38	7.80	15.97	9.58
chemical structures of identified components					

t_R: retention time