Emil Mincsovics, Péter G. Ott, Ágnes Alberti, Andrea Böszörményi, Éva B. Héthelyi, Éva Szőke, Ágnes Kéry, Éva Lemberkovics És Ágnes M. Móricz: *In-situ* clean-up and OPLC fractionation of chamomile flower extract to search active components by bioautography. Journal of Planar Chromatography - Modern TLC 26 (2013) 172-179. DOI: 10.1556/JPC.26.2013.2.12
http://akademiai.com/content/8572q6r58650q753/?p=a6a6ad0d651f4cc387a8e297cd497bfd&pi=12

In-situ Clean-up and OPLC Fractionation of Chamomile Flower Extract Searching Active Components by Bioautography

Key Words
Chamomile (*Matricaria recutita* L.)
Antibacterial activity
OverPressured Layer Chromatography, OPLC
Combined on-line/off-line OPLC
GC-MS
HPLC-MS/MS
Off-line OPLC-MS

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Summary
Bioassay-guided isolation of antibacterial components of chamomile flower methanol extract was performed by OPLC with on-line detection, fractionation combined with sample clean-up in-situ in the adsorbent bed after sample application. The antibacterial effect of the fractions and the separated compounds remained on the adsorbent layer (do not overrun during OPLC separation) was tested with direct bioautography (DB) against the bioluminescent *Pseudomonas savastanoi* pv. *maculicola* and *Vibrio fischeri.* The fractions with great biologically activity were analysed by SPME-GC-MS and LC-MS/MS and the two active uneluted compounds were characterized by OPLC-MS using interface. Mainly essential oil components, coumarins, flavonoids, phenolic acids and fatty acids were identified in the fractions.

1 Introduction

Matricaria recutita L. (syn. *Chamomilla recutita* L.), Asteraceae, a native plant in Europe has been known as a medicinal plant for several thousands of years. The flower heads are used

both internally and externally to treat different infections, indigestion and cramps due to its antiinflammatory, sedative, analgesic, spasmolytic, antioxidant and antimicrobial properties [1-3]. These beneficial effects have been related to different classes of biologically active, pharmaceutically interesting substances such as essential oil components, organic acids and phenolic components (coumarins, phenolic acids, flavonoids) [4-11].

Several in vitro studies showed the antibacterial effect of *M. recutita* essential oil against many bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus mutans* and *Streptococcus salivarius* [12-15]. The ethanolic extracts of chamomile exhibit stronger antibacterial activity than aqueous ones [3]. The hydroalcoholic (42%) extract exhibited antibacterial activity against Gram-positive (*Staphylococcus aureus*, *Streptococcus mutans*, group B *Streptococcus*, and *Streptococcus salivarius*) as well as Gram-negative (*Klebsiella pneumonia, Escherichia coli, Bacillus megaterium* and *Leptospira icterohaemorrhagiae*) bacteria strains [16]. The methanolic extract, enriched in phenolic acids and flavonoids, inhibited the growth of *S. aureus* [14] and had selective inhibitory activity on the growth of colon and cervical carcinoma cell lines, without hepatotoxicity [7]. It seems that phenolic compounds were responsible for this property. Several studies reported the antibacterial effect of individual compounds detected in chamomile [15, 16]. Some components of 50% hydroalcoholic chamomile extract that showed inhibitory action against luminescent gene-tagged *Pseudomonas savastanoi* pv. *maculicola*, *Bacillus subtilis* and *Vibrio fischeri* have been separated, detected and identified in our laboratory using OPLC, TLC/OPLC-direct bioautography (DB) and GC-MS or SPME-GC-MS [17, 18].

OPLC [19] in mixed operating mode (off-line sample application and on-line detection) can be used for fractionation and isolation of separated components from different matrices [18, 20-22]. The off-line sample application makes sample preparation possible directly in the adsorbent layer by washing the uninterested compounds off, using a direction opposite to that of the mobile phase that leaves the required components on the start point [22]. With this process we can decrease the load of the adsorbent, resulting in better separation, and clearer fractions. OPLC coupling with DB [17, 18, 20, 23] enables the bioassay guided isolation of bioactive compounds [17, 18, 20].

To identify the isolated molecules, other off-line hyphenated techniques are needed. In the case of chamomile flower extracts, the main volatile components like the sesquiterpenoids ((-)-alpha-bisabolol, bisabolol-oxides, trans-beta-farnesene, spathulenol) and the polyines (cis- and trans-spiroethers), also the coumarin herniarin are easily identifiable by GC-MS or SPME-GC-MS [4, 5, 11, 17]. The other chamomile coumarin component umbelliferone can be detected also by GC-MS but it has low sensitivity [18]. Other ingredients of chamomile extracts as organic acids and phenolic substances are usually characterized by LC-MS(/MS) [7-10].

The aim of this study was the isolation and characterisation of chamomile components having an antibacterial effect against *Pseudomonas savastanoi* pv. *maculicola* and/or *Vibrio fischeri* using OPLC, DB, GC-MS, LC-MS/MS and off-line OPLC-MS.

2 Experimental

2.1 Materials

Aluminum foil-backed normal particle silica gel $60F_{254}$ plates (TLC, #5554 from Merck, Darmstadt, Germany) were used for TLC separation, as well as for OPLC but in this case the layers were sealed on all four edges. All used solvents were analytical grade purchased from Reanal (Budapest, Hungary).

2.2 Preparation of plant extracts

1.5 g of dried (at room temperature) chamomile (*Matricaria recutita* L.; collected in the end of June 2012, in Harta, in the Great Plain, Hungary) flowers were macerated for 48 h with 10 mL of methanol in a 20 mL screw-capped glass vial. Samples were put into an ultrasonic bath for 2x30 s, and filtered (0.45 μm, Nylon).
Essential oils were obtained by water steam distillation for 3 h, using 10 g of dried, powdered plant material using the apparatus prescribed in the seventh Hungarian Pharmacopoeia.

2.3 OPLC and TLC separations

Methanol extract of chamomile was fractionated by the use of Personal OPLC BS50 system (OPLC-NIT, Budapest, Hungary) in mixed operating mode (off-line sample application and on-line separation/detection/fraction collection) [19, 22] on TLC layer sealed at all four edges and previously washed in the system with 1.5 mL/min (20 mL) acetonitrile-water 85:15 (v/v). 0.6 mL (12 mg) extract was applied in a 16 cm wide band at 3 cm from the lower edge. In-situ clean-up was carried out from the outlet side of the chamber by hexane-chloroform-acetonitrile, 47.5:47.5:5 (v/v), using the TEST menu at 5 MPa external pressure and 1 mL/min for 10 minutes. Without release the external pressure the separation was followed with the automatic step-wise gradient separation process using the chamber inlet and the following parameters: flow-rate 1 mL/min, rapid eluent flash 10 μL (note: the layer is totally wetted), eluent A hexane-chloroform-acetonitrile, 42.5:42.5:15 (v/v) 22 mL, eluent B hexane-chloroform-acetonitrile, 32.5:32.5:35 (v/v) 22 mL and eluent C acetonitrile-water, 95:5 (v/v) 7 mL.
The detection was achieved using an on-line coupled flow-through UV detector at the wavelength of 300 nm, respectively. The peaks collected were concentrated by cold air stream. The separated components remained on the adsorbent layer (do not overrun during OPLC separation) were visualized by UV illuminations (254 and 366 nm), vanillin-sulfuric acid reagent and also by direct bioautography.
The concentrated fractions were chromatographed in unsaturated chamber at room temperature on TLC layer with the appropriate above mentioned mixtures of hexane-chloroform-acetonitrile used for their overrun. The developed plates were dried by a cold air stream using a hair-drier (5 min) and the chromatograms were visualised under UV illumination at 254 and 365 nm as well as in DB systems.

2.4 In vitro antibacterial test of the fractions

DB was applied to check the antibacterial activity of the chamomile fractions obtained by OPLC separation after UV detection. The *in vitro* test was performed using two organisms: the Gram-negative, luminescence tagged plant pathogenic bacterium *Pseudomonas syringae* pv. *maculicola* (Psmlux) [24] and the Gram-negative, naturally luminescent marine bacterium *Vibrio fischeri* (Lumistox test strain, Hach-Lange Ltd.).
Psmlux bacterial cells were grown at 28.5 °C in King's B broth [25], well aerated in an orbital shaker, until they reached the late exponential phase, corresponding to an optical density of 1.2. *V. fischeri* strain was grown in the dark shaking at 28.5 °C until it reached an optical density of 2.4 at 600 nm, using the following liquid medium (slightly modified recipe of NCAIM, Corvinus University, Budapest; ingredients are expressed in g dm^{-3}): pepton 5, yeast extract 5, meat extract 6, NaCl 24, $MgSO_4$ 3.4, $MgCl_2 \times 6H_2O$ 5.3, KCl 0.7, and $CaCl_2$ 0.1.
The fractions were developed by TLC, and the dried chromatoplates were dipped into the cell suspension for 10 s and immediately put into a closed transparent glass cage, in which the bacteria cells receive the appropriate humidity and air. The images of the bioluminescent light of the bacterial cells in the bioautogram were taken by a computer-controlled cooled CCD camera (IS-4000, Alpha Innotech, San Leandro, USA). The darker areas indicate the lack of metabolic activity, which in turn depends on viability.

2.5 Solid phase microextraction (SPME)-GC-MS conditions

Air-dried chamomile flowers (0.5 g) or 50-200 μL extract were put into vials (20 mL headspace) sealed with a silicon/PTFE septum prior to SPME-GC-MS analysis. Sample preparation using the static headspace solid phase microextraction (sHS-SPME) technique was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 μM StableFlex polydimethyl siloxane/divinyl benzene (PDMS/DVB) SPME fibre (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min at 100ºC, extraction was performed by exposing the fibre to the headspace of a 20 mL vial containing the plant material for 10 min at 100ºC. The fibre was then immediately transferred to the injector port of the GC-MS, and desorbed for 1 min at 250ºC. The SPME fibre was cleaned and conditioned in a Fibre Bake-out Station in pure nitrogen atmosphere at 250ºC for 15 min after desorption.

The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Agilent HP-5MS capillary column (30 m × 250 μm × 0.25 μm). The GC oven temperature was programmed to increase from 60ºC (3 min isothermal) to 200ºC at 8ºC/min (2 min isothermal), from 200–230ºC at 10ºC/min (5 min isothermal) and finally from 230–250ºC at 10 ºC/min (1 min isothermal). High purity helium was used as carrier gas at 1.0 mL/min (37 cm/s) in constant flow mode. The injector temperature was 250°C and the split ratio was 1:50. The mass selective detector was equipped with a quadrupole mass analyser and was operated in electron ionization mode at 70 eV in full scan mode (41–500 amu at 3.2 scan/s). The data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with the data of authentic standards, and the NIST 05 library was also consulted.

2.6 LC–MS conditions

The chromatographic separation was performed on an Agilent 1100 HPLC system equipped with G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector (DAD) (Agilent Technologies, Waldbronn, Germany). Samples were separated on a Zorbax SB C18 (Agilent Technologies, Santa Clara, CA, USA) (150 ×3.0 mm I.D., 3.5 μm particle size) column, maintained at 25 °C. Eluent A was formic acid in water (0.5%, v/v), eluent B was acetonitrile. The following gradient program was applied, at a flow rate of 0.4 mL/min: 0 min 90:10 (A:B, v/v), 25 min 15:85 (A:B, v/v), 26 min 0:100 (A:B, v/v), 29 min 0:100 (A:B, v/v), 30 min 90:10 (A:B, v/v). All aqueous solvents were filtered through MF-Millipore (Millipore, Billerica, MA, USA) (0.45 μm, mixed cellulose esters) membrane filters. Chromatograms were acquired at 280 nm. Injection volume was 5 μL. Before injection all samples were filtered through Sartorius (Goettingen, Germany) Minisart RC15 (0.2 μm) syringe filter.

Mass spectrometric analyses were performed with an Agilent 6410B triple quadrupole equipped with an electrospray ionization source (ESI) (Agilent Technologies, Palo Alto, CA, USA). ESI conditions were as follows: temperature: 350 °C, nebulizer pressure: 45 psi, N_2 drying gas flow rate: 9 L/min, fragmentor voltage: 100 V, capillary voltage: 3500 V, collision energy was changed between 5 eV and 50 eV, according to the differences in structures. High purity nitrogen was used as collision gas. Full mass scan spectra were recorded in negative ionization mode over the range of m/z 50–1000 Da (1 scan/s). The Masshunter B.01.03 software was used for data acquisition and qualitative analysis. Compounds were tentatively characterized by comparison of their retention time, UV and mass spectral data with those from the literature.

2.7 Off-line OPLC–MS conditions

OPLC-MS experiments were performed on an Agilent 1200 HPLC system (Waldbronn, Germany) (equipped with a degasser and a binary gradient pump) coupled to a TLC-MS interface (Camag, Muttenz, Switzerland) and an Agilent 6410B triple quadrupole equipped with an electrospray ionization source (ESI) (Agilent Technologies, Palo Alto, CA, USA).
Eluent A was trifluoroacetic acid in water (0.1%, v/v), eluent B was acetonitrile. The following isocratic eluent system was applied at a flow rate of 1.0 mL/min: 5:95 (A:B, v/v).
ESI conditions were as follows: temperature: 350 °C, nebulizer pressure:40 psi, N_2 drying gas flow rate: 13 L/min, fragmentor voltage: 135 V, capillary voltage: 4000 V. Collision energy was changed between 10 eV and 45 eV, in order to obtain as much structural information as it was possible. High purity nitrogen was used as collision gas. Full mass scan spectra were recorded in positive ionization mode over the range of m/z 50–1000 Da (1 scan/s). The Masshunter B.01.03 software was used for data acquisition and qualitative analysis. Compounds were tentatively characterized by comparison of their mass spectral data with those from the literature.

3 Results and Discussion

Bioassay-guided isolation of antibacterial chamomile components was based on OPLC separation with on-line detection and fractionation combined with previous sample clean-up in-situ in the adsorbent bed after sample application. The steps of this procedure are drawn in Figure 1. The first step was a partial pre-wetting of the adsorbent layer (Fig. 1, a1) between the edge of the layer and the sample application band. The aim is to fill up the "dead" area behind the trough, which leads the components to leave the adsorbent layer. With this process the zone behind the trough can be prevented against stucking of any components in it, otherwise the stucked compounds could be detected continuously during the separation/detection/ fraction collection (Fig. 1, b). During the in-situ sample clean-up (2nd step, Fig. 1, a2) the development, the mobile phase flow was in the opposite direction, from outlet to inlet of the chamber. In this step the load of the adsorbent can be decreased for the fractionation (3rd step, Fig. 1, b), what is done in the normal direction of the mobile phase. The fraction -1 was collected during the in-situ sample clean-up and 26 fractions (fractions 1-26) were obtained by a step-wise gradient OPLC separation (Figure 2).
For bioassay the high throughput TLC/OPLC-DB was applied to monitor the antibacterial activity of the fractions and the separated compounds remained on the adsorbent layer. The most prospective fractions and compounds were characterized by GC-MS, LC-MS/MS, TLC-MS.
According to the SPME-GC-MS results (Table 1), the same volatile components (except the chamazulen) were detectable in the essential oil and in the dried chamomile. The methanol extracted many essential oil components as well as the coumarin herniarin from the dried chamomile flower, which components were collected mainly (during sample clean-up) into the fraction -1 and also a small portion of them could be detected in fraction 1. In the fractions 2-26 there were no components detectable by SPME-GC-MS. The antibacterial chamomile components of fraction -1, as the cis-, trans spiroethers, the alpha-bisabolol, the herniarin, and the bisabolol oxides (Figure 3) have been investigated and identified in our lab earlier [17, 18], that is why they were collected mainly into one fraction during the in-situ sample preparation process.
The fractions generally were more active against *V. fischeri* than Psmlux. In Figure 4 the antibacterial effect of the fractions 7-18 is shown against both bacteria. The LC-MS/MS analysis of the methanol extract of dried chamomile flower (Figure 5, Table 2) demonstrated that the extract contains mainly flavonoids, phenolic acids and fatty acids. In the highlighted

active fractions there were some flavonoids and phenolic acids tentatively characterized (Table 3). Unfortunately no main components were detected in some fractions having great antibacterial effect. It seems that for more information further investigations are required, which needs bigger amount of sample, therefore for later OPLC fractionation the use of a preparative adsorbent layer is preferred. Alternatively, removal of disturbing matrix constituents resultant from the adsorbent layer (probably from binding material) or employment of other ionization techniques may provide a solution.
Two of the compounds those remained on the adsorbent layer after fractionation (eluent C) were active against both Psmlux and *V. fischeri* bacteria and were also visible after the use of the vanillin-sulphuric acid reagent (Figure 6). The investigation of these two active components by off-line OPLC-MS exhibited parent ions at m/z values of 387 and 439, at the lower and higher R_F, respectively (Figure 7). For their identification further investigations are needed.

4 Conclusions

The applicability of the complex system containing OPLC with on-line detection and fractionation, in-situ sample clean-up in the planar layer adsorbent bed, DB, TLC-MS, SPME-GC-MS, and LC-MS/MS for the bioassay-guided isolation and characterization of bioactive compounds from a plant matrix was demonstrated.
The fractions having antibacterial activity against Psmlux and *V. fischeri* contained essential oil components, coumarins, flavonoids, phenolic acids, and fatty acids, which generally have antimicrobial activity [26-28].

Acknowledgements

The authors are grateful to Jun Fan (John Innes Center, Department of Disease and Stress Biology, Norwich, UK) for luminescent *Pseudomonas syringae* pv. *maculicola*. This work was partially supported by OTKA grant no. PD83487 and K101271, and Á.M. Móricz was supported by Bolyai grant.

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Legends:

Figure 1 Schematic drawing of in-situ clean-up (a) and on-line OPLC separation/detection fraction collection (b).
a1, partial pre-wetting of the layer, a2, partial elution of sample
1, adsorbent layer, 2, sealed edge, 3, eluent inlet trough, 4, eluent outlet trough, 5, band shaped sample, 6, front of partial pre-wetting behind the sample, 7, wetted layer, 8, sample remained uneluted after clean-up step, 9, eluted part of sample during the clean-up, I, eluent inlet side of the chamber, O, eluent outlet side of the chamber, D, detector, FC, fraction collector.

Figure 2 OPLC fractionation of methanol chamomile flower extract and the 26 collected fractions. Chromatographic conditions see in text, section 2.3.

Figure 3 Detection of the components of fraction -1 collected during the sample preparation. A/B – The developed layer (hexane-chloroform-acetonitrile, 47.5:47.5:5, v/v) under UV light λ = 254/365 nm; C – Bioautogram using *Pseudomonas maculicola* (dark spot = inhibition zone); D – Bioautogram using *Vibrio fischeri* (dark spot = inhibition zone). (The components: a = *cis*-spiroether, b = *trans*-spiroether, c = (–)-alpha-bisabolol, d = herniarin, e = bisabolol oxides)

Figure 4 Antibacterial activity of the fractions 7-18 developed by hexane-chloroform-acetonitrile, 42.5:42.5:15, v/v against *Vibrio fischeri* and *Pseudomonas maculicola*.

Figure 5 TIC (A) and UV (B) chromatograms of chamomile methanolic extract. Detection wavelength, 280 nm Numbering of peaks refers to data shown in Table 2. Chromatographic conditions see in text, section 2.6 LC–MS conditions.

Figure 6 Detection of the components remained on the adsorbent layer after OPLC fractionation. A – Bioautogram using *Vibrio fischeri* (dark spot = inhibition zone); B – Bioautogram using *Pseudomonas maculicola* (dark spot = inhibition zone); C – Visualization with vanillin-sulfuric acid reagent. D/E – The developed layers under UV light λ = 254/365 nm. The arrows show the bands of the two antibacterial components.

Figure 7 TIC chromatograms from OPLC-MS experiments: (A) TIC chromatogram of the two compounds remained on the adsorbent layer (after subtraction of the background), (B) TIC chromatogram of the background, sampling was performed around the same R_F value, as that of the two compounds of interest.

Table 1. Identified compounds in chamomile flower and extracts by SPME-GC-MS

	t_R(min)	essential oil	dried flower	MeOH extract	OPLC Fraction -1	OPLC Fraction 1
Monoterpenes						
p-cymene	7,893	+	+	-	-	-
cineol (eucalyptol)	8,070	+	+	-	-	-
β-ocimene	8,324	+	+	-	-	-
artemisia keton	8,557	+	+	-	-	-
camphor	10,687	+	+	-	-	-
borneol	11,011	+	+	-	-	-
α-terpineol	11,213	+	+	-	-	-
Sesquiterpenes						
β-caryophyllene	15,435	+	+	-	-	-
β-farnesene	15,806	+	+	+	-	-
caryophyllene oxide	16,127	+	+	+	-	-
germacrene D	16,405	+	+	+	-	-
β-selinene	16,549	+	+	+	-	-
α-farnesene	16,641	++	++	+	-	-
trans-nerolidol	17,539	+	+	+	-	-
spathulenol	17,918	+	+	+	-	-
farnesene-epoxide	18,453	+	+	+	+	-
α-bisabolol oxide B	18,986	+	++	++	+	+
α-bisabolol	19,381	+	++	++	+	+
herniarin	20,102	-	-	+	+	-
chamazulene	20,122	+	-	-	-	-
bisabolol oxide A	20,322	+	++	++	++	+
cis en-in-dicycloether	22,262	+	+	+	++	++
trans en-in-dicycloether	22,537	-	+	+	++	++

Abbreviations: - not detectable, + detectable, ++ large amount

Table 2. LC–MS/MS data of tentatively identified compounds from chamomile methanolic extract

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Tentative identification [a]
1	5.8	314	369	161, 133	Umbelliferon hexoside
2	6.2	292, 315	641	179, 135	Caffeic acid hexoside
3	6.4	238, 326	353	191, 171	Caffeoylquinic acid
4	7.4	232, 304	355	193, 149, 134	Ferulic acid hexoside isomer
5	8.3	260, 358	479	317, 207, 163, 113	Myricetin hexoside
6	9.0	238, 296, 320	355	193, 149, 134	Ferulic acid hexoside isomer
7	9.3	256, 370	463	301, 151, 121	Quercetin hexoside
8	9.5	258, 364	447	285, 175	Luteolin hexoside
9	10.0	242, 300, 326	515	379, 311, 243, 175	Dicaffeoylquinic acid isomer
10	10.2	324	161	133, 105, 89, 77	Umbelliferon
11	10.4	266, 329	431	363, 295, 269, 211, 159	Apigenin hexoside
12	10.7	245, 328	515	353, 191, 179, 175, 173	Dicaffeoylquinic acid isomer
13	11.2	242, 315	517	323, 281, 193, 179, 161, 134	Ferrulic acid / Caffeic acid derivative
14	12.1	270, 330	473	269	Apigenin acetylhexoside isomer
15	12.8	266, 282, 330	355	151, 113	Not identified
			473	269	Apigenin acetylhexoside isomer
16	14.6	234, 297	785	665, 545, 243, 145	Hydroxycinnamic acid derivative
17	15.1	268, 336	329	229, 211, 193, 183, 171	Flavonoid
			269	225, 151, 149, 117	Apigenin
18	16.6	257	441	395, 327, 305, 175	Hydroxybenzoic acid derivative
19	18.7	257, 272, 351	373	358, 343, 328, 300	Flavonoid
			535	520, 491	Flavonoid
20	20.5	242, 317	545	481, 205, 169	Hydroxycinnamic acid derivative
21	22.0	-	295	221, 159	Fatty acid
22	23.5	-	295	277, 221, 159	Fatty acid
23	24.5	-	295	277, 221, 177, 159, 115	Fatty acid
24	24.9	-	394	226, 196, 180	Not identified
25	25.5	-	297	249, 197, 183, 141	Fatty acid
26	27.2	-	295	277, 221, 193, 177, 161, 159	Fatty acid
27	29.9	-	277	233, 209, 138	Not identified

[a] Peak numbers and retention times (t_R) refer to chromatograms shown in **Fig. 5.**

Table 3. LC-MS data and tentative identification of compounds in some highlighted fractions obtained from chamomile methanolic extract

Fraction	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Tentative identification
1	393	325, 257	Not idendified
5	279	-	Not idendified
7	277	233, 209, 138, 97	Not idendified
11	161	133, 117, 105, 89	Umbelliferon
12	353	213, 189, 179, 163, 149, 135	Caffeoylquinic acid
	179	135, 91	Caffeic acid
16	329	269, 233, 141, 125	Flavonoid
	365	231, 174, 161	Not idendified
17	329	269, 233	Flavonoid
20	295	221, 177, 159, 133, 115	Not idendified
	371	325, 309, 289	Hydroxycinnamic acid derivative
	473	269	Apigenin acetylhexoside
21	297	251, 183	Fatty acid
22	329	299, 293, 285, 271, 257, 243, 235, 199	Flavonoid
	293	249, 185, 141	Fatty acid
	295	277, 195	Fatty acid
	297	279, 183	Fatty acid
23	295	277, 221, 177, 161, 159	Fatty acid
	293	275, 235, 183	Fatty acid
25	269	225, 183, 149, 117	Apigenin
	311	197, 175, 171, 139	Fatty acid
	355	311, 267	Not idendified
26	311	293, 275, 201, 185, 171	Fatty acid
	379	311, 293, 193, 171	Fatty acid
	269	225, 183, 149, 117	Apigenin

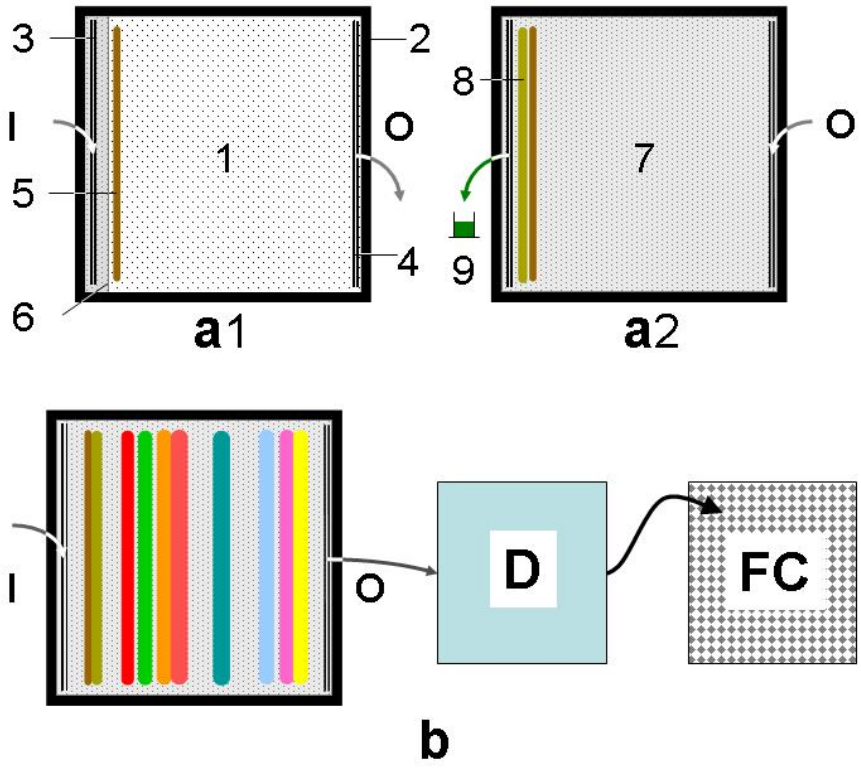

Figure 1

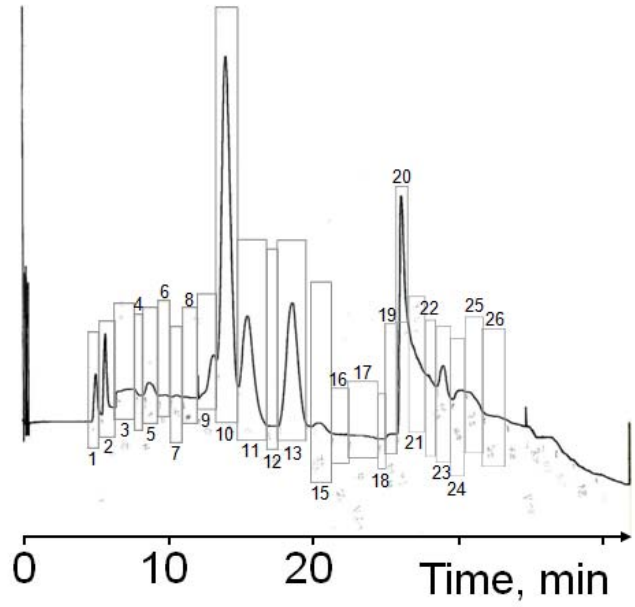
1
2
3
4
5
6
7
8
9
10
11
12
13
15
16
17
18
19
20
21
22
23
24
25
26
0
10
20
Time, min

Figure 2

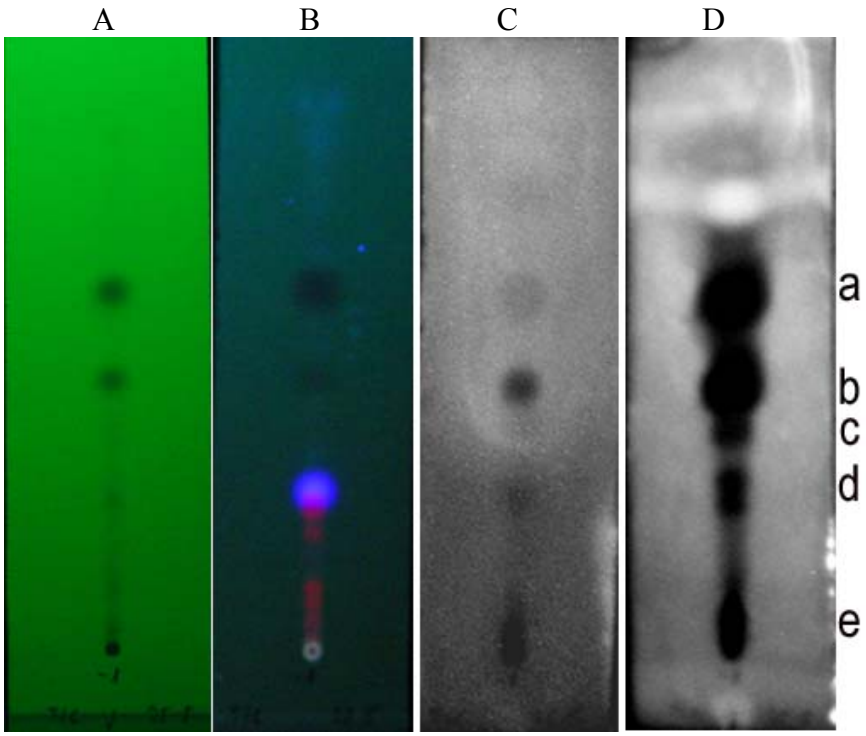

Figure 3

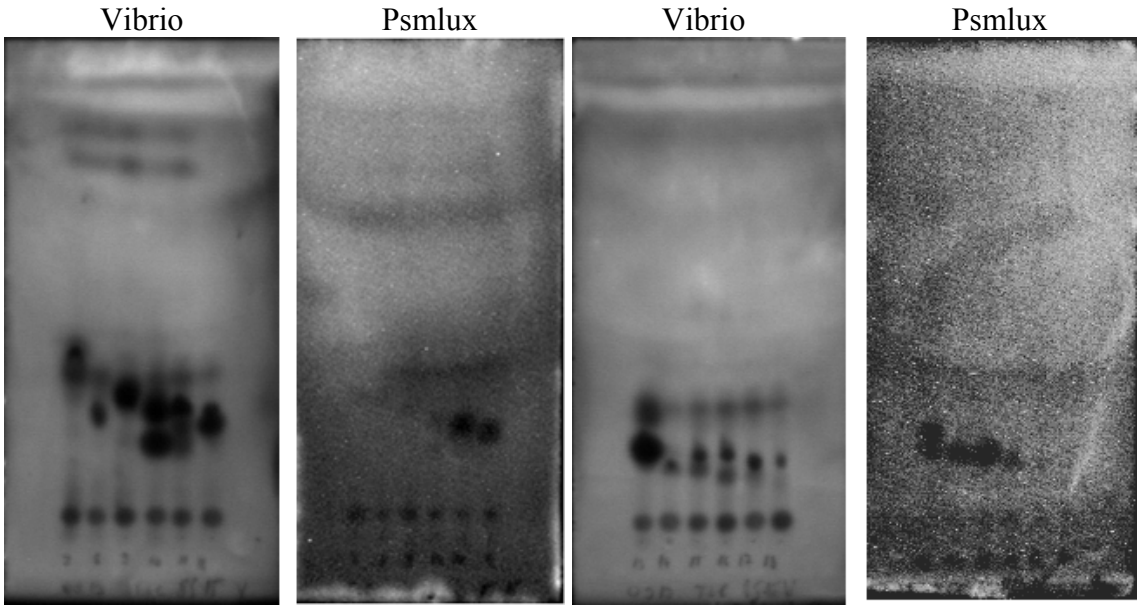
Vibrio
Psmlux
Vibrio
Psmlux
7 8 9 10 11 12
7 8 9 10 11 12
13 14 15 16 17 18
13 14 15 16 17 18

Figure 4

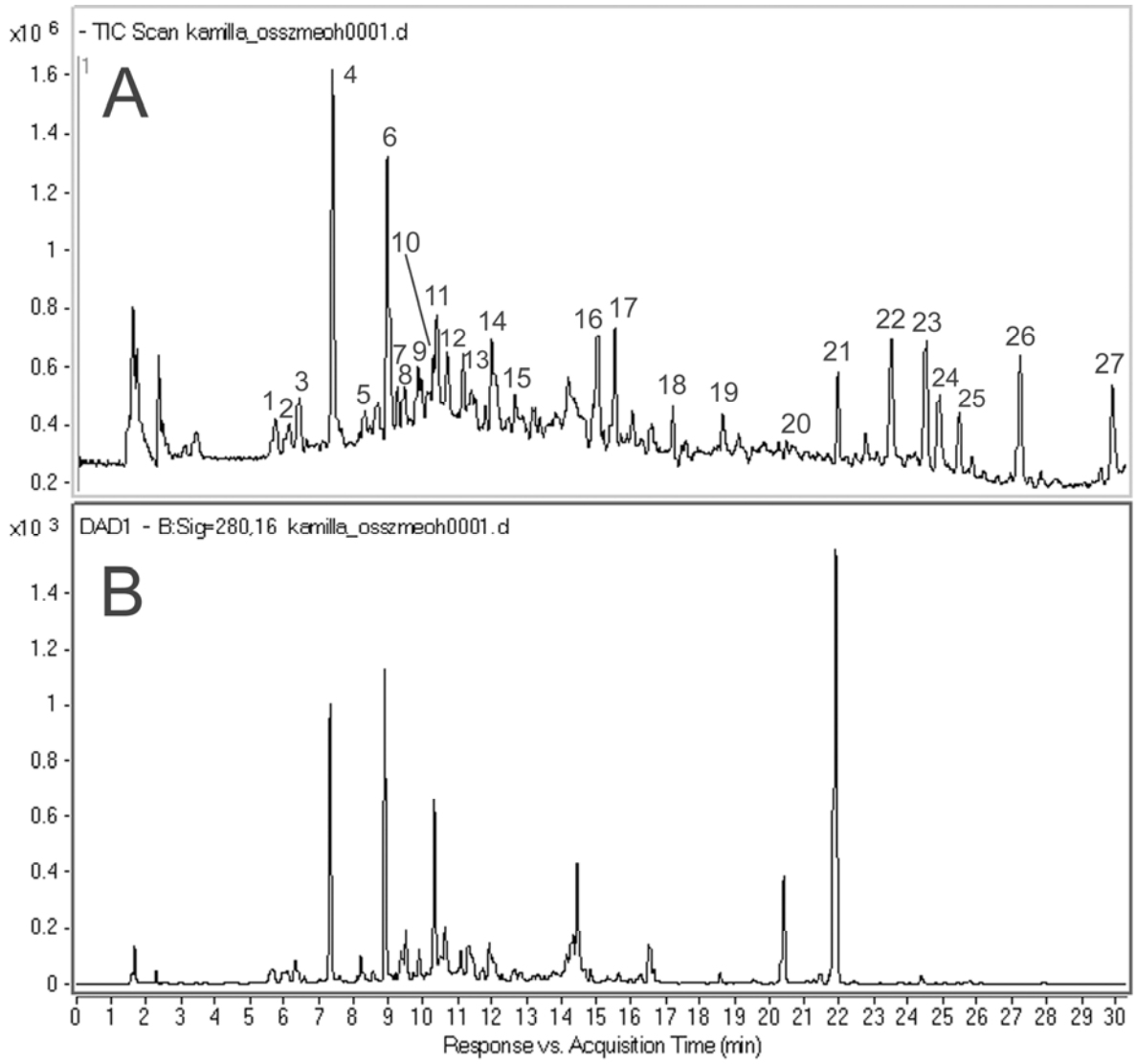
x10 6
- TIC Scan kamilla_osszmeoh0001.d
A
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
x10 3
DAD1 - B:Sig=280,16 kamilla_osszmeoh0001.d
B
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Response vs. Acquisition Time (min)

Figure 5

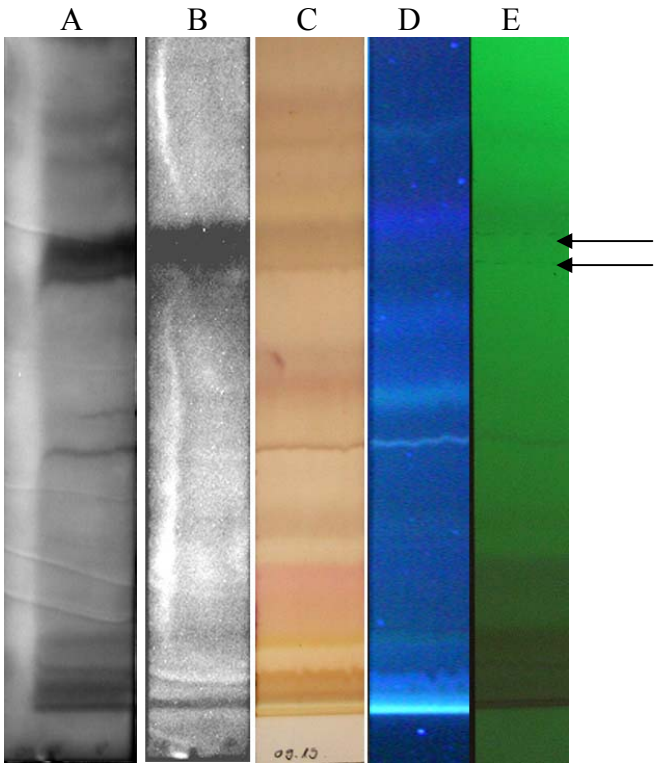

Figure 6

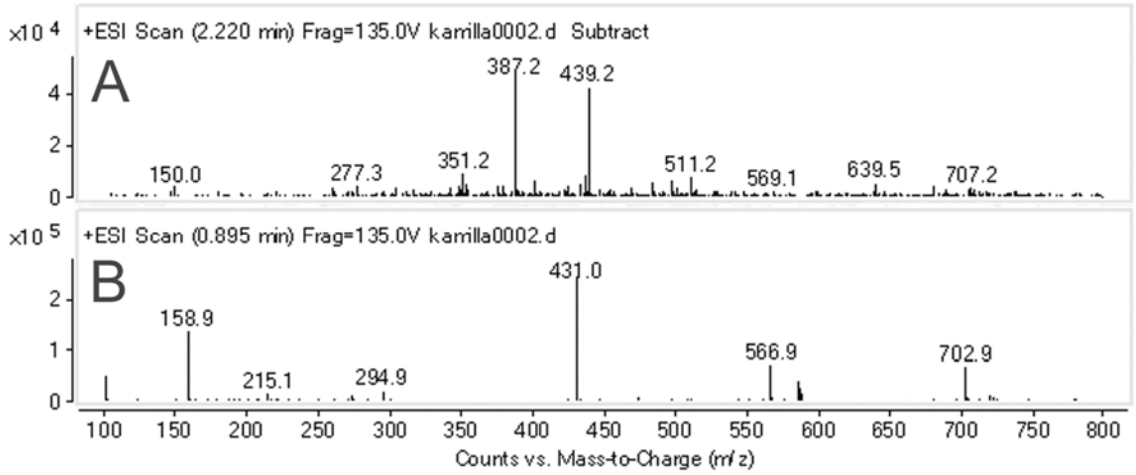
x10 4
+ESI Scan (2.220 min) Frag=135.0V kamilla0002.d Subtract
A
387.2
439.2
150.0
277.3
351.2
511.2
569.1
639.5
707.2
x10 5
+ESI Scan (0.895 min) Frag=135.0V kamilla0002.d
B
431.0
158.9
566.9
702.9
215.1
294.9
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800
Counts vs. Mass-to-Charge (m/z)

Figure 7