1-1-2012

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## Keywords

gypsywort, fatty, alkanes, lycopus, oils, europaeus, volatile, l, gaussian, wax, its, distribution, like


## Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences


## Publication Details

Radulovic, N., Denic, M., Stojanovic-Radic, Z. & Skropeta, D. (2012). Fatty and volatile oils of the gypsywort lycopus europaeus L. and the gaussian-like distribution of its wax alkanes. JAOCS, 89 (12), 2165-2185.

# Fatty and volatile oils of the gypsywort *Lycopus europaeus* L. and the Gaussian-like distribution of its wax alkanes

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**Abstract**

The detailed analyses of the volatile essential oil and lipid profiles of the aerial parts from the blooming and fruit forming stages of both ripe and unripe fruit of *Lycopus europaeus* (Lamiaceae) are presented. Both of these profiles are distinguished by components with a restricted occurrence in the Plant Kingdom. These rare compounds include (*E*)-hotrienol in the volatiles, numerous unusual fatty acids (such as very long chain, odd-numbered and branched-chain) in the bound lipids and a high amount of *iso*- and *anteiso*-alkanes in the epicuticular waxes. Furthermore, a Gaussian-like distribution of the relative amounts of the epicuticular wax alkanes was observed. These normal distributions could be interpreted as the end result of the

work of elongase enzyme systems where the Gaussian parameter μ should match the length of the "ideal" fatty acid biosynthesized and σ would represent the error of this enzyme system. These curve parameters were shown to have a close relationship with ACL and CPI values usually utilized to describe the natural distribution of wax alkanes. The screening of *L. europaeus* essential oil for its *in vitro* antimicrobial activity showed that this oil possesses selectivity towards two Gram negative strains, *E. coli* and *K. pneumoniae*.

**Key words:** *Lycopus europaeus*, Lamiaceae, essential oil, (*E*)-hotrienol, alkanes, bound fatty acids, antimicrobial activity, Gaussian distribution

## Introduction

*Lycopus* L. is a genus of about a dozen species of flowering plants in the family Lamiaceae. *Lycopus europaeus* L., also known as gypsywort, is a perennial plant native to both Europe and Asia, and introduced in the United States. The plant's juice yields a black dye, supposedly used by gypsies to tan their skin in order to mimic Egyptians in Europe, and hence the common name of gypsywort. *L. europaeus* is reputed to have medicinal qualities, often attributed to its phenolic compounds [1-3], and has been used traditionally as an astringent, cosmetic, narcotic and refrigerant [4]. Extracts from *Lycopi europaei herba* are traditionally used in patients with slight hyperthyroidism with vegetative-nervous disturbances as well as in tenseness and pain of the mammary gland [5]. A recent investigation showed a clear reduction of hyperthyroid symptoms in rats, particularly of cardiac symptoms and body temperature, following treatment with an extract of *L. europaeus* [6].

Recently, we also found an antimicrobial phenolic abietane-type diterpene, euroabienol, possessing an unusual oxygenation pattern of the C ring, in relatively high amount (1%, w/w) in the fruit of this plant species [3]. This was surprising, as the plant was repeatedly shown to accumulate several different highly oxygenated isopimarane [7-9] and aliphatic diterpenes [10, 11] in its aerial parts (leaves). The striking differences observed in the chemistries of the plant fruit and aerial parts might also appear in other biosynthetically, biologically and chemotaxonomically important classes of plant metabolites, such as the fatty oils (acylglycerols), alkanes and volatiles oils, although this is relatively underexplored. The fatty acid content of the fruit of *L. europaeus* has been previously investigated [12, 13], however, only limited data (i.e. the most abundant acids) were reported. The leaf wax alkanes were previously characterized during a chemotaxonomic study of Lamiaceae [14]. The essential oil of *L. europaeus* was also studied on one previous occasion [15]. Most of the above-mentioned studies dealing with the lipid constituents of this taxon were undertaken 30-40 years ago, whereas today's cutting-edge, modern analytical techniques enable studies to be performed in much greater detail. Herein, we present a comparative detailed study of the lipid (surface alkane, bound fatty acid and essential oil) profiles of *L. europaeus* fruit and aerial parts, along with screening for their *in vitro* antimicrobial activity to determine if some of these constituents may contribute to the plant's renowned ethnopharmacological uses. It is anticipated that more detailed lipid profiles of this plant species will also provide new insights into the biosynthesis of the abovementioned metabolites.

## Materials and Methods

Materials

Plant material was collected in the blooming and fruit forming stages during the summer and autumn of 2009 from wetlands in western Niš, Serbia. Voucher specimens were deposited in the Herbarium of the Faculty of Science and Mathematics, University of Niš, under the accession numbers DM0609 and DM0709. The *in vitro* antimicrobial activity was tested against a panel of laboratory control strains belonging to the American Type Culture Collection (Maryland, USA) including Gram-positive: *Staphylococcus aureus* ATCC 27853, *Clostridium perfringens* ATCC 19404, *C. sporogenes* ATCC 19411, *Bacillus subtilis* ATCC 6633, *Sarcina lutea* ATCC 9341, and *Micrococcus flavus* ATCC 10240; Gram negative: *Escherichia coli* ATCC 25922, *E. coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 10031, *Proteus vulgaris* ATCC 8427, and *Salmonella enterica* ATCC 13076; and yeasts *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763. All clinical isolates, *Staphylococcus aureus*, *E. coli* and *K. pneumonia*, as well as *E. coli* 95, were kindly provided by the Institute of Public Health (Kragujevac, Serbia) and the Institute of Immunology and Virology ‘‘Torlak’’ (Belgrade, Serbia), respectively, and stored in the microbiological collection at the Microbiology Laboratory (Department of Biology, Faculty of Science and Mathematics, University of Niš, Serbia). The testing was also performed against the following molds *Aspergillus restrictus*, *A. fumigatus*, *Penicillium chrysogenum* and *Acremonium chrysogenum* (all isolates), which were obtained from mattress dust and identified by Dr. B. Ranković (Department of Biology, Faculty of Science, University of Kragujevac, Serbia). All chemicals and solvents used were of analytical or pharmaceutical grade. Diethyl ether ($Et_2O$), dichloromethane ($CH_2Cl_2$), *n*-hexane, methanol (MeOH), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and anhydrous magnesium sulphate

($MgSO_4$), as well as, lauric, myristic, palmitic, stearic, linoleic, linolenic and oleic acid, were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Sulphuric acid, dimethyl sulfoxide (DMSO), *tert*-butanol ($^tBuOH$), dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), potassium (K), sodium (Na) and anhydrous calcium chloride ($CaCl_2$) were supplied by Merck (Darmstadt, Germany). Triphenyl tetrazolium chloride (TTC) and Mueller Hinton agar (MHA) were also supplied by Merck (Darmstadt, Germany), while Sabouraud's dextrose agar (SDA) was obtained from Difco Laboratories (Detroit, Michigan, USA). Microtitre plates were from Carl Roth GmbH + Co.KG (Karlsruhe, Germany) whereas amoxicillin and nystatin, used as positive control in the antimicrobial assays, were obtained from Galenika (Belgrade, Serbia). Chromatographic separations were carried out using silica gel 60 (particle size distribution 40-63 μm) purchased from Merck (Darmstadt, Germany). All synthetic procedures were carried out under anhydrous conditions.

## Methods

### *Essential oils - extraction and identification of constituents*

Fresh plant material (aerial parts) was subjected to hydrodistillation with *ca.* 2.5 L of distilled water for 2.5 h using a Clevenger-type apparatus to produce yellowish essential oils with a strong characteristic green-grassy odor in the following yields (%, w/w, typical values): 0.01% essential oil A (from the blooming stage, three batches of around 250 g per sample), and 0.006% essential oil B (from the fruit forming stage, three batches of around 100 g per sample). The obtained oils were separated by extraction with $Et_2O$, dried over anhydrous $MgSO_4$, and the solvent

evaporated under a stream of nitrogen at room temperature, in order to exclude any loss of the essential oil, and stored at −18 °C until further analysis. Once the oil yields were determined, the residue was exposed to vacuum at room temperature for a short period to eliminate the solvent completely. The pure oil was then measured on an analytical balance and multiple gravimetric measurements were taken during 24 h to ensure that all of the solvent had evaporated.

Qualitative analysis of the essential oils constituents was based on the comparison of their linear retention indices (RI) relative to the retention times of $C_7$-$C_{34}$ *n*-alkanes on a DB-5MS column [16] with those reported in the literature [17], and by comparison of their mass spectra with those of authentic standards as well as those from Wiley 6, NIST07, MassFinder 2.3, and a homemade MS library of spectra corresponding to pure substances and components of known essential oils, and, wherever possible, by co-injection with an authentic sample.

*Lipids (alkanes and bound fatty acids) – extraction and identification*

The surface fruit waxes were washed off by ultra-sound assisted extraction of the intact unripe (25 g, three batches, sample C) and ripe fruits (25 g, three batches, sample D) with dichloromethane for the duration of 30 min. The wax washings were then filtered through a Syringe Econofilter (25/0.45 μm RC, Agilent Technologies, Santa Clara, California, USA) to remove any insoluble material, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to yield 16±3 mg and 19±2 mg of surface waxes, respectively. A portion of the washings, as well as a concentrated sample of solvent, were subjected to GC-MS analysis to identify any possible contamination that could arise from the use of the described procedures and/or solvents.

The fruit were dried, crushed and submitted to ultra-sound assisted dichloromethane extraction for 2 h. The crude extracts (C and D, respectively) obtained after removal of the solvent (2.5 g and 4.0 g, typical values) were subjected to "dry flash" column chromatography on silica gel (40-63 μm) using *n*-hexane-EtOAc (v/v) mixtures of increasing polarity for elution. The bound fatty acids were collected in the 5% EtOAc fractions. Solvent removal under reduced pressure yielded 1.1 g and 3.0 g (typical values) of acylglycerides, respectively.

The *n*-alkanes were identified by GC co-injection of authentic standards (a mixture of $C_7$-$C_{34}$ *n*-alkanes acquired from Sigma-Aldrich, USA) with the alkane fractions. Branched *iso*- and *anteiso*-alkanes were mutually distinguished, as well as from the *n*-alkanes, by their differences in mass spectral fragmentation patterns since scission occurs at adjacent bonds to the tertiary carbon atoms, yielding ions of variable intensities for the two classes of isomers. Both branched isomeric series are cleaved predominately at the C2-C3 linkage, giving major peaks at $[M - C_3H_7]^+$ and $[M - C_2H_5]^+$ for the *iso*- and *anteiso*-isomers, respectively [18]. This difference was used, in conjunction with the comparison of GC data with those reported in the literature [19], to identify 2- and 3-methylalkanes.

In order to determine the bound fatty acid profile, acylglyceride fractions were submitted to alkaline transesterification (MeONa/MeOH and $^t$BuOK/$^t$BuOH). Qualitative analysis of the methyl and *tert*-butyl esters obtained after alkaline transesterification, was achieved in a similar manner as for the alkanes by GC co-injection of available standards and comparison of RI values, as well as mass spectral fragmentation patterns (e.g. ions having *m/z* 74 for methyl esters, correspond to the $[CH_2{=}C(OH){-}OCH_3]^+$ fragment).

*Alkaline transesterification using MeONa/MeOH*

A solution of MeONa was prepared by dissolving 500 mg of metallic sodium in anhydrous MeOH (20 mL), which was then cooled to room temperature. A portion of the acylglyceride fractions (*ca.* 500 mg) dissolved in MeOH was added with stirring to this solution, brought to reflux and quenched with excess ice-water. This was followed by immediate extraction of the reaction mixture with $Et_2O$, the organic layers were combined, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to yield an oily residue.

*Alkaline transesterification using $^t$BuOK/$^t$BuOH*

A solution of $^t$BuOK was prepared by dissolving 500 mg of metallic potassium in anhydrous $^t$BuOH (20 mL) under reflux. The acylglycerides (500 mg) were dissolved in 20 mL of $Et_2O$ and 2 mL of the prepared $^t$BuOK solution was cautiously added with stirring. The reaction mixture was refluxed for 2 h followed by work up as described for the methyl esters.

*General procedure for the synthesis of fatty acid ester standards*

Fatty acid (1 *eq*.), dicyclohexylcarbodiimide (DCC, 1.1 *eq*.), alcohol (MeOH or $^t$BuOH, 3 *eq*.), 4-dimethylaminopyridine (DMAP, 0.1 *eq*.) and 10 mL of anhydrous $CH_2Cl_2$ were mixed and stirred for 4 h under anhydrous conditions. The precipitated dicyclohexylurea (DCU) was filtered

off (with the addition of *n*-pentane causing further precipitation). The removal of the solvent and excess alcohol under vacuum gave the appropriate fatty acid esters.

*GC and CG-MS analysis*

Analyses of the essential oils, alkanes and fatty acid esters were carried out by GC and GC/MS. The GC/MS analyses were performed in triplicate on a Hewlett-Packard 6890N gas chromatograph equipped with fused silica capillary columns DB-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 $\mu$m, Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 and 290 °C, respectively. Oven temperature was raised from 70 to 290 °C at a heating rate of 5 °C/min and then isothermally held for 10 min. As a carrier gas helium at 1.0 mL/min was used. The samples, 1 $\mu$L of the oil, alkanes and fatty acid esters solutions in $Et_2O$ (1 mg/mL), were injected in a pulsed split mode (the flow was 1.5 mL/min for the first 0.5 min and then set to 1.0 mL/min throughout the remainder of the analysis; split ratio 40:1). Mass selective detector was operated at the ionization energy of 70 eV, in the 35–650 amu range and scanning speed of 0.34 s. GC (FID) analysis was carried out under the same experimental conditions using the same columns as described for the GC/MS. The percentage composition was computed from the GC peak areas without the use of correction factors.

*IR measurements*

The IR measurements (ATR-attenuated total reflectance) were carried out using a Thermo Nicolet model 6700 FTIR instrument (Waltham, USA).

Alkane fraction (sample C) - FTIR-ATR (neat) $cm^{-1}$: 2956 (asymmetric stretching of $CH_3$-groups, $\nu_{as}(CH_3)$), 2916 ($\nu_{as}(CH_2)$), 2847.8 (symmetric stretching of -$CH_2$- groups, $\nu_s(CH_2)$), 1461 (scissoring of -$CH_2$- groups, $\delta_{sc}(CH_2)$), 1377 ($\delta_s(CH_3)$), 730 and 719 (rocking of -$CH_2$-groups, $\delta_r(CH_2)$); Acylglycerides (sample C) - FTIR-ATR (neat) $cm^{-1}$: 3010 (C–H stretching in olefins ($\nu$(=CH)), 2922 ($\nu_{as}(CH_2)$), 2854 ($\nu_s(CH_2)$), 1742 (broad C=O stretching ($\nu$(C=O)), suggesting the presence of different CO), 1462 ($\delta_{sc}(CH_2)$), 1376 ($\delta_s(CH_3)$), 1159 (C–O stretching, $\nu$(C–O)), 1098, 719 ($\delta_r(CH_2)$), 591; Methyl esters (sample C) - FTIR-ATR (neat) $cm^{-1}$: 3010 ($\nu$(=CH)), 2925 ($\nu_{as}(CH_2)$), 2853 ($\nu_s(CH_2)$), 1739.4 ($\nu$(C=O)), 1436 ($\delta_{sc}(CH_2)$), 1362 ($\delta_s(CH_3)$), 1196, 1170 ($\nu$(C–O)), 986, 723 ($\delta_r(CH_2)$), 591.

Alkane fraction (sample D) - FTIR-ATR (neat) $cm^{-1}$: 2955 ($\nu_{as}(CH_3)$), 2915 ($\nu_{as}(CH_2)$), 2848 ($\nu_s(CH_2)$), 1472 ($\delta_{as}(CH_3)$), 1462 ($\delta_{sc}(CH_2)$), 1377 ($\delta_s(CH_3)$), 1260, 1096, 1022, 803, 729 and 719 ($\delta_r(CH_2)$); Acylglycerides (sample D) - FTIR-ATR (neat) $cm^{-1}$: 3011 ($\nu$(=CH)), 2925 ($\nu_{as}(CH_2)$), 2853 ($\nu_s(CH_2)$), 1742 ($\nu$(C=O)), 1461 ($\delta_{sc}(CH_2)$), 1376 ($\delta_s(CH_3)$), 1239, 1159 ($\nu$(C–O)), 1097, 721 ($\delta_r(CH_2)$), 591; Methyl esters (sample D) - FTIR-ATR (neat) $cm^{-1}$: 3012 ($\nu$(=CH)), 2929 ($\nu_{as}(CH_2)$), 2854 ($\nu_s(CH_2)$), 1732 ($\nu$(C=O)), 1437 ($\delta_{sc}(CH_2)$), 1170 ($\nu$(C–O)), 978, 568; *tert*-Butyl esters (sample D) - FTIR-ATR (neat) $cm^{-1}$: 3013 ($\nu$(=CH)), 2922 ($\nu_{as}(CH_2)$), 2851 ($\nu_s(CH_2)$), 1731 ($\nu$(C=O)), 1632, 1584, 1456 ($\delta_{sc}(CH_2)$), 1368 ($\delta_s(CH_3)$), 1153 ($\nu$(C–O)), 985, 714 ($\delta_r(CH_2)$), 511.

*Antimicrobial assay*

The antimicrobial activity was evaluated using a microdilution broth method [20]. Minimum inhibitory concentration (MIC) determination was performed by a serial dilution method in 96 well microtitre plates. Microorganisms were cultured at 37 ºC in MHA for bacteria and SDA for yeasts (30 ºC). Bacterial suspensions were made from overnight broth cultures and their turbidity was standardized to 0.5 McFarland. Absorbance was measured on a spectrophotometer (UV-VIS 1610, Shimadzu, Japan). The final density of bacterial and yeast's inocula was $5 \times 10^5$. Suspensions of the molds were made in sterile saline and their turbidity confirmed by viable counting in a Thoma chamber, where the final size of the inoculums in SDA broth was $1 \times 10^4$.

A stock solution of the essential oil A was prepared in 10% DMSO (v/v) and serial dilutions prepared in the range of 10.00 to 0.019 mg/mL. The inoculum was added to all wells and the plates were incubated at 37 ºC for 24 h (bacteria) or at 30 ºC (*C. albicans*) and 28 ºC (molds) for 48 h. Amoxicillin and nystatin served as positive controls, while the corresponding solvent was used as the negative control. One inoculated well was included without the test oil to verify the adequacy of the broth for microorganism growth, while another non-inoculated well, free of the antimicrobial agent, was used to ensure medium sterility.

The bacterial growth was determined by adding 20 μL of 0.5% TTC aqueous solution [21]. MIC was defined as the lowest concentration of oil that inhibited visible growth (red colored pellet on the bottom of the wells after the addition of TTC). To determine minimal bactericidal/fungicidal concentrations (MBC/MFC), broth was taken from each well without visible growth and inoculated in MHA for 24 h at 37 ºC for bacteria or in SDA for 48 h at 28 ºC (molds) and 30 ºC (yeast). The MBC/MFC is defined as the lowest concentration of the essential oil at which inoculated microorganisms were 99.9% killed. The experiments were performed in quadruplicate and mean values are presented.

*Statistical analysis*

A one-way ANOVA test was used to evaluate statistically significant differences among the mean values, using Minitab release version 14 (Minitab Inc., State College, USA). In all tests, the significance level at which the critical value differences were evaluated was 5%.

## Results and discussion

*Volatiles of* L. europaeus *aerial parts*

Table 1 lists the identified compounds in both samples of *L. europaeus* essential oils along with the summation of the compounds according to their respective compound classes (percentage and number of detected constituents). A total of 185 and 122 constituents were identified in the oils and accounted for 88.7% and 93.9% of the total oil amount, respectively. The terpenoid fractions represented 81.3% and 80.4% of the oils unevenly distributed between mono-, sesqui- and diterpenoids. More than 50% of the two oils were sesquiterpenoids (56.7% and 54.2%) with (*E*)-β-caryophyllene as the major compound (13.9% and 25.7%) in both cases. Other important contributors were shyobunol (9.7% in sample A oil, but not detected in sample B) and caryophyllene oxide at 2.4% and 12.5% in samples A (blooming stage) and B (fruit-forming stage), respectively. In addition to this rather unequal distribution of the main sesquiterpenoids, the other significant feature that distinguished the two samples was the inverted ratio of the mono- and diterpenoids. The diterpenoid fraction was dominated by (*E*,*E*)-geranyl linalool and

(*E*)-phytol in the blooming stage (15.6% against 1.6%), whereas monoterpenoids prevailed in the fruit-forming stage (24.6% against 8.8%). These differences could be attributed to the fact that the oils had been isolated from plant material collected in different phenological phases, hence, containing different plant organs. The monoterpenoid fraction was characterized by a high content of oxygenated acyclic derivatives among which the most predominant one was (*E*)-hotrienol, with 4.5% and 13.7% in oils A and B, respectively. This rather rare monoterpene has an attractive fruity smell [22], however, it is usually available in only trace amounts from natural sources by hydrodistillation as it is glycoside-bound [23, 24]. There are several previous reports on plant species whose essential oil have (*E*)-hotrienol as one of the main constituents including the ornamental plants and trees: *Tilia cordata* [25], *Chimonanthus praecox* [26] and *Garcinia macrophylla* [27]. Moreover, the pleasant green-grassy odor of the essential oils herein originated from the so call "green leaf" volatiles, with a content of around 4% in both cases. Finally, the minor constituents were long chain *n*-alkanes, as well as some *iso*- and *anteiso*-alkanes, followed by long chain linear aldehydes and fatty acid esters.

The only previous study of *L. europaeus* essential oil [15] resulted in the identification of only 13 compounds, all of which were monoterpenoids. There are evident differences between the results of our study and those previously reported, expectedly in the number, but also in the identity of the detected metabolites. In fact, Sharipov and co-workers had classified *L. europaeus* as a mono- and bicyclic monoterpene-, as well as oxygenated acyclic monoterpene-, containing plant. However, none of those mono- and bicyclic monoterpenes (such as α-pinene, camphene, γ-terpinene etc.) was found in our samples. Geraniol and nerol represent the only common features of the currently investigated oils and the one previously described. The dissimilarities in the volatile profiles of *L. europaeus* can support the existence of different chemotypes of this taxon

or it may be that the more sophisticated analysis utilized at present provides more detailed data. However, other factors that might result in the alteration of the volatile profile of *L. europaeus* such as harvest season, plant infection, insect attack etc. should also not be dismissed.

*Antimicrobial activity of* L. europaeus *essential oil*

Sample A oil was obtained in sufficient quantity to screen for its *in vitro* antimicrobial activity against fifteen strains of bacteria and six fungal strains using a microdilution assay. The results obtained are listed in Table 2. As it can be seen, *L. europaeus* essential oil manifested a wide range of antimicrobial activities – from being completely inactive to a moderate activity when compared to that of the standard used as the positive control (e.g. amoxycillin or nystatin). The analyzed sample showed particular selectivity towards two Gram negative bacteria strains, *E. coli* (isolate) and *Klebsiella pneumoniae* (ATCC 10031), where the greatest reduction of bacterial growth gave a MIC value of 0.156 mg/mL, albeit still significantly higher than the MIC value of amoxycillin (0.0025 mg/mL). The lowest MBC value (0.312 mg/mL) was again observed in the case of *E. coli* 95 (i.e. the clinical isolate that was a more resistant strain than the ATCC one). On the other hand, this oil was completely inactive against two bacterial strains, *Salmonella enterica* and *Micrococcus flavus*, at the tested concentrations. The tested essential oil also manifested a broad spectrum of antifungal activity with greatest sensitivity to the fungi *Aspergillus fumigatus* (MIC = 0.625 mg/mL; MFC = 5.000 mg/mL). Overall, the activity of *L. europaeus* oil was slightly higher towards bacteria than to fungal strains.

*Surface alkane profiles of the fruit of* L. europaeus

The content and composition of wax alkanes of *L. europaeus* fruit are given in Table 3. The analysis allowed the identification of 43 components accounting for 98.6% (sample C – unripe fruit) and 99.0% (sample D – ripe fruit) of the total alkane fractions. *n*-Alkanes were dominant in both samples with 85.0% and 82.3% for samples C and D respectively, followed by *anteiso*- (7.1-7.6%) and *iso*-alkanes (5.7-7.1%). The latter have a rather restricted occurrence in higher plants and are mostly contained in significantly lower amounts than those reported here [28]. One notable exception is the tobacco plant, *Nicotiana tabacum*, which can have approximately equivalent amounts of *anteiso*-, *iso*-, and *n*-alkanes [29]. Recent environmental studies have relied on the large abundance of *anteiso*- and *iso*-alkanes in tobacco to detect cigarette smoke pollution in indoor and urban aerosols [29, 30]. *Lycopus europaeus*, along with some other species of Lamiaceae [28], fall between most higher plants and tobacco in terms of their content of *anteiso*- and *iso*-alkanes.

The *n*-alkane distribution reflects the conventional higher plant pattern with an odd carbon predominance centered at *n*-$C_{29}$, with an average chain length (ACL) of 29.52 and 28.93, accompanied by high carbon preference index (CPI) values of 6.94 and 6.72. CPI and ACL were calculated according to a modified formula given in reference [28]. The *anteiso*-alkanes have a predominant even-numbered distribution (ACL = 28.80 and 27.96), whereas the *iso*-alknes (ACL = 29.17 and 28.81) are mainly odd-numbered. Similar patterns have been reported previously for Lamiaceae species belonging to the genus *Micromeria* and others [28] and reflect the small differences in biosynthetic pathways of these compounds. In general, biosynthesis of major wax components occurs *via* sequential elongation of a primer with $C_2$ units derived from malonyl-CoA [31]. In this condensation-elongation process, acyl chains of up to $C_{16}$ and $C_{18}$ formed by *de*

*novo* synthesis are further extended to $C_{30}$ or higher by a second elongation system. Finally, modification of the acyl chain gives products including alkanes, aldehydes, primary alcohols, alkyl esters, secondary alcohols, ketones and various polyoxygenated compounds. The type of primer is the factor that dictates the alkane distribution; e.g. the *n*- and *iso*-alkanes have an odd numbered predominance with even number $C_2$-acetyl-CoA (derived from pyruvate) and $C_4$-isobutyryl-CoA (derived from valine) units as precursors, while the odd numbered $C_5$-2-methylbutyryl-CoA primer (derived from isoleucine) for *anteiso*-alkanes favors even numbered homologues [32]. The previously reported *L. europaeus* leaf alkane profile [14] was characterized with a similar predominant odd numbered distribution (CPI = 6.73), but with the maximum at $C_{31}$, shifted by one $C_2$ unit.

*Bound fatty acid profile of* L. europaeus *fruit*

The bound fatty acids of the fruit of *L. europaeus* were converted to methyl esters by alkaline transesterification and analyzed by GC-MS. A total of 23 and 30 fatty acid methyl esters were identified in samples C (unripe) and D (ripe) respectively. These represented 96.2 and 98.9% of the total amount of detected fatty acid esters, as listed in Table 4 in order of elution from a DB-5MS column. To improve the resolution of some partially overlapped peaks in the GC data (e.g. methyl esters of oleic and linoleic acids), the bound fatty acids of *L. europaeus* fruit (sample D) were transformed to *tert*-butyl esters by alkaline transesterification (Table 5). A previous study showed that some unsaturated fatty acids (such as oleic, vaccenic and petroselinic acids) could not be separated by GC as their methyl esters, but separation of their *n*-butyl esters was feasible [34]. Herein, GC-MS analysis of the *tert*-butyl derivatives proved to be particularly suitable for

characterizing and quantification of two unsaturated fatty acids (oleic and linoleic), while keeping the other ones well resolved. From Table 5, it can be seen that the increment in the values of retention indices of *tert*-butyl compared to the corresponding methyl esters (ΔRI = RI(RCOO$^t$Bu) – RI(RCOOMe)) was relatively constant and varied between 125 and 132 units. This study also provides the first report of the mass spectral data of *tert*-butyl esters of most fatty acids, along with some branched fatty acid methyl esters (Table 5 and 4, respectively). Note, however, that the mass spectral data of *tert*-butyl palmitate was published previously (Wiley 9 Registry of Mass Spectral Data).

Unsaturated fatty acids dominated over saturated ones in the bound fatty acid profile of *L. europaeus* fruit (e.g. 85.8 and 91.7% *vs* 10.4 and 7.2% in samples C and D, respectively). The qualitative and quantitative composition of the total fatty acids of *L. europaeus* fruit shows that this species is similar to other higher plants in the predominance of ubiquitous fatty acids, such as palmitic, linoleic, and linolenic acid, in its lipids. Together, these fatty acids accounted for about 90% of all fatty acids. This is consistent with the general view that higher plants contain, for the most part, $C_{16}$–$C_{18}$ even-numbered *n*-fatty acids [33]. However, *L. europaeus* fruit lipids also included numerous unusual fatty acids, such as very long-chain ($C_{20}$–$C_{28}$), odd-numbered ($C_{15}$-$C_{23}$), as well as branched-chain *iso*- ($C_{16}$ and $C_{20}$-$C_{24}$) and *anteiso*- ($C_{15}$ and $C_{23}$) fatty acids, which were detected in low amount. These infrequently encountered fatty acids were not observed in the two previous studies dealing with the fatty acid composition of *L. europaeus* fruits [12, 13].

Saturated or unsaturated very long chain fatty acids with a chain longer than 20 carbon atoms are rare, although they have been reported in microbes, insects, plants and animals, their organs or products such as meibomian gland and wool wax, and in humans suffering from

pathobiochemical diseases (e.g. inability of α- and β-oxidation of fatty acids) [35]. The branched *iso*- and *anteiso*-fatty acids also have a very restricted distribution in higher plants. The most common one is 14-methylpalmitic acid, which has been identified in gymnosperm leaves (where it represents one of the chemotaxonomical biomarkers), and in the wood of several pines and conifer needles, as well as in *Ginkgo biloba* seed oil [36]. By contrast, the branched-chain fatty acids of the *iso*- and *anteiso*- series occur widely in bacteria and have a great value in bacterial systematics where they are used as an aid in their identification [37]. Additionally, it is well established that a major aspect of the cryotolerant physiology of some bacterial species, such as *Listeria*, relates, is the predominance of low freezing-point branched-chain fatty acids in the cell membrane, which permits the maintenance of membrane function at low temperatures [38]. Furthermore, there is a hypothesis that the formation of saturated very long chain and branched fatty acids in alpine plants is related to a protective function of waxes under extreme environmental conditions [33].

The *iso*-fatty acids had a predominant even-numbered distribution in the samples investigated, whereas the *anteiso*-fatty acids were mainly odd-numbered. As expected, this is opposite to the observed distributions of *iso*- and *anteiso*-alkanes, but consistent with the previously mentioned biosynthetic pathway of the major wax components where a decarboxylation of an even-numbered fatty acid gives the corresponding odd-numbered alkane and *vice versa*. Another fact, that is also consistent with this biosynthetic pathway, is the absence of fatty acids that are expected to be the precursors of the most abundant detected alkanes, as well as the presence of only trace amounts of the alkanes that should have the main fatty acids as the decarboxylation substrates.

*Gaussian-like distribution of* L. europaeus *wax alkanes*

A partial GC trace of the alkane fraction of ripe *L. europaeus* fruits is presented in Fig. 1, where a Gaussian-like distribution for the relative amounts of *n*-alkanes ($C_{23}$-$C_{33}$ with a step of 2 carbons) can be readily observed. The *anteiso*- (3-methyl) and *iso*- (2-methyl) alkanes also have similar homolog distributions, which take up the same region of the chromatogram as *n*-alkanes. The Gaussian distribution is usually referred to as a normal distribution or as "normal law of error" which can be interpreted in a manner that the mean of this distribution is an ideal at which nature is aiming, and observations to either side to the mean represent an error (a deviation from nature's ideal) [39]. A normal distribution is often used as a first approximation to describe real-valued random variables that cluster around a single mean value. Thus, the observed *L. europaeus* alkane distributions might reflect, in a highly informative (statistical) manner, the work of an elongase-modification system producing wax alkanes from *de novo* fatty acid precursors. The biosynthesis is believed to proceed through two key steps – elongation of stearic or palmitic acids with "acetate" units and the modification of the obtained very long-chain fatty acids (leading to net decarboxylation) resulting in odd-numbered alkanes. It is still unclear which factor dictates the chain length distribution of these aliphatic wax compounds. There is some evidence indicating that the modifying reactions (e.g. aldehyde reductase) do not have chain length specificities [40]. Recently, an understanding of how the final acyl chain length is specified by the condensing enzyme, which catalyses the first reaction of the microsomal fatty acid elongation system, is starting to emerge [41]. It is supposed (based on results obtained in yeasts) that the determining factor is the depth of a hydrophobic pocket where the very long

chain fatty acid sits during elongation, so once the length of the growing acyl chain exceeds the depth of the pocket, further elongation is not possible [42]. One can speculate that the elongase enzyme would catalyze best the onset of a single fatty acid precursor to alkanes (corresponding to the depth of the mentioned hydrophobic pocket) and that the other elongation products obtained in this way are to be regarded as expected statistical errors (accidental occurrences) that are to obey a normal law distribution. Hence, the parameters of the experimentally fitted Gaussian distributions for *n*- and branched alkanes convey the data (mean value, μ, and standard deviation, σ) that can be interpreted as – μ being the length of the ideal fatty acid, the one nature intended to produce (encoded in the genetic material), and σ the error of this elongation enzyme system. Interestingly, this error is, for the four alkane distributions of *L. europaeus* (Fig. 2), *ca.* two acetate (1.7-2.5) units and can be connected to another feature on this elongation system. It was found that the interaction of the terminal methyl group with a certain lysine residue in the lumen part of the helix of this enzyme marks the end of the elongation process [42]. Enzymes with an altered position of the lysine that are either one loop of the helix closer or more distant from the active site of the elongation were also fully operational but gave products that differed in four carbon units (two acetate units) from the unaltered one. This was explained by the fact that one helical turn (5.4 Å) is in excellent agreement with the length of a four-carbon unit along a saturated hydrocarbon chain (5.3 Å). It is remarkable that the σ of normal alkane distributions (giving usually the 95% of the area under the bell-shaped curve (i.e. the radius of a 95 percent confidence interval) at about twice the standard deviation) and the outcome of such enzyme modifications would give the same number of acetate units, indicating that the one proximal and one distal loop amino acid residues from the key lysine one also play a significant role in determining the final length of the fatty acid to be biosynthesized. If several elongation enzymes

with different key lysine positions were operational one should obtain a set of superimposed Gaussian distribution patterns that would not necessarily result in the global Gaussian-like but most probably deformed Gaussian distribution. It would then appear that an organism is genetically predetermined by the depth of the hydrophobic pocket in the enzyme membrane complex and that the distribution around this ideal (mean) would always have to correspond to the most probable errors two acetate units apart. This hypothesis is well sustained not only by *L. europaeus* but by also a number of other Lamiaceae members, as well as those from other plant families. The mentioned four distributions of *L. europaeus* seed wax alkanes are presented in Fig.2 where it can be noted that the parameter μ (expressed as a total number of $C_2$ units added to the appropriate starting precursor during elongation) also varies for *ca*. 2 acetate units (from 12.34 for *antheiso*- to 14.06 for odd-numbered *n*-alkanes). Furthermore, Fig. 3 provides some examples of Gaussian-like distributions of other plant wax *n*-alkanes, which are characterized by different σ and μ parameters. The leaf *n*-alkanes of *L. europaeus*, and two other taxa of the genus *Lycopus* (*L. exaltatus* and *L. americanus*, Fig. 3a) display distributions with σ of *ca*. 2 acetate units, but centered at μ = 15 [14]. The odd-numbered *n*-alkanes in some members of the genus *Achillea* (Asteraceae, Fig. 3b) [44] show good and mutually uniform Gaussian-like distributions characterized with the same σ and μ values (*ca*. 2 and 14 $C_2$ units, respectively). However, for another two genera of the family Lamiaceae, *Salvia* [14] and *Micromeria* [28] (Fig. 2c and 2d, respectively), these parameters infragenetically vary considerably e.g.: *M. cristata* (σ = 1.66, μ = 14.91) has a high-quality Gaussian-like distribution, while *S. sclarea* (σ = 1.92, μ = 15.30), *S. verticilata* (σ = 1.44, μ =15.48) and *M. thymifolia* (σ = 1.81, μ = 15.32) display rather deformed Gaussian distributions that are readily recognizable by the absence of a clear maximum, as there are two predominant, equally abundant *n*-alkanes. The parameter most influenced by these

deformations is μ. The presence of two (or more) main wax alkanes could be explained by an existence of at least two different elongases. This touches an important question in wax biosynthesis, which is still unanswered, concerning the number of the enzymes involved in the elongation of $C_{18}$–$C_{36}$ acyl groups [41]. But it is quite interesting that in most cases when μ is closer to a round number the value of $R^2$ (the square of the correlation coefficient between the observed and modeled (predicted) data values) tends towards 1 (>0.999) suggesting that only one enzyme is operational.

Finally, the common parameters for describing alkane distributions in samples of natural origin, ACL and CPI, could also be expressed, in a more precise manner, by analogous parameters of the corresponding Gaussian distributions. Hence, the mean value (μ) corresponds to ACL (ACL = 2*μ + 1), while the ratio of the areas under the Gaussian curves of odd and even *n*-alkanes (Ao/Ae) should match to CPI. For example, the distributions of the ripe-seed wax odd- and even-numbered *n*-alkanes (Fig. 2) have the following Gaussian parameters: μ = 14.06, σ = 1.73, $R^2$ = 0.995, and μ = 13.36, σ = 1.96, $R^2$ = 0.991, respectively, with Ao/Ae = 6.08. Thus, the calculated ACL and CPI from this data are 29.12 and 6.08, respectively, and these fit nicely with those already given above (28.93 and 6.72, respectively) obtained using the classical formula [28].

Up to now, the Gaussian distributions of alkanes were studied in the context of the influence of the statistical parameter σ on the disorder appearance, the structure, and the mechanical properties (the storage modulus and the loss factor) of waxes [44]. The obtained results show, among other things, an increase of the amount of amorphous phase and the number of crystallized phases with an increase of the standard deviation σ. Furthermore, the analyses revealed that the amount of the amorphous phase increases with the mass of *iso*-alkanes added to

the synthetic waxes, but the effect of the *iso*-alkanes is not important until 12%, as branched alkanes in a small proportion can occupy voids or interlamellar spaces of the lattice. For higher quantities, the disorder increases sharply [45]. It seems that in real wax mixtures a formation of the amorphous phase (composed by linear and nonlinear alkanes) is very important as it promotes the formation of a single solid solution or prevents the appearance of other solid solutions [44]. Since the epicuticular *n*-alkanes of ripe *L. europaeus fruits* have a narrow distribution (with σ value of *ca.* 2) and the content of branched alkanes is close to 12% they probably form a single solid crystalline surface with a low percent of amorphous zones (reported to be higher than 11.3% [44]).

## Conclusion

This work provides detailed chemical data on the volatile and lipid (alkanes and bound fatty acids) profiles of *L. europaeus*, which were characterized by components with a restricted occurrence in higher plants. This includes (*E*)-hotrienol in the essential oil and numerous unusual fatty acids, such as very long chain, odd-numbered, as well as branched-chain *iso*- and *anteiso*-alkanes, the latter found in the wax or bound lipids of the plant's fruit. Antimicrobial screening of *L. europaeus* essential oil revealed a selective action of the tested oil towards two Gram-negative strains, *E. coli* (isolate) and *K. pneumoniae* (ATCC 10031). A feature of the epicuticular wax alkane profiles is their Gaussian-like appearance. We propose that these normal distributions are the result of elongase enzyme systems. The parameters μ should match the length of the ideal fatty acid biosynthesized and σ would represent the error of this enzyme

system. These curve parameters were shown to excellently correspond to ACL and CPI values usually utilized to describe the natural distribution of wax alkanes.

**Acknowledgements**

Financial support of this work by the Ministry of Education and Science of Serbia is gratefully acknowledged (Project No. 172061).

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**Table 1** Percentage composition of the essential oils of two samples of *L. europaeus*

| RI[a] | Compound | Content % | | Class | Method of identification |
|---|---|---|---|---|---|
| | | Sample A[b] | Sample B[c] | | |
| 730 | 3-Methyl-1-butanol | tr[d] | n.d.[e] | HT | RI, MS, CoI[f] |
| 733 | 2-Methyl-1-butanol | tr | n.d. | HT | RI, MS, CoI |
| 762 | 1-Pentanol | tr | n.d. | GL | RI, MS, CoI |
| 765 | (*Z*)-2-Penten-1-ol | tr | n.d. | GL | RI, MS |
| 800 | Octane | tr | n.d. | O | RI, MS, CoI |
| 830 | 4-Methyl-1-pentanol | tr | n.d. | O | RI, MS |
| 839 | Furfural | tr | n.d. | GL | RI, MS, CoI |
| 844 | (*E*)-3-Hexen-1-ol | tr | 0.1±0.01[g] | GL | RI, MS, CoI |
| 850 | (*Z*)-3-Hexen-1-ol | 3.1±0.20 | 1.5±0.07 | GL | RI, MS, CoI |
| 858 | (*Z*)-2-Hexen-1-ol | tr | 0.6±0.04 | GL | RI, MS, CoI |
| 861 | 1-Hexanol | 0.2±0.01 | 0.6±0.03 | GL | RI, MS, CoI |
| 886 | 2-Butylfuran | tr | n.d. | GL | RI, MS |
| 944 | 3-Ethyl-1-octene | n.d. | tr | O | MS |
| 968 | Benzaldehyde | tr | tr | O | RI, MS, CoI |
| 975 | 1-Octen-3-one | tr | n.d. | GL | RI, MS |
| 977 | 1-Octen-3-ol | 0.6±0.04 | 0.7±0.03 | GL | RI, MS |
| 983 | 6-Methyl-5-hepten-2-one | tr | 0.1±0.01 | CR | RI, MS |
| 984 | 2-Octanone | tr | n.d. | GL | RI, MS |

| | | | | | |
|---|---|---|---|---|---|
| 990 | 2-Pentylfuran | tr | tr | GL | RI, MS |
| 992 | 6-Methyl-5-hepten-2-ol | tr | tr | CR | RI, MS |
| 997 | 3-Octanol | 0.1±0.01 | 0.1±0.01 | GL | RI, MS |
| 1004 | (*Z*)-3-Hexenyl acetate | tr | n.d. | GL | RI, MS, CoI |
| 1026 | 2-Ethyl-1-hexanol | tr | n.d. | O | RI, MS, CoI |
| 1033 | (*Z*)-β-Ocimene | 0.1±0.01 | 0.2±0.01 | MT | RI, MS |
| 1038 | 2,6,6-Trimethylcyclohexanone | n.d. | tr | CR | RI, MS |
| 1045 | (*E*)-β-Ocimene | tr | 0.1±0.01 | MT | RI, MS |
| 1046 | Phenylacetaldehyde | 0.1±0.01 | 0.6±0.03 | O | RI, MS, CoI |
| 1051 | 2,6-Dimethyl-2,6-octadiene[h] (*syn*.[i] Deoxygeraniol) | n.d. | 0.2±0.01 | MT | MS |
| 1066 | (*E*)-2-Octen-1-ol | tr | n.d. | GL | RI, MS |
| 1068 | 1-Octanol | tr | tr | GL | RI, MS, CoI |
| 1070 | Acetophenone | tr | n.d. | O | RI, MS, CoI |
| 1093 | 4,8-Dimethyl-1,3,7-nonatriene (Isomer 1[h]) | tr | n.d. | MT | RI, MS |
| 1100 | Undecane | tr | tr | O | RI, MS, CoI |
| 1105 | (*E*)-Hotrienol | 4.5±0.28 | 13.7±0.75 | MT | RI, MS |
| 1106 | Nonanal | tr | tr | GL | RI, MS |
| | (*E*)-4,8-Dimethylnona-1,3,7- | | | MT | RI, MS |
| 1113 | triene | 0.1±0.01 | 0.2±0.01 | | |
| 1117 | 3-Octyl acetate | tr | 0.1±0.01 | GL | RI, MS |

| | | | | | |
|---|---|---|---|---|---|
| | 2,6-Dimethyl-1,3,5,7- | | | MT | MS |
| 1123 | octatetraene[h] | tr | n.d. | | |
| 1129 | (*Z*)-Epoxy-ocimene | tr | n.d. | MT | RI, MS |
| 1131 | (3*E*,5*E*)-2,6-Dimethyl-1,3,5,7-octatetraene | tr | n.d. | MT | RI, MS |
| 1139 | (*E*)-Epoxy-ocimene | tr | n.d. | MT | RI, MS |
| 1143 | *exo*-Isocitral | tr | tr | MT/CR | RI, MS |
| 1154 | (2*E*,6*Z*)-2,6-Nonadienal | tr | tr | GL | RI, MS |
| 1160 | (*Z*)-Isocitral | 0.1±0.01 | 0.3±0.01 | MT/CR | RI, MS |
| 1163 | Lavandulol | tr | n.d. | MT | RI, MS |
| 1170 | 1-Nonanol | tr | n.d. | GL | RI, MS |
| 1175 | (3*E*,5*Z*)-1,3,5-Undecatriene | tr | n.d. | O | RI, MS |
| 1176 | Borneol | n.d. | 0.2±0.01 | MT | RI, MS, CoI |
| 1179 | (*E*)-Isocitral | 0.1±0.01 | 0.2±0.01 | MT/CR | RI, MS |
| 1180 | 1-Decen-3-ol | tr | n.d. | GL | RI, MS |
| 1184 | (*Z*)-3-Hexenyl butanoate | tr | n.d. | GL | RI, MS, CoI |
| 1184 | Terpinen-4-ol | tr | tr | MT | RI, MS, CoI |
| 1185 | 1,3,5-Undecatriene[h] | tr | n.d. | O | MS |
| 1189 | 1-Phenylethyl acetate | tr | n.d. | O | RI, MS |
| 1196 | (*E*)-3-Hexenyl butanoate | tr | n.d. | GL | RI, MS, CoI |
| 1198 | α-Terpineol | tr | 0.1±0.01 | MT | RI, MS, CoI |
| 1200 | Dodecane | 0.1±0.01 | 0.1±0.01 | O | RI, MS, CoI |

| | | | | | |
|---|---|---|---|---|---|
| 1200 | Methyl salicylate | n.d. | tr | O | RI, MS, CoI |
| 1202 | Safranal | tr | tr | CR | RI, MS |
| 1206 | Decanal | tr | 0.1±0.01 | GL | RI, MS, CoI |
| 1222 | β-Cyclocitral | 0.1±0.01 | 0.2±0.01 | CR | RI, MS |
| 1232 | 2,3-Epoxygeranial | tr | n.d. | MT | MS |
| | (*Z*)-3-Hexenyl 2- | | n.d. | GL | RI, MS, CoI |
| 1234 | methylbutanoate | tr | | | |
| 1239 | Neral | 1.5±0.09 | 3.5±0.22 | MT | RI, MS, CoI |
| 1250 | Geraniol | tr | n.d. | MT | RI, MS, CoI |
| 1258 | (2,6,6-Trimethyl-1-cyclohexen- | tr | tr | CR | RI, MS |
| | 1-yl)-acetaldehyde | | | | |
| 1263 | (*E*)-2-Decenal | tr | 0.1±0.01 | GL | RI, MS |
| 1269 | Geranial | 2.4±0.20 | 5.9±0.29 | MT | RI, MS, CoI |
| 1282 | Lavandulyl acetate | tr | n.d. | MT | RI, MS |
| 1286 | Bornyl acetate | n.d. | tr | MT | RI, MS, CoI |
| 1293 | Dihydroedulan I | tr | n.d. | CR | RI, MS |
| 1300 | Tridecane | 0.1±0.01 | 0.2±0.01 | O | RI, MS, CoI |
| 1309 | Undecanal | tr | tr | GL | RI, MS |
| 1321 | (2*E*,4*E*)-2,4-Decadienal | tr | n.d. | GL | RI, MS |
| 1350 | α-Cubebene | tr | tr | S | RI, MS |
| 1358 | (*E*)-Solanone | tr | n.d. | O | RI, MS |
| 1366 | (*E*)-2-Undecenal | tr | tr | GL | RI, MS |

| | | | | | |
|---|---|---|---|---|---|
| 1373 | α-Ylangene | tr | tr | S | RI, MS |
| 1380 | α-Copaene | 0.1±0.01 | 0.3±0.01 | S | RI, MS |
| 1381 | (*E*)-β-Damascenone | tr | tr | CR | RI, MS |
| 1388 | β-Bourbonene | 0.3±0.02 | 1.6±0.08 | S | RI, MS |
| 1390 | β-Cubebene | n.d. | 0.3±0.01 | S | RI, MS |
| 1392 | β-Elemene | 0.6±0.03 | n.d. | S | RI, MS |
| 1395 | (*Z*)-Isoeugenol | tr | n.d. | O | RI, MS |
| 1396 | 4-(2,2-Dimethyl-6-methylenecyclohexyl)-2-butanone (*syn*. dihydro-γ-ionone) | n.d. | 0.1±0.01 | CR | RI, MS |
| 1400 | Tetradecane | tr | n.d. | O | RI, MS, CoI |
| 1409 | (*Z*)-Caryophyllene | tr | 0.8±0.04 | S | RI, MS |
| 1411 | (*E*)-β-Damascone | tr | tr | CR | RI, MS |
| 1411 | α-Gurjunene | 1.0±0.07 | n.d. | S | RI, MS |
| 1427 | (*E*)-Caryophyllene | 13.9±0.85 | 25.7±1.34 | S | RI, MS, CoI |
| 1430 | Acora-3,5-diene | tr | n.d. | S | RI, MS |
| 1432 | γ-Elemene | tr | n.d. | S | RI, MS |
| 1434 | β-Copaene | 0.1±0.01 | 0.3±0.01 | S | RI, MS |
| 1438 | α-Guaiene | tr | tr | S | RI, MS |
| 1448 | Geranyl acetone | 0.9±0.06 | 5.6±0.34 | CR | RI, MS |
| 1449 | *cis*-Muurola-3,5-diene | tr | n.d. | S | RI, MS |

| | | | | | |
|---|---|---|---|---|---|
| | 6,10-Dimethyl-5,9-undecadien- | | | CR | MS |
| 1451 | 2-ol[h] | 0.2±0.01 | 1.3±0.07 | | |
| 1453 | *trans*-Muurola-3,5-diene | tr | tr | S | RI, MS |
| 1456 | *allo*-Aromadendrene | tr | n.d. | S | RI, MS |
| 1461 | α-Humulene | 1.3±0.11 | 1.7±0.10 | S | RI, MS, CoI |
| 1463 | 9-*epi*-(*E*)-Caryophyllene | 0.5±0.04 | tr | S | RI, MS |
| 1466 | *cis*-Muurola-4(14),5-diene | tr | n.d. | S | RI, MS |
| 1476 | *trans*-Cadina-1(6),4-diene | tr | n.d. | S | RI, MS |
| 1478 | γ-Muurolene | 0.5±0.04 | 0.2±0.01 | S | RI, MS |
| 1480 | (*E*)-β-Ionone | tr | 0.1±0.01 | CR | RI, MS, CoI |
| 1482 | *ar*-Curcumene | n.d. | tr | S | RI, MS |
| 1485 | Germacrene D | 1.2±0.09 | 1.0±0.06 | S | RI, MS, CoI |
| 1489 | *cis*-β-Guaiene | 0.1±0.01 | tr | S | RI, MS |
| 1494 | β-Selinene | 0.1±0.01 | n.d. | S | RI, MS |
| 1496 | *trans*-β-Muurola-4(14),5-diene | 0.1±0.01 | tr | S | RI, MS |
| 1499 | *epi*-Cubebol | tr | 0.3±0.01 | S | RI, MS |
| 1500 | Bicyclogermacrene | 0.9±0.06 | n.d. | S | RI, MS |
| 1501 | α-Muurolene | 0.5±0.03 | tr | S | RI, MS |
| 1511 | β-Curcumene | n.d. | tr | S | RI, MS |
| 1512 | (*Z*)-γ-Bisabolene | n.d. | tr | S | RI, MS |
| 1512 | Germacrene A | 0.3±0.01 | n.d. | S | RI, MS |
| 1517 | γ-Cadinene | 0.6±0.04 | 0.3±0.01 | S | RI, MS |

| | | | | | |
|---|---|---|---|---|---|
| 1518 | Cubebol | n.d. | tr | S | RI, MS |
| 1519 | *endo*-1-Bourbonanol | n.d. | tr | S | RI, MS |
| 1522 | δ-Cadinene | 2.9±0.20 | 0.8±0.05 | S | RI, MS |
| 1524 | *trans*-Calamenene | tr | tr | S | RI, MS |
| 1527 | Zonarene | tr | n.d. | S | RI, MS |
| 1534 | 10-*epi*-Cubebol | 0.2±0.01 | tr | S | RI, MS |
| 1536 | *trans*-Cadina-1,4-diene | tr | n.d. | S | RI, MS |
| 1540 | α-Cadinene | 0.1±0.01 | tr | S | RI, MS |
| 1545 | α-Calacorene | tr | tr | S | RI, MS |
| 1550 | Hedycaryol | tr | tr | S | RI, MS |
| 1551 | Elemol | 0.1±0.01 | 0.1±0.01 | S | RI, MS |
| 1551 | *cis*-Muurol-5-en-4β-ol | tr | n.d. | S | RI, MS |
| 1555 | Isocaryophyllene oxide | n.d. | 0.2±0.01 | S | RI, MS |
| 1557 | *cis*-Muurol-5-en-4α-ol | tr | n.d. | S | RI, MS |
| 1564 | Germacrene B | 0.3±0.02 | tr | S | RI, MS |
| 1566 | β-Calacorene | tr | n.d. | S | RI, MS |
| 1574 | (3*E*,7*E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene | tr | tr | S | RI, MS |
| 1576 | Palustrol | 0.8±0.05 | n.d. | S | RI, MS |
| 1582 | Germacrene D-4-ol | 3.9±0.30 | 0.4±0.02 | S | RI, MS |
| 1588 | Caryophyllene oxide | 2.4±0.18 | 12.5±0.75 | S | RI, MS, CoI |

| | | | | | |
|---|---|---|---|---|---|
| 1600 | Viridiflorol | tr | n.d. | S | RI, MS |
| 1600 | Guaiol | 1.4±0.09 | 1.0±0.05 | S | RI, MS |
| 1602 | Humulene epoxide I | tr | tr | S | RI, MS |
| 1611 | Ledol | 2.6±0.22 | n.d. | S | RI, MS |
| 1616 | Humulene epoxide II | tr | 0.7±0.04 | S | RI, MS |
| 1619 | 1,10-di-*epi*-Cubenol | 0.8±0.05 | n.d. | S | RI, MS |
| | (6*E*,8*Z*)-4,6,8-Megastigmatrien- | | | CR | RI, MS |
| 1625 | 3-one | tr | tr | | |
| 1630 | Muurola-4,10(14)-dien-1-β-ol | 0.1±0.01 | 0.3±0.01 | S | RI, MS |
| 1631 | 1-*epi*-Cubenol | 0.2±0.01 | tr | S | RI, MS |
| | Caryophylla-4(12),8(13)-dien- | | | S | RI, MS |
| 1639 | 5α-ol | 0.5±0.03 | 0.8±0.05 | | |
| | Caryophylla-4(12),8(13)-dien- | | | S | RI, MS |
| 1642 | 5β-ol | 0.7±0.06 | 2.5±0.14 | | |
| 1646 | *epi*-α-Cadinol (*syn*. τ-cadinol) | 1.2±0.08 | 0.4±0.02 | S | RI, MS |
| 1648 | *epi*-α-Murrolol (*syn*. τ-muurolol) | 1.8±0.15 | tr | S | RI, MS |
| 1651 | α-Muurolol (*syn*. torreyol) | tr | tr | S | RI, MS |
| 1659 | α-Cadinol | 3.6±0.20 | 0.6±0.02 | S | RI, MS |
| 1669 | Bulnesol | 0.6±0.03 | 0.1±0.01 | S | RI, MS |
| | 14-Hydroxy-9-*epi*-(*E*)- | | | S | RI, MS |
| 1672 | caryophyllene | tr | 0.9±0.05 | | |
| 1689 | Germacra-4(15),5,10(14)-trien- | 0.7±0.04 | 0.4±0.02 | S | RI, MS |

| | | | | | |
|---|---|---|---|---|---|
| | 1α-ol | | | | |
| 1702 | Shyobunol | 9.7±0.66 | n.d. | S | RI, MS |
| 1710 | (2*E*,6*Z*)-Farnesal | 0.2±0.01 | tr | S | RI, MS |
| 1716 | Pentadecanal | tr | 0.3±0.01 | O | RI, MS |
| 1738 | (2*E*,6*E*)-Farnesal | tr | n.d. | S | RI, MS |
| 1745 | Mint sulfide | tr | n.d. | S | RI, MS |
| 1771 | Benzyl benzoate | tr | n.d. | O | RI, MS, CoI |
| 1788 | Phenanthrene | tr | n.d. | O | RI, MS, CoI |
| 1817 | Hexadecanal | tr | tr | O | RI, MS, CoI |
| 1823 | Isopropyl tetradecanoate | tr | n.d. | O | RI, MS, CoI |
| 1841 | Hexahydrofarnesyl acetone | 0.1±0.01 | 0.1±0.01 | CR | RI, MS |
| 1881 | 1-Hexadecanol | 0.1±0.01 | tr | O | RI, MS, CoI |
| 1909 | (5*E*,9*E*)-Farnesyl acetone | 0.6±0.04 | 0.6±0.03 | CR | RI, MS |
| 1925 | Methyl hexadecanoate | tr | tr | O | RI, MS, CoI |
| 1960 | (*Z*,*Z*)-Geranyl linalool | 0.5±0.03 | tr | D | RI, MS |
| 1961 | Hexadecanoic acid | tr | n.d. | O | RI, MS, CoI |
| 1986 | (*E*,*Z*)-Geranyl linalool | 0.2±0.01 | n.d. | D | RI, MS |
| 2015 | Unidentified 1[j] | 1.7±0.10 | n.d. | D | |
| 2025 | (*E*,*E*)-Geranyl linalool | 7.6±0.50 | 1.1±0.06 | D | RI, MS |
| 2084 | 1-Octadecanol | 0.2±0.02 | n.d. | O | RI, MS, CoI |
| 2097 | Methyl (9Z)-9-octadecenoate (*syn*. methyl oleate) | n.d. | tr | O | RI, MS, CoI |

| | | | | | |
|---|---|---|---|---|---|
| 2100 | Heneicosane | tr | tr | O | RI, MS, CoI |
| 2105 | Unidentified 2[k] | 0.9±0.06 | n.d. | D | |
| 2110 | (*E*)-Phytol | 4.7±0.28 | 0.5±0.02 | D | RI, MS |
| 2200 | Docosane | n.d. | tr | O | RI, MS, CoI |
| 2225 | Eicosanal | tr | tr | O | RI, MS |
| 2263 | 2-Methyldocosane | tr | n.d. | O | RI, MS |
| 2300 | Tricosane | 0.1±0.01 | tr | O | RI, MS, CoI |
| 2400 | Tetracosane | tr | tr | O | RI, MS, CoI |
| 2430 | Docosanal | tr | n.d. | O | RI, MS |
| 2463 | 2-Methyltetracosane | tr | n.d. | O | RI, MS |
| 2500 | Pentacosane | 0.2±0.01 | tr | O | RI, MS, CoI |
| 2572 | 3-Methylpentacosane | tr | n.d. | O | RI, MS |
| 2600 | Hexacosane | tr | tr | O | RI, MS, CoI |
| 2633 | Tetracosanal | tr | n.d. | O | RI, MS |
| 2662 | 2-Methylhexacosane | tr | n.d. | O | RI, MS |
| 2700 | Heptacosane | 0.2±0.01 | 0.1±0.01 | O | RI, MS, CoI |
| 2773 | 3-Methylheptacosane | tr | n.d. | O | RI, MS |
| 2800 | Octacosane | tr | tr | O | RI, MS, CoI |
| 2811 | (all *E*)-Squalene | tr | n.d. | O | RI, MS, CoI |
| 2863 | 2-Methyloctacosane | tr | n.d. | O | RI, MS |
| 2900 | Nonacosane | 0.2±0.01 | 0.2±0.01 | O | RI, MS, CoI |
| 2973 | 3-Methylnonacosane | tr | n.d. | O | RI, MS |

| | | | | | |
|---|---|---|---|---|---|
| 3000 | Triacontane | tr | tr | O | RI, MS, CoI |
| 3100 | Hentriacontane | 0.2±0.01 | tr | O | RI, MS, CoI |
| 3200 | Dotriacontane | tr | n.d. | O | RI, MS, CoI |
| 3300 | Tritriacontane | tr | n.d. | O | RI, MS, CoI |
| | Total | 88.7 (185)[l] | 93.9 (122) | | |
| | Terpenoids (T) | 81. 3 (95) | 80.4 (68) | | |
| | Hemiterpenoids (HT) | tr (2) | n.d. | | |
| | Monoterpenoids (MT) | 8.8 (20) | 24.6 (14) | | |
| | Hydrocarbons | 0.2 (6) | 0.7 (4) | | |
| | Oxygenated derivatives | 8.6 (14) | 23.9 (10) | | |
| | Acyclic | 8.8 (18) | 24.3 (10) | | |
| | Sesquiterpenoids (S) | 56.9 (67) | 54.2 (51) | | |
| | Hydrocarbons | 25.4 (37) | 33.0 (27) | | |
| | Oxygenated derivatives | 31.5 (30) | 21.2 (24) | | |
| | Diterpenoids (D) | 15.6 (6) | 1.6 (3) | | |
| | “Green leaf” volatiles (GL) | 4.0 (29) | 3.9 (15) | | |
| | Carotenoid derived compounds (CR) | 1.9 (14) | 8.1 (15) | | |
| | Others (O) | 1.5 (47) | 1.5 (24) | | |

a - Retention indices on a DB-5MS column calculated against a series of co-injected *n*-alkanes ($C_{12}$-$C_{34}$);

b - Essential oil obtained from fresh plant material (aerial parts) collected in the blooming stage;

c - Essential oil obtained from fresh plant material (aerial parts) collected in the fruit forming stage;

d - trace (<0.05%);

e - not detected;

f - RI - Constituent identified by retention index matching ; MS - Constituent identified by mass spectra comparison; CoI - The identity of the constituent was additionally confirmed by co-injection of an authentic sample;

g - standard error;

h - Correct (stereo)isomer unknown;

i - synonym;

j - MS(EI, 70 eV), *m/z* (rel. int, %): 270 (0.6), 245 (0.3), 218 (9.1), 203 (2.0), 189 (0.8), 175 (5.5), 161 (2.5), 147 (13.2), 134 (10.0), 121 (7.2), 107 (24.5), 93 (13.8), 81 (29.3), 71 (100), 69 (71.8), 55 (11.7), 43 (22.6), 41 (37.5);

k - MS(EI, 70 eV), *m/z* (rel. int, %): 288 (0.2), 270 (1.1), 255 (0.4), 245 (0.5), 227 (0.5), 218 (2.2), 203 (2.9), 190 (1.8), 175 (4.7), 159 (3.7), 147 (6.3), 133 (18.3), 123 (17.4), 107 (37.5), 94 (38.1), 81 (35.7), 71 (79.1), 69 (100), 55 (15.9), 43 (25.5), 41 (43.7);

l - Number in brackets represents the number of compounds belonging to that class.

**Table 2** Minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) of the *Lycopus europaeus* essential oil (mg/mL)

| **Bacterial strain** | | MIC (mg/mL) | MBC/MFC (mg/mL) | Amoxycillin (μg/mL) | Nystatin (μg/mL) |
|---|---|---|---|---|---|
| *Gram negative* | | | | | |
| *E. coli* | Isolate | 0.156 | 0.312 | 2.50 | NT[a] |
| *E. coli* | ATCC 25922 | 5.00 | 5.00 | 5.00 | NT |
| *E. coli* | ATCC 8739 | 5.00 | >10.0 | 5.00 | NT |
| *E. coli* | Torlak 95 | 5.00 | >10.0 | 5.00 | NT |
| *K. pneumoniae* | ATCC 10031 | 0.156 | 2.50 | 5.00 | NT |
| *K. pneumoniae* | Isolate | 5.00 | 10.0 | 5.00 | NT |
| *P. vulgaris* | ATCC 8427 | 5.00 | 10.0 | 5.00 | NT |
| *S. enterica* | ATCC 13076 | /[b] | / | 5.00 | NT |
| *Gram positive* | | | | | |
| *S. aureus* | ATCC 25923 | 5.00 | 5.00 | 2.50 | NT |
| *S. aureus* | Isolate | 2.50 | >10.0 | 2.50 | NT |
| *C. perfringens* | ATCC 19574 | 1.25 | 1.25 | 5.00 | NT |
| *C. sporogenes* | ATCC 19404 | 2.50 | 10.0 | 2.50 | NT |
| *S. lutea* | ATCC 9341 | 5.00 | >10.0 | 1.75 | NT |
| *M. flavus* | ATCC 10240 | / | / | 5.00 | NT |
| *B. subtilis* | ATCC 6633 | 5.00 | 5.00 | 1.75 | NT |
| ***Fungal strains*** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| *P. chrysogenum* | Isolate | 10.0 | 10.0 | NT | 0.0390 |
| *A. restrictus* | Isolate | 5.00 | 10.0 | NT | 0.0780 |
| *A. chrysogenum* | Isolate | 10.0 | 10.0 | NT | 0.0390 |
| *A. fumigates* | Isolate | 0.625 | 5.00 | NT | 0.0390 |
| *C. albicans* | ATCC 10231 | 10.0 | 10.0 | NT | 2.50 |
| *S. cerevisiae* | ATCC 9763 | 2.50 | 5.00 | NT | 1.75 |

a - not tested;

b - not active at the tested concentrations.

**Table 3** Fruit alkanes of *L. europaeus*

| RI[a] | Compound | Content/% | | Class |
|---|---|---|---|---|
| | | Sample C[b] | Sample D[c] | |
| 1200 | Dodecane[d] | tr[e] | tr | N |
| 1300 | Tridecane[d] | tr | 0.4±0.03[f] | N |
| 1391 | 1-Tetradecene | tr | tr | U |
| 1400 | Tetradecane[d] | tr | tr | N |
| 1592 | 1-Hexadecene | tr | tr | U |
| 1600 | Hexadecane[d] | tr | tr | N |
| 1700 | Heptadecane[d] | tr | tr | N |
| 1793 | 1-Octadecene | tr | tr | U |
| 1800 | Octadecane[d] | tr | tr | N |
| 1900 | Nonadecane[d] | tr | tr | N |
| 1993 | 1-Eicosene | tr | n.d.[g] | U |
| 2000 | Eicosane[d] | tr | tr | N |
| 2100 | Heneicosane[d] | tr | tr | N |
| 2200 | Docosane[d] | tr | n.d. | N |
| 2293 | 1-Tricosene | n.d. | tr | U |
| 2300 | Tricosane[d] | 0.5±0.04 | 1.2±0.08 | N |
| 2393 | 1-Tetracosene | n.d. | tr | U |
| 2400 | Tetracosane[d] | tr | tr | N |
| 2461 | 2-Methyltetracosane | tr | tr | I |

| | | | | |
|---|---|---|---|---|
| 2500 | Pentacosane[d] | 1.7±0.12 | 3.9±0.24 | N |
| 2561 | 2-Methylpentacosane | tr | tr | I |
| 2570 | 3-Methylpentacosane | 0.5±0.04 | 1.0±0.07 | A |
| 2600 | Hexacosane[d] | 0.8±0.06 | 1.5±0.10 | N |
| 2660 | 2-Methylhexacosane | 0.7±0.06 | 1.5±0.11 | I |
| 2670 | 3-Methylhexacosane | tr | tr | A |
| 2700 | Heptacosane[d] | 10.6±0.59 | 14.3±0.78 | N |
| 2761 | 2-Methylheptacosane | tr | tr | I |
| 2771 | 3-Methylheptacosane | 2.5±0.15 | 3.3±0.19 | A |
| 2800 | Octacosane[d] | 3.9±0.23 | 4.2±0.24 | N |
| 2808 | Squalene[d] | 0.8±0.06 | 2.1±0.12 | O |
| 2861 | 2-Methyloctacosane | 2.0±0.12 | 2.4±0.14 | I |
| 2871 | 3-Methyloctacosane | tr | tr | A |
| 2900 | Nonacosane[d] | 30.9±1.50 | 31.1±1.51 | N |
| 2960 | 2-Methylnonacosane | tr | tr | I |
| 2971 | 3-Methylnonacosane | 2.0±0.12 | 2.1±0.13 | A |
| 3000 | Triacontane[d] | 4.2±0.24 | 3.3±0.19 | N |
| 3061 | 2-Methyltriacontane | 1.9±0.11 | 2.0±0.13 | I |
| 3100 | Hentriacontane[d] | 25.1±1.31 | 17.1±0.91 | N |
| 3161 | 2-Methylhentriacontane | tr | tr | I |
| 3171 | 3-Methylhentriacontane | 1.4±0.10 | 1.2±0.08 | A |
| 3200 | Dotriacontane[d] | 2.1±0.14 | 1.3±0.09 | N |

| | | | | |
|---|---|---|---|---|
| 3261 | 2-Methyldotriacontane | 1.1±0.08 | 1.2±0.08 | I |
| 3271 | 3-Methyldotriacontane | tr | n.d. | A |
| 3300 | Tritriacontane[d] | 5.2±0.31 | 3.9±0.24 | N |
| 3371 | 3-Methyltritriacontane | 0.7±0.05 | tr | A |
| | Total | 98.6 (43)[h] | 99.0 (43) | |
| | Alkanes | 97.8 (38) | 97.0 (37) | |
| | *n*-Alkanes (N) | 85 (21) | 82.3 (21) | |
| | *Iso*-alkanes (I) | 5.7 (9) | 7.1 (9) | |
| | *Anteiso*-alkanes (A) | 7.1 (8) | 7.6 (7) | |
| | Alkenes (U) | tr (4) | tr (5) | |
| | Others (O) | 0.8 (1) | 2 (1) | |

a - Retention indices on a DB-5MS column calculated against a series of co-injected *n*-alkanes ($C_{12}$-$C_{34}$);

b - The dichloromethane washings of intact unripe *L. europaeus* fruits;

c - The dichloromethane washings of intact ripe *L. europaeus* fruits;

d - The identity of the constituent, based on mass spectral comparison and RI matching, was additionally confirmed by co-injection of an authentic sample;

e - trace (<0.05%);

f - standard error;

g - not detected;

h - Number in brackets represents the number of compounds belonging to that class.

**Table 4** Fatty acid composition of the fruits of *L. europaeus* (methyl esters)

| RI[a] | Compound | Content/% | | Class | Method of identification |
|---|---|---|---|---|---|
| | | Sample C[b] | Sample D[c] | | |
| 1524 | Methyl dodecanoate (methyl laurate) | tr[d] | n.d.[e] | NM | RI, MS, CoI[f] |
| 1724 | Methyl tetradecanoate (methyl myristate) | tr | tr | NM | RI, MS, CoI |
| 1795 | Methyl 12-methyltetradecanoate | tr | n.d. | AM | RI, MS |
| 1824 | Methyl pentadecanoate | tr | tr | NM | RI, MS |
| 1886 | Methyl 14-methylpentadecanoate | tr | n.d. | IM | RI, MS |
| 1902 | Methyl (9*Z*)-9-hexadecenoate (methyl palmitoleate) | tr | tr | UM | RI, MS |
| 1924 | Methyl hexadecanoate (methyl palmitate) | 5.6±0.30[g] | 4.2±0.24 | NM | RI, MS, CoI |
| 1995 | Methyl 14-methylhexadecanoate | tr | n.d. | AM | RI, MS |
| 1999 | Methyl (9*E*)-9-heptadecenoate | tr | tr | UM | RI, MS |
| 2024 | Methyl heptadecanoate | 0.2±0.02 | tr | NM | RI, MS |
| 2089 | Methyl (9*Z*,12*Z*)-9,12-octadecadienoate (methyl linoleate) + methyl (*Z*)-9-octadecenoate (methyl oleate) | 38.1±1.90 | 31.5±1.593 | UM | RI, MS, CoI |
| 2098 | Methyl (9*Z*,12*Z*,15*Z*)-9,12,15- | 47.2±2.28 | 58.5±2.75 | UM | RI, MS, CoI |

| | | | | | |
|---|---|---|---|---|---|
| | octadecatrienoate (methyl linolenate) | | | | |
| 2102 | Methyl (*E*)-9-octadecenoate (methyl elaidate) | n.d. | 1.4±0.10 | UM | RI, MS |
| 2125 | Methyl octadecanoate (methyl stearate) | 3.3±0.19 | 2.4±0.15 | NM | RI, MS, CoI |
| 2196 | Methyl 16-methyloctadecanoate | tr | tr | AM | RI, MS |
| 2200 | Methyl (*Z*)-9-nonadecenoate | tr | n.d. | UM | RI, MS |
| 2224 | Methyl nonadecanoate | tr | tr | NM | RI, MS |
| 2288 | Methyl 18-methylnonadecanoate[h] | 0.1±0.01 | tr | IM | RI, MS |
| 2292 | Methyl (11*Z*,14*Z*)-11,14-eicosadienoate | n.d. | tr | UM | RI, MS |
| 2298 | Methyl (*Z*)-9-eicosenoate | 0.5±0.04 | 0.3±0.03 | UM | RI, MS |
| 2326 | Methyl eicosanoate (Arachidic acid methyl ester) | 0.8±0.06 | 0.4±0.03 | NM | RI, MS |
| 2398 | Methyl 18-methyleicosanoate[i] | tr | tr | AM | RI, MS |
| 2426 | Methyl heneicosanoate | tr | n.d. | NM | RI, MS |
| 2489 | Methyl 20-methylheneicosanoate[j] | tr | tr | IM | RI, MS |
| 2526 | Methyl docosanoate (methyl behenate) | 0.3±0.02 | 0.2±0.02 | NM | RI, MS |
| 2598 | Methyl 20-methyldocosanoate[k] | tr | n.d. | AM | RI, MS |
| 2627 | Methyl tricosanoate | tr | tr | NM | RI, MS |
| 2689 | Methyl 22-methyltricosanoate[l] | tr | tr | IM | RI, MS |

| | | | | | |
|---|---|---|---|---|---|
| 2728 | Methyl tetracosanoate (methyl lignocerate) | 0.1±0.01 | tr | NM | RI, MS |
| 2928 | Methyl hexacosanoate | tr | n.d. | NM | RI, MS |
| 3128 | Methyl octacosanoate | tr | n.d. | NM | RI, MS |
| | Total | 96.2 (30)[m] | 98.9 (23) | | |
| | Saturated fatty acid methyl esters | 10.4 (23) | 7.2 (15) | | |
| | Normal (NM) | 10.3 (14) | 7.2 (10) | | |
| | *Anteiso* (AM) | tr (5) | tr (2) | | |
| | *Iso* (IM) | 0.1 (4) | tr (3) | | |
| | Unsaturated fatty acid methyl esters (UM) | 85.8 (7) | 91.7 (8) | | |

a - Retention indices on a DB-5MS column calculated against a series of co-injected *n*-alkanes ($C_{12}$-$C_{34}$);

b - Methyl esters obtained from alkaline transesterification (MeONa/MeOH) of the acylglyceride fraction of the unripe fruit dichloromethane extract;

c - Methyl esters obtained from alkaline transesterification (MeONa/MeOH) of the acylglyceride fraction of the ripe fruit dichloromethane extract;

d - trace (<0.05%);

e - not detected;

f - RI - Constituent identified by retention index matching (the retention indices, used for RI matching, of methyl esters of branched *iso*- and *anteiso*-fatty acids, since unavailable from the literature, were calculated using the following simple expression RI (iso- or anteiso-RCOOMe) = RI(*n*-RCOOMe) - ΔRI, where ΔRI was 38 and 29 for *iso*- and *anteiso*- isomers, respectively; MS - Constituent identified by mass spectra comparison; CoI - The identity of the constituent was additionally confirmed by co-injection of an authentic sample;

g - standard error;

h - MS(EI, 70 eV), *m/z* (rel. int, %): 326 (15.2), 283 (7.9), 199 (7.3), 143 (17.9), 129 (11.1), 111 (12), 97 (18.5), 87 (74), 74 (100), 55 (44.1), 44 (46.1);

i - MS(EI, 70 eV), *m/z* (rel. int, %): 340 (15.8), 241 (15.3), 191 (13.6), 143 (22.1), 129 (14.3), 97 (19.8), 87 (70.8), 74 (100), 57 (62.3), 44 (69);

j - MS(EI, 70 eV), *m/z* (rel. int, %): 354 (16.3), 143 (18.2), 133 (17), 95 (21.8), 87 (78.9), 74 (99.8), 69.1 (29.9), 55 (54), 44 (100);

k - MS(EI, 70 eV), *m/z* (rel. int, %): 143 (17.2), 133 (27.3), 96 (32.4), 87 (36.9), 83 (39.4), 74 (49.7), 55 (30.6), 44 (100);

l - MS(EI, 70 eV), *m/z* (rel. int, %): 191 (33.2), 133 (28.7), 96 (31.8), 91 (38.7), 87 (20.6), 74 (46.5), 55 (22.6), 44 (100).

m - Number in brackets represents the number of compounds belonging to that class.

**Table 5** Fatty acid composition of the fruits of *L. europaeus* (*tert*-butyl esters)

| RI[a] | ΔRI[b] | Compound | Sample D[c] (content/%) | Class |
|---|---|---|---|---|
| 1856 | 132 | *tert*-Butyl tetradecanoate[d,e] | tr[f] | NB |
| 1900 | 130 | *tert*-Butyl (9*Z*)-9-hexadecenoate[g] | tr | UB |
| 2054 | 130 | *tert*-Butyl hexadecanoate (*tert*-butyl palmitate)[d] | 3.5±0.23[h] | NB |
| 2125 | 130 | *tert*-Butyl 14-methylhexadecanoate[i] | tr | AB |
| 2154 | 129 | *tert*-Butyl heptadecanoate[j] | tr | NB |
| 2219 | 130 | *tert*-Butyl (9*Z*,12*Z*)-9,12-octadecadienoate (*tert*-butyl linoleate)[d,k] | 28.7±1.42 | UB |
| 2223 | 130 | *tert*-Butyl (9*Z*)-9-octadecenoate (*tert*-butyl oleate)[d,l] | 1.7±0.12 | UB |
| 2228 | 130 | *tert*-Butyl (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienoate (*tert*-butyl linolenate)[d,m] | 49.6±2.38 | UB |
| 2232 | 130 | *tert*-Butyl (9*E*)-9-hexadecenoate (*tert*-butyl elaidate)[n] | 0.4±0.03 | UB |
| 2254 | 129 | *tert*-Butyl octadecanoate (*tert*-butyl stearate)[d,o] | 2.3±0.14 | NB |
| 2325 | 129 | *tert*-Butyl 16-methyloctadecanoate[p] | tr | AB |
| 2353 | 129 | *tert*-Butyl nonadecanoate[q] | tr | NB |
| 2416 | 128 | *tert*-Butyl 18-methylnonadecanoate[r] | tr | IB |
| 2426 | 128 | *tert*-Butyl (9*Z*)-9-eicosenaote[s] | 0.2±0.02 | UB |
| 2453 | 127 | *tert*-Butyl eicosanoate[t] | 0.3±0.02 | NB |
| 2525 | 127 | *tert*-Butyl 18-methyleicosanoate[u] | tr | AB |
| 2553 | 127 | *tert*-Butyl heneicosanoate[v] | tr | NB |
| 2615 | 126 | *tert*-Butyl 20-methylheneicosanoate[w] | tr | IB |

| | | | | |
|---|---|---|---|---|
| 2652 | 126 | *tert*-Butyl docosanoate[x] | 0.2±0.02 | NB |
| 2725 | 127 | *tert*-Butyl 20-methyldocosanoate[y] | tr | AB |
| 2853 | 125 | *tert*-Butyl tetradecanoate[z] | tr | NB |

| | |
|---|---|
| Total | 86.9 (21)[aa] |
| Saturated fatty acid *t*-butyl esters | 6.3 (15) |
| Normal (NB) | 6.3 (9) |
| *Anteiso* (AB) | tr (4) |
| *Iso* (IB) | tr (2) |
| Unsaturated fatty acid *tert*-butyl esters (UB) | 80.6 (6) |

a - Retention indices on a DB-5MS column calculated against a series of co-injected *n*-alkanes ($C_{12}$-$C_{34}$);

b - The increment in the values of retention indices of *tert*-butyl compared to the corresponding methyl esters (ΔRI = RI(RCOO$^t$Bu) – RI(RCOOMe));

c - *tert*-Butyl esters obtained from alkaline transesterification ($^t$BuOK/$^t$BuOH) of the acylglyceride fraction of the ripe fruit dichloromethane extract;

d - The identity of the constituent, based on mass spectral comparison and RI matching, was additionally confirmed by co-injection of an authentic sample;

e - MS(EI, 70 eV), *m/z* (rel. int, %): 229 (25.2), 211 (7.2), 191 (9.8), 96 (13.7), 57 (100), 56 (34.2), 44 (56.6), 41 (33.5);

f - trace (<0.05%);

g - MS(EI, 70 eV), *m/z* (rel. int, %): 237 (15.9), 191 (8.8), 151 (5.2), 133 (7.3), 111 (8.6), 97 (18), 96 (18.1), 83 (18.1), 73 (9.8), 69 (23), 57 (100), 56 (19.1), 44 (45.2), 41 (46.1);

h - standard error;

i - MS(EI, 70 eV), *m/z* (rel. int, %): 271 (24.9), 269 (2.3), 253 (2.6), 191 (4.0), 177 (3.1), 129 (4.2), 109 (3.5), 96 (9.9), 83 (7.3), 69 (11.5), 57 (100), 56 (32.8), 41 (31);

j - MS(EI, 70 eV), *m/z* (rel. int, %): 271 (16.6), 253 (4), 222 (4.7), 191 (2.6), 163 (3.3), 149 (8.9), 135 (10.8), 121 (16.1), 108 (33.2), 95 (65.7), 81 (65.8), 79 (100), 67 (89.5), 57 (64.5), 55 (78.8), 41 (76.6);

k - MS(EI, 70 eV), *m/z* (rel. int, %): 336 (0.1), 280 (26.3), 279 (18.1), 263 (17.8), 237 (0.5), 223 (1.2), 209 (1.6), 195 (1.5), 182 (2.2), 163 (1.9), 149 (3.6), 137 (7.6), 123 (12.4), 109 (17.4), 95 (35.5), 81 (43.9), 67 (50), 57 (100), 41 (34.2);

l - MS(EI, 70 eV), *m/z* (rel. int, %): 338 (0.1), 282 (2.9), 280 (3.6), 265 (14.4), 235 (1.1), 222 (3.3), 207 (3.2), 193 (1.4), 179 (2), 165 (2.7), 135 (4.2), 125 (4.9), 111 (10.3), 95 (19.9), 83 (22.1), 69 (28.5), 57 (100), 41 (36.6);

m - MS(EI, 70 eV), *m/z* (rel. int, %): 334 (0.1), 278 (14.9), 277 (15.6), 261 (10.8), 235 (1.7), 222 (6), 221 (5), 209 (3), 193 (1.1), 173 (1.9), 163 (2.5), 149 (7.8), 135 (13.2), 121 (15.5), 108 (22.2), 95 (31.1), 79 (54.2), 67 (34.7), 57 (100), 41 (35.8);

n - MS(EI, 70 eV), *m/z* (rel. int, %): 338 (0.1), 282 (2.9), 280 (3.6), 265 (14.4), 235 (1.1), 222 (3.3), 207 (3.2), 193 (1.4), 179 (2), 165 (2.7), 135 (4.2), 125 (4.9), 111 (10.3), 95 (19.9), 83 (22.1), 69 (28.5), 57 (100), 41 (36.6);

o - MS(EI, 70 eV), *m/z* (rel. int, %): 285 (33), 284 (12.1), 267 (7.5), 241 (1.6), 222 (0.4), 185 (1.7), 171 (0.7), 167 (0.3), 143 (0.6), 129 (2.8), 111 (2.3), 97 (4.4), 85 (5), 71 (7.3), 57 (100), 41 (18.7);

p - MS(EI, 70 eV), *m/z* (rel. int, %): 299 (16.5), 281 (6.2), 277 (6.2), 241 (1.6), 191 (2.9), 185 (1.7), 171 (1.7), 163 (1.9), 149 (3.3), 133 (4.9), 121 (5.2), 108 (5.8), 95 (16.6), 79 (22.7), 67 (21), 57 (100), 41 (32.3);

q - MS(EI, 70 eV), *m/z* (rel. int, %): 299 (18.9), 281 (15.1), 261 (6.6), 191 (11.6), 149 (11.3), 133 (13.3), 121 (19.8), 108 (25.1), 95 (58.8), 79 (78.2), 67 (78.4), 57 (100), 44 (76.3), 41 (75.9);

r - MS(EI, 70 eV), *m/z* (rel. int, %): 313 (27.1), 277 (4.3), 191 (4.2), 177 (2.4), 163 (2), 149 (2.4), 133 (4.5), 119 (3.6), 109 (6.4), 95 (13), 79 (13.8), 67 (16.5), 57 (100), 43 (24.7);

s - MS(EI, 70 eV), *m/z* (rel. int, %): 310 (2.3), 293 (12.5), 273 (1), 249 (1.7), 235 (1.6), 221 (1.4), 193 (1.7), 179 (1.6), 165 (1.9), 151 (2.3), 135 (2.6), 125 (5.3), 111 (10.7), 97 (18.2), 83 (20.3), 69 (25.5), 57 (100), 41 (30.8);

t - MS(EI, 70 eV), *m/z* (rel. int, %): 313 (31.3), 295 (5.6), 269 (1), 241 (0.4), 227 (0.5), 213 (0.9), 185 (1.1), 171 (0.8), 157 (0.7), 129 (3.1), 111 (2.3), 97 (5.1), 85 (5.4), 71 (8.2), 57 (100), 41 (19.2);

u - MS(EI, 70 eV), *m/z* (rel. int, %): 327 (27.2), 191 (8), 177 (3.2), 167 (5.2), 133 (7.5), 111 (5.3), 96 (13.2), 83 (12.2), 67 (19.6), 57 (100), 43 (31.8);

v - MS(EI, 70 eV), *m/z* (rel. int, %): 327 (24.7), 191 (23.2), 177 (8.6), 133 (23.8), 117 (12.7), 96 (44.2), 79 (38.3), 67 (39.7), 57 (98.6), 44 (100);

w - MS(EI, 70 eV), *m/z* (rel. int, %): 341 (20.1), 191 (18.6), 147 (8.6), 133 (14.6), 117 (10.6), 96 (30), 79 (27.8), 67 (24.8), 57 (100), 44 (73.5);

x - MS(EI, 70 eV), *m/z* (rel. int, %): 341 (27.3), 323 (5.2), 203 (1.3), 191 (3.6), 177 (2), 163 (1.5), 149 (2.4), 131 (44.4), 117 (11), 101 (12), 95 (11.7), 79 (15.3), 67 (14.3), 57 (100), 41 (28.3);

y - MS(EI, 70 eV), *m/z* (rel. int, %): 355 (20.3), 191 (32.8), 177 (14.7), 147 (12.4), 133 (31.8), 117 (31.3), 105 (16.1), 96 (49), 79 (43.9), 67 (38.9), 57 (77.9), 44 (100);

z - MS(EI, 70 eV), *m/z* (rel. int, %): 369 (16.1), 191 (27.4), 177 (9.8), 147 (11.9), 133 (30.2), 117 (15.5), 96 (50.9), 79 (37.8), 67 (47), 57 (81.8), 44 (100);

aa - Number in brackets represents the number of compounds belonging to that class.

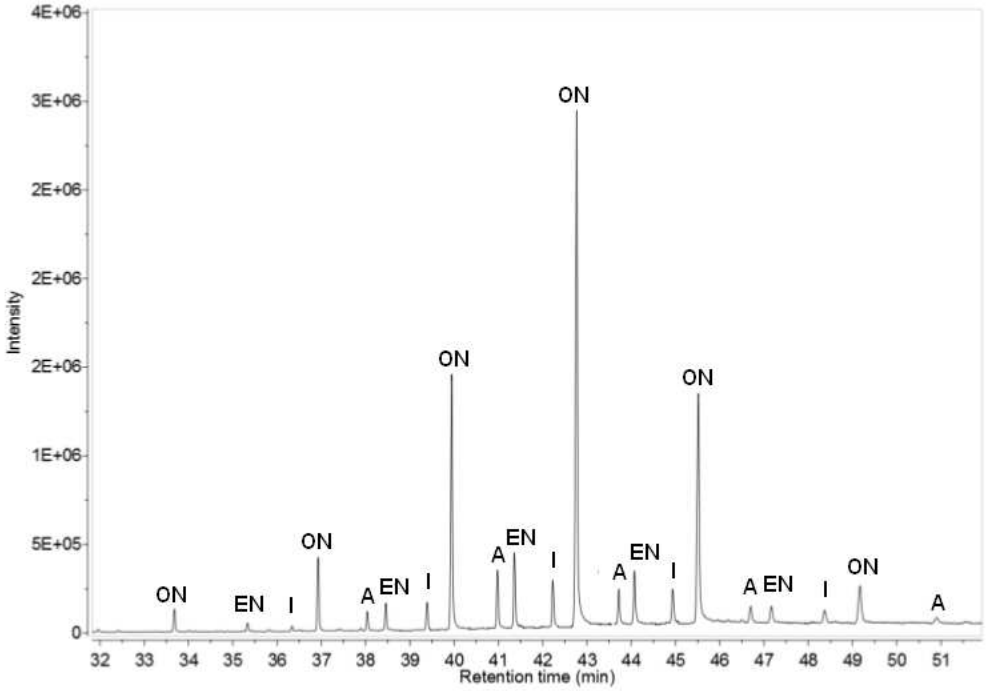


**Fig. 1** The partial GC trace of the alkane fraction of the ripe *L. europaeus* fruits; even-numbered *n*-alkanes (EN), odd-numbered *n*-alkanes (ON), *iso* (I) and *anteiso*-alkanes (A).

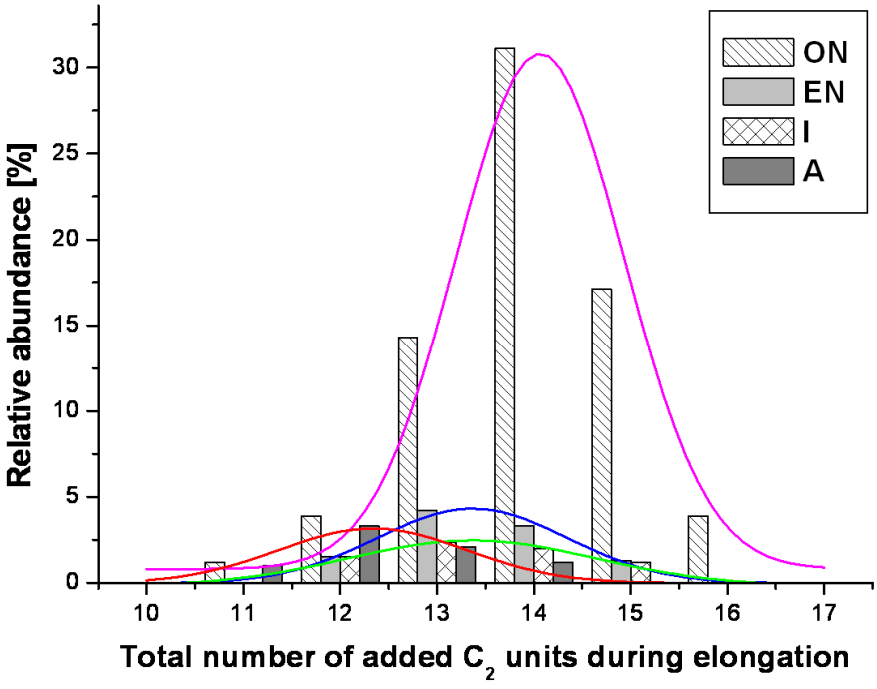


**Fig. 2** The Gaussian-like distributions of odd-numbered *n*-alkanes (ON, magenta curve), even-numbered *n*-alkanes (EN, blue curve), *iso*- (I, green curve) and *anteiso*-alkanes (A, blue curve) of the epicuticular wax fraction of ripe *L. europaeus* fruits expressed as the frequency (relative abundance) of the addition of a specific number of $C_2$ units during elongation.

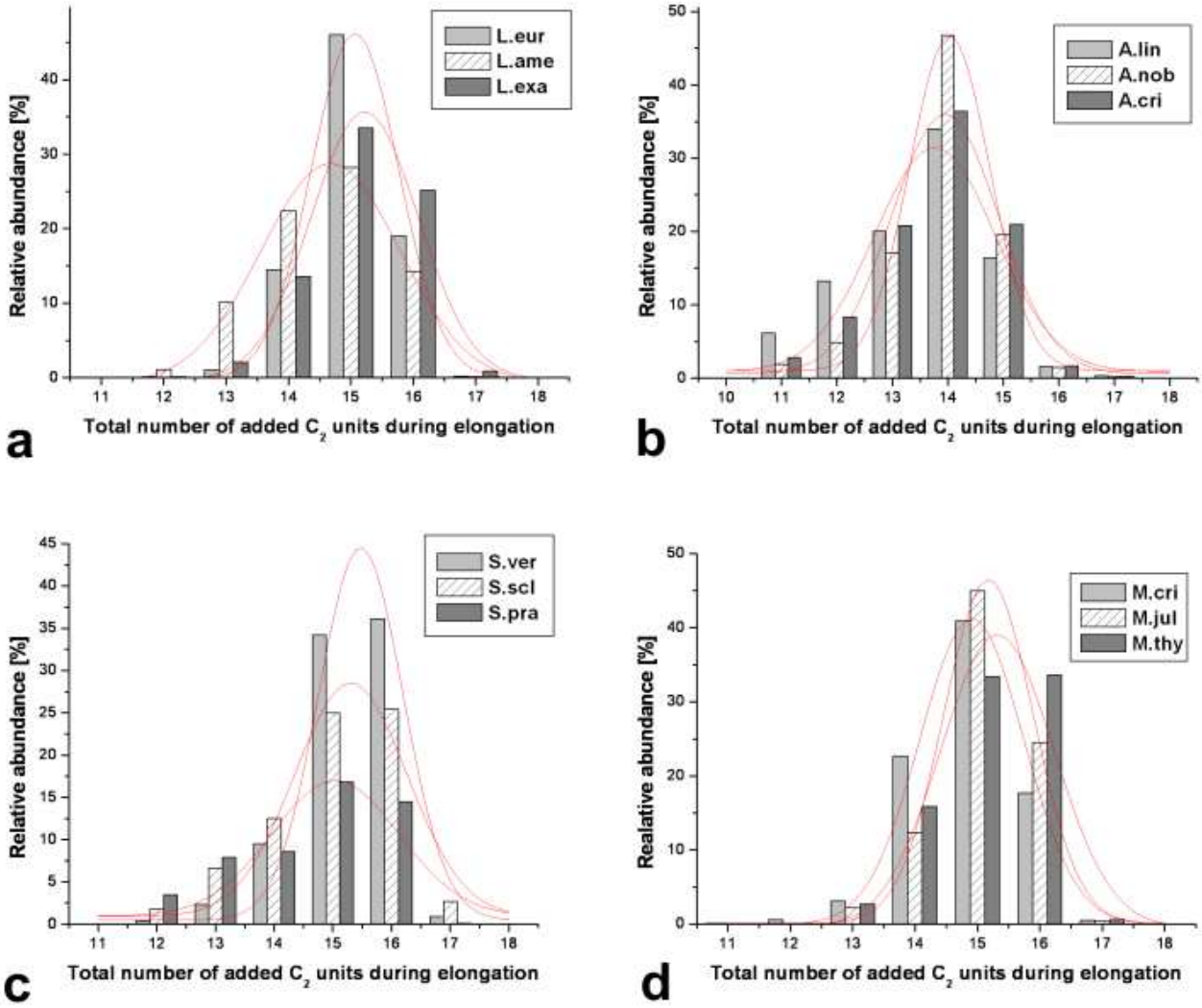


**Fig. 3** The Gaussian-like distributions of various plant wax odd-numbered *n*-alkanes expressed as the frequency (relative abundance) of the addition of a specific number of $C_2$ units during elongation: **a** - *Lycopus europaeus* (L. eur), *L. americanus* (L. ame) and *L. exaltatus* (L. exa); **b** - *Achillea lingulata* (A. lin), *A. nobilis* (A. nob) and *A. crithmifolia* (A. cri); **c** - *Salvia verticillata* (S. ver), *S. sclarea* (S. scl) and *S. pratensis* (S. pra) **d** -. *Micromeria cristata* (M. cri), *M. juliana* (M. jul) and *M. thymifolia* (M. thy).

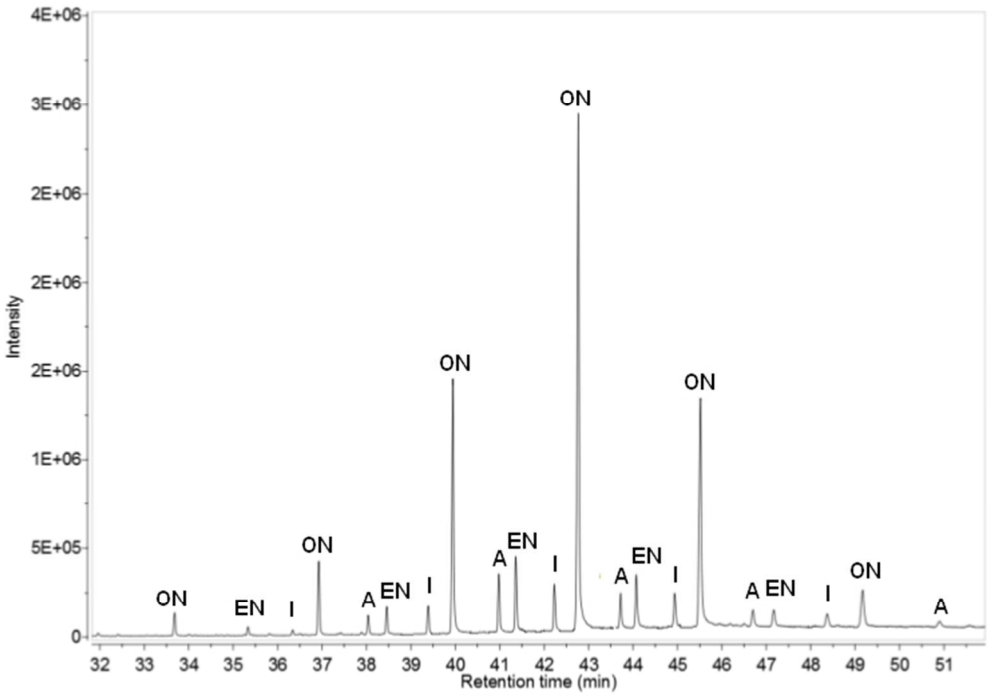


Fig. 1 The partial GC trace of the alkane fraction of the ripe L. europaeus fruits; even-numbered n-alkanes (EN), odd-numbered n-alkanes (ON), iso (I) and anteiso-alkanes (A).
264x184mm (72 x 72 DPI)

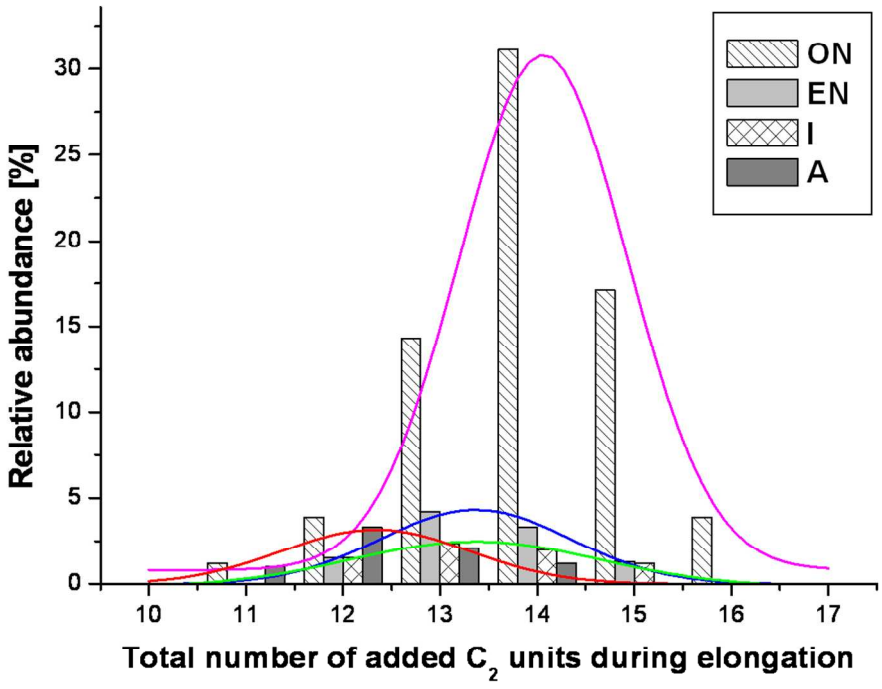


Fig. 2 The Gaussian-like distributions of odd-numbered n-alkanes (ON, magenta curve), even-numbered n-alkanes (EN, blue curve), iso- (I, green curve) and anteiso-alkanes (A, blue curve) of the epicuticular wax fraction of ripe L. europaeus fruits expressed as the frequency (relative abundance) of the addition of a specific number of C2 units during elongation.
104x84mm (300 x 300 DPI)

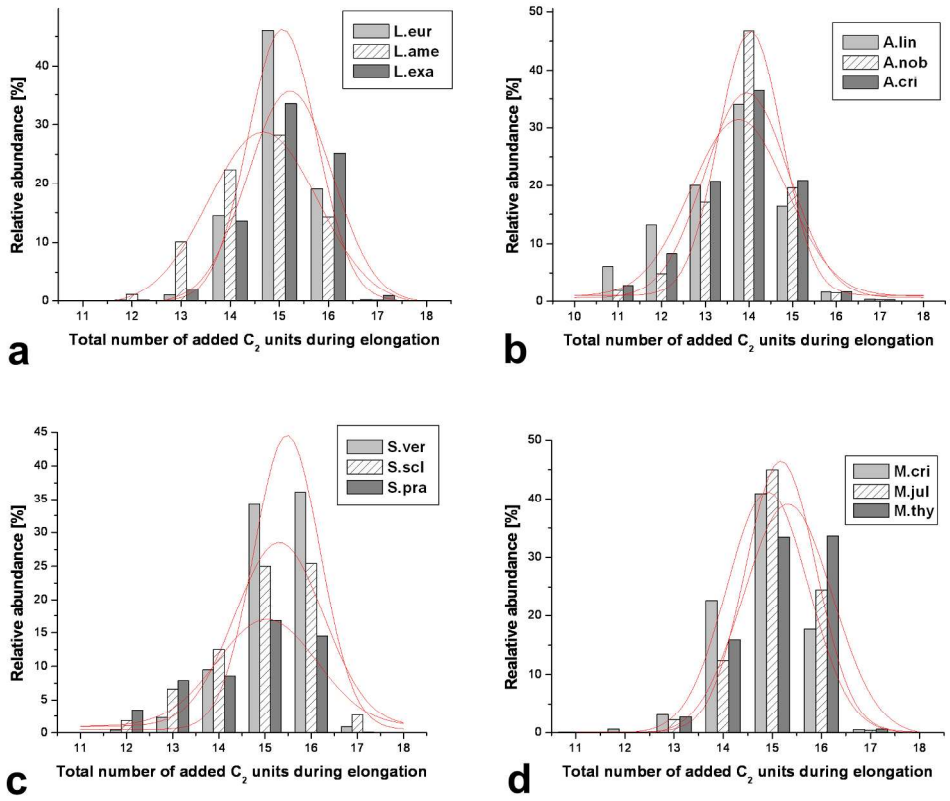


Fig. 3 The Gaussian-like distributions of various plant wax odd-numbered n-alkanes expressed as the frequency (relative abundance) of the addition of a specific number of C2 units during elongation: a - Lycopus europaeus (L. eur), L. americanus (L. ame) and L. exaltatus (L. exa); b - Achillea lingulata (A. lin), A. nobilis (A. nob) and A. crithmifolia (A. cri); c - Salvia verticillata (S. ver), S. sclarea (S. scl) and S. pratensis (S. pra) d -. Micromeria cristata (M. cri), M. juliana (M. jul) and M. thymifolia (M. thy).
645x545mm (96 x 96 DPI)