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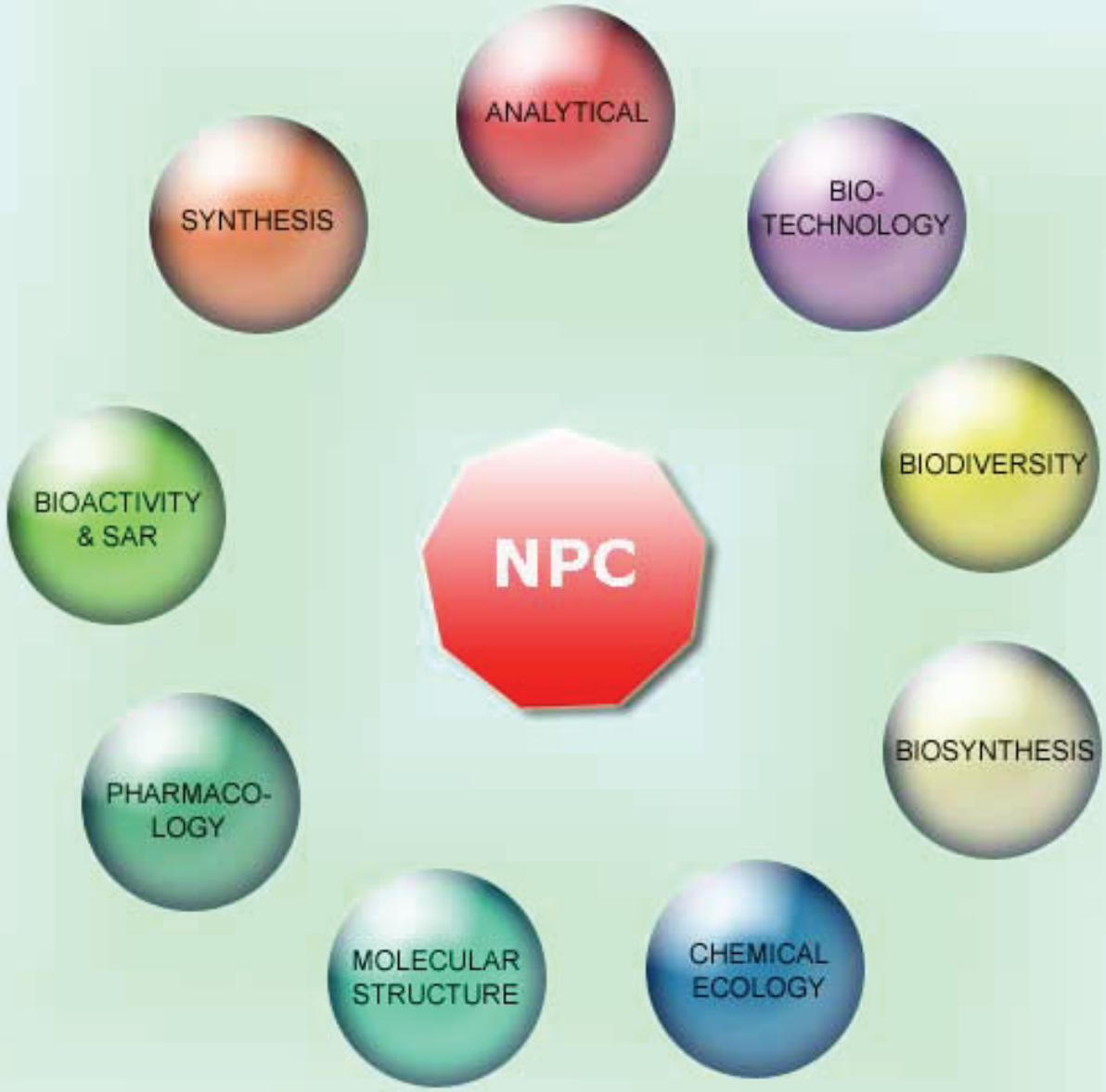

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Quantitative and Physical Evaluation of Patchouli Essential Oils Obtained from Different Sources of *Pogostemon cablin*

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Patchouli essential oil can be obtained from fresh, dried and fermented plant material. It is a highly valuable product in the fragrance industry and its quality changes depending upon raw material age and oil storage. In this work, patchouli essential oils derived from different treatments have been subjected to GC-FID quantitative analysis using an internal standard (ISTD) method with response factors (RF). Samples were obtained from i) fresh plants; ii) hydrodistillation of one year mature and fermented plants; iii) hydrodistillation of one year mature plants; iv) commercial products from Indonesia and Malaysia. Linear Retention Indices (LRI) for both polar and non-polar GC-MS analyses were also measured as a tool for qualitative analysis towards a homologous series of C_7-C_{30} *n*-alkanes. The results obtained confirmed that, in all samples, patchouli alcohol was the main volatile constituent, with higher amount in lab-scale produced oils, compared with commercial samples. Other major compounds, in lab oils and commercial samples respectively, were: δ-guaiene, α-guaiene, pogostol, seychellene and α-patchoulene. Another 36 compounds were also found.

Keywords: Quantitative analysis, Linear retention index (LRI), GC-FID response factor, GC-MS, Patchouli, *Pogostemon cablin*.

Quantification of constituents in essential oil research is a very important tool in order to define a correct chemical amount in the samples. The general procedure adopted when analysing an essential oil by GC techniques is to report the raw area percentage (FID % or MS %). Another common practice is to apply a " semi-quantitation", which means that peak areas are divided by an internal standard peak area. Basically, both procedures assume that the detector response is equal to unity. This assumption is approximately correct when target analytes are hydrocarbons, due to a linear response of the FID detector to the carbon number. On the other hand, when different moieties are present in the molecules to be quantified, such as keto-groups or carboxylic functions, FID response undergoes a shift that needs to be "corrected" through the measurement of response factors, for single compounds [1a-1c]. Quantitative GC analytical methods based on response factors (RF) and internal standard (ISTD) are still lacking for patchouli essential oil as compared with several reports present in the literature based on the sole GC-MS qualitative fingerprinting. Patchouli essential oil (PEO) is dominated by a large amount of oxygenated sesquiterpenes (such as pogostol and patchoulol). The presence of a hydroxyl group in the structure of patchouli alcohol (PA) affects the FID response, for the reasons briefly explained here. The exact mechanism of flame ionization is still not completely understood. It seems likely that upon burning all carbon atoms are converted into methane [2]. Therefore, hydrocarbons respond always in the same manner. When heteroatoms, like oxygen, are present in the organic solute, it becomes necessary to calibrate this fluctuation by using response factors, as previously reported by Costa *et al.* for other plants [1c].

Recently, some analytical procedures have been applied to *Pogostemon* sp. analysis by means of GC-MS coupled with integrated chemometric methods [3]; application of PA external standard calibration [4]; a comparison between supercritical fluid extraction and steam distillation [5]; a fast analysis with a short capillary column [6]; a simple wavelength detection of patchoulol by means of UV-spectrophotometer [7]; a GC-MS analysis with different ionization techniques [8]; HPLC with UV detection at 220nm for mono and sesquiterpenes [9], and a 2D fingerprinting analysis [10]. *P. cablin* essential oil was recently reported to contain 65.8% of sesquiterpene hydrocarbons, followed by oxygenated sesquiterpenes (27.0%), dominated by patchouli alcohol, α-bulnesene (also known as δ-guaiene), α-guaiene, γ-patchoulene and β-patchoulene [11a]. Other *Pogostemon* species have been recently investigated i.e. *P. heyneanus*, whose predominant constituent was acetophenone (51.0%) followed by patchouli alcohol (14.0%) [11b]; and *P. benghalensis*, which showed a completely different composition compared with *P. cablin* (β-caryophyllene, 12.5-15.2%; β-bisabolene, 8-18%; elemol, 4-20%) [11c]. The woody balsamic notes and the strong fixative properties of patchouli oil made it a basic ingredient for certain high value perfumes, due to the presence of patchouli alcohol (PA) and norpatchoulenol (NP) [12a, 12b]. These two compounds have been considered in a study on contact dermatitis as potential allergens [13]. In addition, PEO is not only a fixative material in perfumery, but also an insecticide, flavouring agent and clothing odorant. The traditional uses of *P. cablin* are based on some analgesic, anti-inflammatory and

Table 1: Sample description and physical evaluation of patchouli oil samples analyzed.

No. of Samples	I.D.	Source	Moisture content (%)	Recovery %	Color	Solubility
2	A	Fresh plants [1]	73-75	0.38	Weakly light green and very clear	*n*-Hexane
2	B	Hydrodistillation of the pre-treated material (heating and fermenting in water for 7 days) of 1 year mature and dried plants	10-12	0.64	Light yellowish, clear	*n*-Hexane
2	C (Control)	Hydrodistillation of 1 year mature and dried plants	10-12	1.12	Light yellowish, clear	*n*-Hexane
1	D	Commercial PEO[2, 3], Indonesia	unknown	No data received from supplier	Brownish and slightly cloudy	Dichloromethane. Not soluble in *n*-hexane, turning cloudy
1	E	Commercial PEO[4,5], Malacca, Peninsular Malaysia	unknown	No data received from supplier	Golden yellowish and clear	Dichloromethane. Not soluble in *n*-hexane, turning cloudy

[1]Fresh *P. cablin* leaves and stems (maturity during harvesting was 5 months); [2]Unknown distillation type; [3]oil ageing time: 4 years; [4]Steam distillation; [5]oil ageing time: 3 years

antibacterial effects [4,14]. As reported by Sundaresan *et al.* [15] and Xu *et al.* [3] PA content ranges from 14.6% to 23.3%. However, in some cases it was determined as 60.3% [16a]; 43.60-66.25% [16b]; and 44.35-56.30% [16c]. A standardized grade PEO, besides satisfying specific olfactory needs, must have a PA content between 26-40%, as required by Essential Oil Associations, to enter the global markets. Table 1 reports the list and description of samples analyzed, along with some physico-chemical data. Samples A, B and C were oils produced in the laboratory, whereas D and E were two commercial PEOs produced in Indonesia and Malaysia, respectively.

From morphological observation, sample D was thicker and more brownish than sample E; D oil was older than E oil. Both oils were not fully soluble in *n*-hexane and provided cloudy solution. The solubility of compounds in a non-polar solvent was probably reduced by terpene oxidation, which makes the oil more soluble in a polar solvent (dichloromethane). PEOs from the laboratory (A, B and C) were younger and showed a high solubility in non-polar organic solvents (*n*-hexane). PEO obtained from fresh leaves and stems (A) yielded a very low amount of oil (0.38%). This was due to the higher water content in fresh plant material (73-75%), as can be seen in table 1. However, this result was similar to that reported by Swamy *et al.* although the PA content was much lower (30.31%) [17]. Essential oil yields for B and C samples were 0.64% and 1.12%, respectively. B oil recovery was lower, probably due to volatiles loss during one week of wet fermentation and soaking conditions, after a pre-heating process. Sample C, being one year dried, had a more concentrated essential oil (1.12%). According to Hussin *et al*. [18], PEO recovery from premature and 4 months dried *P. cablin* plants was 0.96%, an amount that increased after 1 year of plant growth. In a research study by Kongkathip *et al.* on 3-9 months dried *P. cablin*, a reduction of the PEO yield from 3.01 to 1.78% was observed [4]. It is a matter of fact, though, that the oil content and its composition are greatly affected by variables such as site of harvest, material and condition of distillation. Evaluation of the PEOs' color was also carried out (Table 1). The main finding was a darker color for commercial samples compared with lab scale produced oils. In total, 44 components have been determined in the GC fingerprint of the PEOs analyzed. Table 2 reports the quantitative data expressed as g/100g, obtained through the application of the internal standard method with FID response factors. The latter have been measured for chemical groups, following the analytical procedure previously published [1c]: selected standard compounds, representative of a chemical class (e.g. limonene for monoterpene hydrocarbons), have been injected at 5 different concentrations with an internal standard (*n*-nonane). When available, more than one compound for each chemical class was injected. Each PEO sample has been run in triplicate and repeatability tested by measuring the %RSD, which was <5% on average, except for some small peaks, such as linalool, where it was raised up to 12%. Linear Retention Indices (LRIs) have also been measured on both polar and nonpolar columns and reported in Table 2. With regard to the LRIs from the polar stationary phase, it must be highlighted that they are characterized by a lower level of repeatability and by a lack of literature data. Lab produced PEO samples and the other two commercial samples showed the same pattern for six major chemical compounds, namely patchouli alcohol, δ-guaiene, α-guaiene, seychellene, pogostol and α-patchoulene. PA content ranges were 51.9-68.0% and 46.3-45.9%, in lab PEOs and commercial samples, respectively. These results demonstrated that *Pogostemon cablin* produces higher amounts of patchoulol compared with previous research that reported 26-40% and 30-40% [19a,19b]. This range far exceeded the one prescribed by the Essential Oil Associations for authentic oil, that is 26-40%, also reported by Burfield [20,21]. Minor compounds (Table 2), such as norpatchoulenol, although present in lower amount, are considered very important to the olfactory character of PEO, as mentioned by Sunderkotter *et al.* in a study on (-)-patchoulol and (+)-norpatchoulenol [22]. These two compounds are estimated as the most important woody markers in mature *P. cablin* material and essential oils and suggest a further chiral investigation on the PEOs here investigated.

A samples presented trace amounts (≥0.01%) of other compounds, such as benzaldehyde, 1-octen-3-ol, 3-octanone, 2-ethyl hexanol, linalool, methyl salicylate and eugenol. Limonene and δ-elemene were found in slightly higher amounts than in the other PEOs. Phytol, a diterpenoid compound, was also observed in the A oil, although in small amount (0.02%) compared with other samples. A contained a higher amount of *cis*-3-hexen-1-ol, already mentioned by Oyen and Dung, which is a leaf alcohol with green and fresh notes that gives floral notes in trace amount [23]. Commercial PEOs, B and C samples had very low amounts of these compounds.

Two interesting unknown compounds (I and II) were found at consistent level: 3.4-9.8% and 4.7%, <0.01% and 0.8-0.9% and 0.6-0.4%, respectively, for both the 6 lab samples and the 2 commercial PEOs. From a literature survey, it seems likely that the mass spectrum (MS) of unknown II corresponds to that reported by Lu *et al.* [14], while no data can be found about unknown I. Further investigation by isolation, purification steps and NMR, will be carried out in order to elucidate their structure.

From the overall observations, it came out that PEOs produced on the lab-scale were richer in patchoulol compared with commercial samples. This finding could be explained by the raw material used, which was 12 months dried. No data are available for commercial samples which relate to the same material age. Also, it must be taken in consideration that hydrodistillation was conducted with a much lower amount of water and plant material (100 g vs 20 Kg on industrial scale). In this study, a new quantitative analytical method was developed for the chemical investigation of *P. cablin* essential oil. The results gathered from this research can be of support to patchouli traders and producers worldwide. Quantification of volatiles was accomplished by accurate calibration based on the internal standard method and FID response factors.

Table 2: Chemical composition of the 8 patchouli essential oil (PEO) samples analyzed. Quantitative values are expressed as g/100g.

				SAMPLE ID							
Compounds	**R.F.**	**LRI $_P$ (Exp.P)**	**LRI $_{NP}$ (ExpNP)**	**A 1**	**A 2**	**B 1**	**B 2**	**C 1**	**C 2**	**D**	**E**
α-Pinene	1.0	1021	932	0.04	0.04	0.02	0.04	0.05	0.07	0.07	0.04
Camphene	1.0	-	949	<0.01	<0.01	0.01	0.02	<0.01	<0.01	0.04	<0.01
Sabinene	1.0	-	972	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
β-Pinene	1.0	1107	978	0.1	0.1	0.06	0.08	0.14	0.17	0.16	0.14
Limonene	1.0	-	1029	0.03	0.03	0.01	0.01	0.01	0.01	0.02	0.01
4,8-Dimethylnona-1,3,7-triene	1.0	-	1113	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
TOTAL MONOTERPENES				***0.20***	***0.20***	***0.10***	***0.25***	***0.20***	***0.25***	***0.29***	***0.29***
Linalool	1.3	1544	1099	0.06	0.06	<0.01	0.01	<0.01	0.01	<0.01	<0.01
α-Terpineol	1.3	-	1196	<0.01	<0.01	0.01	0.02	0.01	0.01	<0.01	<0.01
Geranial	1.3	-	1268	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
TOTAL OXYGENATED MONOTERPENES				***0.07***	***0.07***	***0.03***	***0.03***	***0.02***	***0.02***	***0.02***	***0.02***
δ-Elemene	1.0	1466	1331	0.04	0.03	0.01	0.02	0.02	0.03	0.08	0.03
β-Patchoulene	1.0	1475	1388	0.8	1.0	1.5	1.5	1.2	1.7	0.8	1.5
β-Elemene	1.0	1752	1392	0.7	0.6	0.4	0.6	0.4	0.6	1.3	0.7
Cycloseychellene	1.0	1553	1418	0.2	0.1	0.3	0.4	0.3	0.3	0.6	0.5
(E)-Caryophyllene	1.0	1589	1424	1.2	1.1	1.0	1.3	1.1	1.5	1.1	2.0
α-Guaiene	1.0	1584	1443	3.8	3.4	4.3	5.5	4.6	6.3	5.8	9.4
Seychellene	1.0	1628	1456	2.7	2.3	3.7	4.9	3.7	4.6	7.3	6.2
α-Humulene	1.0	1660	1460	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.4
α-Patchoulene	1.0	1624	1468	1.4	1.2	1.8	2.8	1.9	2.5	4.2	4.0
γ-Guriunene	1.0	-	1473	0.4	0.4	0.7	0.7	0.6	0.9	0.5	1.1
Alloaromadendrene	1.0	-	1478	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.3
β-Selinene	1.0	1698	1492	0.1	0.1	0.2	0.3	0.2	0.2	0.3	0.3
Aciphyllene	1.0	1693	1500	0.8	0.7	1.0	1.1	1.0	1.4	1.4	2.2
δ-Guaiene (α-bulnesene)	1.0	1706	1511	5.8	5.2	6.8	7.4	7.0	9.5	9.2	14.4
Eremophila-1(10),8,11-triene	1.0	-	1514	0.06	0.05	0.03	0.04	0.04	0.04	0.07	0.1
7-*epi*-α-selinene	1.0	-	1524	0.1	0.1	0.1	0.2	0.1	0.2	0.3	0.2
TOTAL SESQUITERPENES				***18.6***	***16.8***	***22.3***	***27.2***	***22.7***	***30.2***	***33.5***	***43.5***
Elemol	1.3	-	1551	0.1	0.1	0.07	0.05	0.07	0.06	0.1	0.09
Norpatchoulenol	1.3	2093	1562	1.5	1.2	1.5	1.9	1.9	1.6	4.0	2.2
Caryophyllene oxide	1.5	1960	1586	0.9	0.8	1.7	2.0	1.8	1.6	4.6	1.8
cis-eudesma-4(15),11-dien-5-ol	1.3	-	1655	0.4	0.4	0.4	0.5	0.4	0.4	<0.01	0.3
Pogostol	1.3	2194	1649	5.7	6.0	5.5	4.6	5.2	4.7	3.5	3.7
Intermedeol	1.3	2236	1651	0.3	0.3	0.2	0.2	0.2	0.2	0.3	0.2
Patchouli alcohol	1.3	2162	1677	64.4	68.0	61.4	50.9	58.1	51.9	46.3	45.9
unknown #1 (oxygenated sesquiterpene)	1.5	-	1711	4.8	3.4	4.3	9.8	7.0	6.6	4.7	<0.01
(E,E)-Farnesal	1.3	-	1740	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.3
α-Costol	1.3	-	1777	0.5	0.5	0.5	0.4	0.4	0.5	0.	0.2
TOTAL OXYGENATED SESQUITERPENES				***79.3***	***81.4***	***76.2***	***71.0***	***75.7***	***68.0***	***64.5***	***54.7***
unknown #2	1.3	-	1774	0.8	0.9	0.80	0.88	0.78	0.80	0.56	0.38
Benzaldehyde	1.3	-	961	0.03	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01
1-Octen-3-ol	1.3	1446	979	0.3	0.2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
2-Ethyl hexanol	1.3	1390	996	0.08	0.07	<0.01	<0.01	<0.01	<0.01	0.01	<0.01
Methyl salicylate	1.6	-	1193	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Eugenol	1.3	-	1353	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Phytol	1.3	-	2110	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
3-Octanone	1.3	-	984	0.01	0.03	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
TOTAL OTHER COMPOUNDS				***1.3***	***1.2***	***0.9***	***0.9***	***0.8***	***0.8***	***0.6***	***0.4***

R.F.: response factor; LRI $_P$: linear retention index from the polar column; LRI $_{NP}$: linear retention index from the non polar column.

Experimental

Plant material and treatments: Patchouli leaves and stems were collected from Aromatic Plant Nursery, Essential Oil Incubator, MARDI, Kuala Linggi Station, Malacca, Malaysia. The material was dried and aged (fermented) for 12 months. The plant maturity of fresh material during harvesting was 5 months. The plants were growing in acid soil type (containing bisulfite), where the condition was suitable for *Pogostemon* sp. and other aromatic crops in Peninsular Malaysia. The plants collected were pre-dried with a commercial solar dryer until less than 10% moisture, then dried plants were kept in polyethylene (PE) bags, and drying continued at ambient temperature prior to essential oil processing. Each bag was able to hold up to 2.5 Kg dried materials. Fresh material collected was kept in a refrigerator at 4^{o}C for 2 days before distillation. A 100 g portion was added to 1.2 L distilled water and subjected to distillation for up to 7 h using a Clevenger apparatus (2 L capacity). A full description of the samples is described in Table 1.

Moisture analysis: The moisture content of the fresh and dried samples (1 g) was determined using an automatic IR-Moisture balance at 105^{o}C until the moisture was completely removed. The time taken to complete the analysis ranged from 6 to 12 mins.

PEO recovery: One hundred g of wet or dried plant material was weighed, added to 1.2 L distilled water and transferred into a 2 L round bottom flask in a Clevenger system. A cooling system was set up prior to heating the flask. Every hour the PEO was collected and pooled in a closed 10 mL bottle until distillation was completed. The entire process lasted 7. The moist PEO was dried using Na_2SO_4, then carefully pipetted into a 1 mL GC vial and weighed.

Sample preparation for GC analysis: The PEOs (0.10 g), were mixed with ISTD (100 µL of a 100,000 ppm stock solution) and 0.8 mL *n*-hexane. For retention indices, a mixture of *n*-alkanes {(C_7:C_{30}; Supelco (PA, USA)} was used.

GC-FID analysis: GC-FID analyses were carried out by means of a GC-2010 system (Shimadzu, Japan) equipped with an SLB-5MS column (30 m × 0.25 mm i.d. × 0.25 µm d_f), supplied by Supelco. Oven temperature program was: from 50°C at 3°C/min to 300°C, then held for 5 mins. Injection temperature was 280°C; injection took place in split mode with a split ratio of 1:100. Carrier gas was helium, with a linear velocity of 30 cm/s, pressure was 99.8 KPa. Detector temperature was 300°C, detection gases were H_2 (40 mL/min), N_2 (40 mL/min) and air (400 mL/min).

GC-MS analysis: GC-MS analyses were carried out with 2 different GCMS-QP2010 systems (Shimadzu) equipped with SLB-5MS (30 m × 0.25 mm i.d. × 0.25 μm d_f) and Supelcowax-10 (30 m × 0.25 mm i.d. × 0.25 μm d_f) columns (Supelco), respectively.

Polar column system: Injector temperature was 250°C, and injection took place in split mode with a split ratio of 1:100. Oven temperature program was: from 50°C at 3°C/min to 280°C, held 5 min. Carrier gas was helium, with a linear velocity of 30 cm/s, pressure was 28.4 KPa. Ion source temperature was 220°C, while interface temperature was 250°C. Scan range was 40-350 m/z.

Non-polar system: Injector temperature was 280°C, and injection took place in split mode with a split ratio of 1:200. Oven temperature program was: from 50°C at 3°C/min to 300°C, held 5 min. Carrier gas was helium, with a linear velocity of 30.0 cm/s, pressure was 26.7 KPa. Ion source temperature was 220°C, while interface temperature was 250°C. Scan range was 40-400 m/z. For mass spectral library matching, FFNSC 1.3, Adams and other homemade databases provided with LRIs were used [24a,24b].

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