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Determination of new retention indices for quick identification of essential oils compounds

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Abstract

The classical methods of chromatographic identification of compounds were based on calculation of retention indices by using different stationary phases.

The aim of the work was to differentiate essential oils extracted from different plant species by identification of some of their major compounds. The method of identification was based on the calculation of new retention indices of essential oils compounds fractionated on a polar chromatographic column with temperature programming system. Similar chromatograms have been obtained on the same column for one plant family with two different temperature gradients allowing the rapid identification of essential oils of different species, sub-species or chemotypes of *Citrus*, *Mentha* and *Thymus*.

Keywords: Gas chromatography; Essential oils; Retention indices; Polar stationary phase

1. Introduction

The use of essential oils is largely widespread in foods, drinks, cosmetics and medicine especially with aromatherapy becoming increasingly popular [1–3].

The essential oils studied here were those extracted from different species of *Citrus*, *Mentha* and *Thymus*.

Several therapeutic effects of *Citrus*, such as sedative, anti-inflammatory, anti-coagulant, anti-infectious and anti-spasmodic activities have been reported [4]. *Mentha* and their essential oils were largely used in foods and beverages but possess also interesting pharmaceutical potencies like anti-bacterial, tonic, anti-inflammatory or expectorant activities [4]. *Thymus vulgaris*, also known as common thyme, has long been used as a source of the essential oil. Diverse applications in pharmacy and medicine of the plants and their essential oils have been found in addition to their numerous traditional uses [5]. Actually, the *Thymus vulgaris* essential oils were reported to have anti-microbial, anti-infectious, anti-oxidant or spasmolytic activities [4,6].

All these plant families were divided into species, sub-species and/or chemotypes leading to a great number of essential oils with complex and variable chemical composition. The essential oils of different species of a same plant have often chemical and biochemical properties quite different. Thus, knowledge of the exact composition of essential oils seems to be evident for an aromatherapy worthy of the name, the opposite could led to therapeutic mistakes. Therefore, we have developed a new method of chromatographic indexation to allow a rapid identification of essential oils compounds characterising a particular species, sub-species or chemotype.

Several methods using relative retention indices were developed in order to reproduce the identification of compounds in gas chromatography. Generally, the retention values were expressed in relation to standards not present in material characteristics.

Isothermal retention index databases were widely referenced since it was proposed by Kovats [7]. Kovats method and other usual methods used linear *n*-alkanes as standards and were based on linearity between the carbon atoms number and the logarithm

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of specific retention volume or retention time on low polar stationary phases [8–10]. However, the complexity of natural essential oils induced to analyse them in temperature-programmed conditions instead of isothermal conditions [11]. Van den Dool and Kratz [12] proposed a generalization of the retention index system including linear temperature-programmed gas chromatography as follows:

$$I_x = 100\left[\frac{t_x - t_n}{t_{n+1} - t_n} + n\right]$$

where I_x is the temperature-programmed retention index, t_n, t_{n+1} and t_x the retention time (in minute) of the two n-alkanes containing n and $n+1$ carbons and of the compound of interest, respectively.

However, we could not transpose all these methods in this work to analyses in gradient temperature mode on polar stationary phase because of the lack of solubility of n-alkanes in numerous polar phases and moreover the lack of linearity of these n-alkanes in these chromatographic conditions. In addition, the logarithm transposition (isothermal method) led to a loss of sensibility.

In the chromatographic double indexation method, fatty acid methyl esters (FAME) from C_5 to C_{20} were used as soluble references in polar columns. Two sufficiently different temperature gradients led to modification in elution on the same column. Direct use of elution temperature as experimental parameter allows to reach a greater sensibility.

Practically, chromatographic analysis of a complex mixture like an essential oil is easier using a column at two different gradient temperatures instead of two different columns [13–15].

Formula used for retention indices calculation is:

$$I = 100\left[n + \frac{T_r - T_n}{T_{n+1} - T_n}\right]$$

n is the carbon atoms number of the FAME before the peak of the analysed compound, T_r the elution temperature of the analysed compound, T_n the elution temperature of the FAME before the peak of the analysed compound and T_{n+1} is the elution temperature of the FAME after the peak of the analysed compound.

2. Experimental

2.1. Materials

All essential oils (*Citrus aurantium* ssp. *aurantium*, *Citrus aurantium* ssp. *bergamia*, *Citrus limetta*, *Citrus hystrix*, *Citrus limon*, *Citrus medica* var. *vulgaris*, *Citrus paradisii*, *Citrus reticulata*, *Mentha arvensis*, *Mentha citrata*, *Mentha longifolia*, *Mentha piperata*, *Mentha pullegium*, *Mentha spicata* ssp. *viridis*, *Mentha suaveolens menthofuranolifera*, *Thymus vulgaris carvacroliferum*, *Thymus vulgaris thymoliferum*, *Thymus vulgaris linaloliferum*, *Thymus vulgaris thujanoliferum*, *Thymus vulgaris geranioliferum*, *Thymus vulgaris paracymeniferum*) were supplied by Aromalys S.A. (accredited in pharmacy) and have not been modified by the producer.

Table 1
Characteristics of *Citrus*, *Mentha* and *Thymus vulgaris* plants and parts used for essential oils extractions

Latin name	Parts used
Citrus aurantium ssp. *aurantium*	Leaves
Citrus aurantium ssp. *aurantium*	Flowers
Citrus aurantium ssp. *bergamia*	Zests
Citrus limetta	Zests
Citrus hystrix	Zests
Citrus limon	Zests
Citrus medica var. *vulgaris*	Zests
Citrus paradisii	Zests
Citrus reticulata	Zests
Citrus reticulata	Leaves
Mentha arvensis	Herbs
Mentha citrata	Herbs
Mentha longifolia	Herbs in flower
Mentha piperita	Aerial parts
Mentha pullegium	Herbs in flower
Mentha spicata ssp. *viridis*	Herbs
Mentha suaveolens menthofuranolifera	Herbs in flower
Thymus vulgaris carvacroliferum	Herbs
Thymus vulgaris thymoliferum	Herbs
Thymus vulgaris linaloliferum	Herbs
Thymus vulgaris thujanoliferum	Herbs
Thymus vulgaris geranioliferum	Herbs
Thymus vulgaris paracymeniferum	Herbs

Table 1 described the characteristics of each plant, *Citrus*, *Mentha* and *Thymus*, from which essential oils were extracted. Fig. 1 showed a total ion current chromatogram (TIC) obtained from the essential oil of leaves of *Citrus aurantium* ssp. *aurantium*, major peaks were identified and their retention time was indicated.

tert-Butylmethylether (TBME) was purchased from Fluka and fatty acid methyl esters (FAME) from C5 to C20 were obtained from Sigma.

2.2. Standard compounds

Camphene, camphor, carvacrol, *cis*-carveol, *trans*-carveol, carvone, β-caryophyllene, caryophyllene oxyde, citronnellal, citronnellol, *para*-cymene, dihydrocarvone, eucalyptol, *trans*-farnesol, *cis*-geraniol, geranyl acetate, α-humulene, isopulegol, linalol, linalyl acetate, menthol, menthone, menthyl acetate, methyl anthranilate, nerol, *trans*-nerolidol, 3-octanol, octen-1-ol-3, α-pinene, pulegone, α-terpinene, γ-terpinene, terpinen-1-ol-4, terpinolene, thymol, verbenone were purchased from Fluka. Isomenthone, limonene, piperitone, sabinene were obtained from Extrasynthèse. Citronellyl acetate and β-pinene were purchased from Acros. Borneol, limonene *cis*-oxyde, limonene *trans*-oxyde, β-myrcene, neryl acetate, α-terpineol were obtained from Sigma–Aldrich.

2.3. Gas chromatography

The analyses were performed on a Hewlett-Packard Model 5890 A gas chromatograph equipped with a Hewlett-Packard Model 7673 automatic sampler, a split/splitless injec-

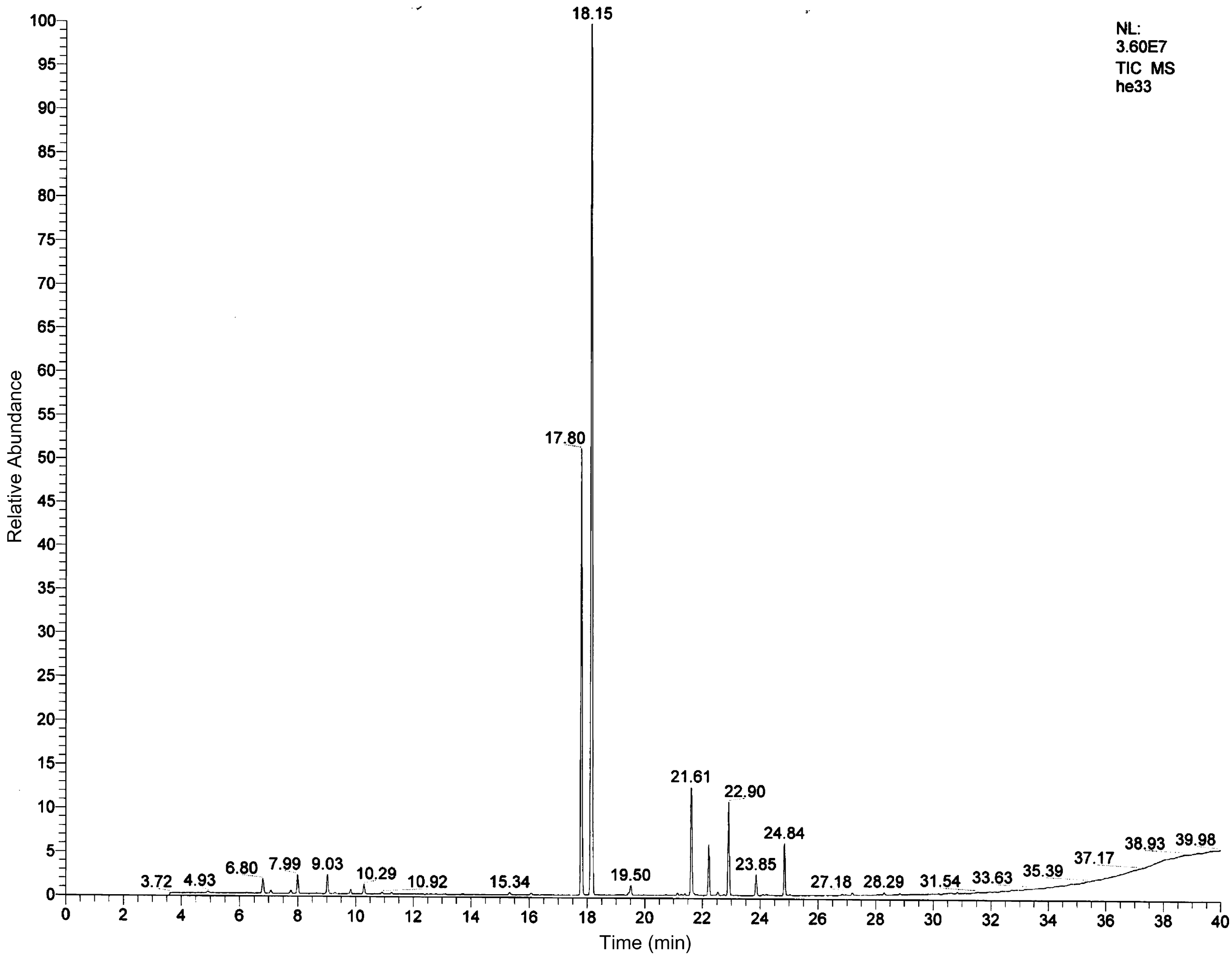

Fig. 1. Total ion current chromatogram (TIC) of *Citrus aurantium* ssp. *aurantium* (leaves) essential oil, name and retention time (min) of compounds: β-pinene (6.80), β-myrcene (7.99), limonene (9.03), linalol (17.80), linalyl acetate (18.15), α-terpineol (21.61), neryl acetate (22,22), geranyl acetate (22.90), nerol (23.85), *cis*-geraniol (24.84).

tor, an ionisation flame detector and a Hewlett-Packard 5895 A GC ChemStation software for acquisition and data treatments.

Separation of essential oils compounds were carried out on a 25 m × 0.25 mm, wall-coated open tubular (WCOT) CP-Wax 52 CB capillary column (film thickness: 0.2 μm). The volume injected was 1 μl and the split ratio was 1/75. The oven was programmed to rise from 50 to 250 °C at 6 °C/min or at 10 °C/min and then held at 250 °C for 5 min. The carrier gas was helium at a flow rate of 0.8 ml/min and the injector and detector temperatures were 260 °C. All analyses were performed at constant flow.

2.4. Mass spectrometry

The GC–MS analyses were performed on a TRACE GC 2000 series gas chromatograph interfaced to a TRACE MS mass spectrometer (ThermoQuest, Milan, Italy) operating in the electron impact mode (70 eV) and equipped with NIST libraries. The same operating conditions were used as for the GC-FID analyses.

2.5. Experimental conditions

The FAME were first injected in triplicate in the chromatograph and their temperature elution was calculated according the gradient temperature program applied.

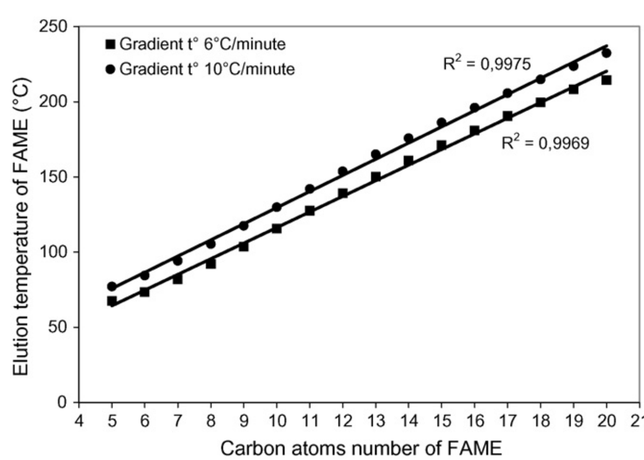

Fig. 2. Linearity of FAME on polar stationary phase at two temperature gradients.

Table 2
Retention indices at 6°/min (I_6) and at 10°/min (I_{10}) of *Citrus* species essential oils

Compounds	1		2		3		4		5		6		7		8		9		10	
	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}
α-Pinene							536				535	539	535	539	536	539	536	539	536	540
β-Pinene	623	628	623	628	623	627	623	628			623	627	623	627			623	628	623	628
Sabinene			634	638			634	638	634	637	634	637							635	638
β-Myrcene	675	675	674	675	675	675	675	675					674	675	675	675	675	675		
Limonene	714	717	714	718	714	718	715	719			716	719	715	718	716	719	716	720	714	718
γ-Terpinene			763	764	760	763	760	763					760	763			760	763	760	764
para-Cymene					785	789	785	789			785	788	785	788	785	789	785	789	785	789
Limonene *cis*-oxyde											957	963			958	964				
Limonene *trans*-oxyde															970	976				
Citronnellal									989	991										
Linalol	1049	1046	1048	1046	1048	1046			1048	1045			1048	1045			1048	1045	1048	1045
Linalyl acetate	1063	1063	1061	1062	1062	1062							1061	1062						
Isopulegol									1071	1077										
α-Bergamotene							1094	1099												
Citronellyl acetate									1163	1163										
α-Terpineol	1197	1199	1196	1199																
neryl acetate	1226	1226	1225	1226			1225	1226												
β-Bisabolene							1231	1235			1233	1238	1233	1238						
Carvone															1239	1249				
Geranyl acetate	1255	1255	1254	1255																
Citronnellol									1259	1257										
δ-Cadinene										1265										
Nerol	1294	1293	1293	1293																
trans-Carveol															1330	1333				
cis-Geraniol	1338	1337	1337	1338																
cis-Carveol															1360	1363				
Caryophyllene oxyde															1486	1503				
trans-Nerolidol			1524	1523																
Methyl anthranilate																			1574	1589
trans-Farnesol			1827																	

1, *Citrus aurantium* ssp. *aurantium* (leaves); 2, *Citrus aurantium* ssp. *aurantium* (flowers); 3, *Citrus aurantium* ssp. *bergamia*; 4, *Citrus limetta*; 5, *Citrus hystrix*; 6, *Citrus limon*; 7, *Citrus medica* var. *vulgaris*; 8, *Citrus paradisii*; 9, *Citrus reticulata* (zests); 10, *Citrus reticulata* (leaves).

Table 3
Retention indices at 6°/min (I_6) and at 10°/min (I_{10}) of *Mentha* species essential oils

Compounds	1		2		3		4		5		6		7	
	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}
α-Pinene	536	539											535	539
Camphene													582	587
β-Pinene	623	627									623	627	623	627
β-Myrcene					674	675								
Limonene	714	717	714	717	714	717	714	717	714	717	714	718	714	717
Eucalyptol					723	727					723	728	723	727
para-Cymene					785	788								
3-Octanol	898	895												
Menthone	977	985			976	984	977	985					976	984
Isomenthone	1004	1013			1004	1012	1003	1012					1004	1013
Linalol			1050	1047	1050	1043							1048	1046
Linalyl acetate			1058	1063										
Terpinen-1-ol-4					1107	1111								
Menthyl acetate	1071	1074					1070	1074						
Isopulegol	1078	1083												
β-Caryophyllene	1109		1109	1119		1119								1119
Dihydrocarvone					1117	1126					1117	1126		
Menthol	1143	1144	1141	1143			1143	1145					1141	1143
Pulegone					1154	1164	1154	1165	1157	1167	1154	1165	1154	1165
α-Humulene									1179	1190				
α-Terpineol			1197	1199										
Borneol													1202	1207
Germacrene D	1217	1228			1217	1226							1217	1228
Neryl acetate			1226	1226										
Piperitone					1234	1244	1233	1245						
Carvone					1239	1249					1241	1251		
Geranyl acetate			1255	1255										
Chavicol methyl ether					1327	1334								
Verbenone									1422	1437				
Thymol					1662	1662								

1, *Mentha arvensis*; 2, *Mentha citrata*; 3, *Mentha longifolia*; 4, *Mentha piperita*; 5, *Mentha pullegium*; 6, *Mentha spicata* ssp. *viridis*; 7, *Mentha suaveolens menthofuranolifera*.

The essential oils were diluted in TBME (1%, v/v) and then injected in triplicate in the chromatograph. Their temperature elution and their retention indices were then calculated at the two gradient temperature programs.

3. Results and discussion

In order to show the usefulness of FAME as reference compounds for polar stationary phase, Fig. 2 illustrates their linearity at the two temperature gradients. A linear relationship between the carbon atoms number and the elution temperature of each FAME (from C5 to C20) was obtained. This relation was verified for the two oven temperature programs.

The aim of this study was to identify some of the major compounds present in essential oils by calculating the retention indices of each of them. The calculation formula of these indices was described in the introduction part.

The hypothesis of the work was to enable us to differentiate qualitatively the species, sub-species and chemotypes of genus *Citrus*, *Mentha* and *Thymus* by using gas chromatography and FID detection.

The identification of compounds was confirmed at first by chromatographic injection of analytical standard compounds and comparison of their retention times with those obtained for essential oils. A second confirmation was performed by gas chromatography coupled to mass spectrometry to make sure of our double chromatographic indexation method. When standard compounds were not commercially available, GC–MS allowed us to check compounds identity using the NIST libraries.

The comparison with traditional retention indices methods was not justified because the methods were very different. Indeed, it was particularly difficult to rediscover the compounds when changing the stationary phases. The important deviation of retention times with usual methods was not suitable for complex mixtures such as essential oils. The "soft" method described here maintained the elution order of the compounds analysed.

A list of identified compounds and their retention indices (means of triplicates, CV < 2%) were proposed for the essential oils of *Citrus*, *Mentha* and *Thymus* in Tables 2–4, respectively. All compounds for which the peak area represented less than 1% of total area (FID response) were eliminated.

Comparison of results obtained by injecting standard compounds in gas chromatography, by analysing essential oils in mass spectrometry and by applying the chromatographic double

Table 4
Retention indices at 6°/min (I_6) and at 10°/min (I_{10}) of *Thymus* species essential oils

Compounds	1		2		3		4		5		6	
	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}	I_6	I_{10}
α-Pinene							528	532	528	531		
α-Thuyene	531		532									
Sabinene							627	630				
β-Myrcene	667	668	668	668			668	668				
α-Terpinene	687	691	687	691			687	691				
Limonene							707	710	707	710		
Eucalyptol	719	723	719	723					720	723	719	723
γ-Terpinene	751	755	751	755	751	755	751	755			751	755
para-Cymene	777	781	778	782	777	781	777	781	777	782	778	782
Terpinolene							789	792				
Octen-1-ol-3	955	953	955	953							955	953
trans-4-Thujanol							974	975			973	974
Camphor											1023	1035
Linalol	1051	1049	1051	1049	1053	1051	1056	1058	1051	1049	1051	1049
Linalyl acetate					1060	1060						
β-Caryophyllene	1094	1104	1094	1104	1095	1105	1094	1104	1094	1104	1094	1104
Terpinen-1-ol-4	1107	1111					1107	1111	1106	1111	1107	1111
Borneol	1199	1205					1199		1199	1204	1200	1205
Geranyl acetate	1252	1252							1252	1253		
cis-Geraniol	1342	1340							1342	1341		
Myrcen-8-yl acetate							1260	1261				
Myrcen-8-ol							1376	1375				
Caryophyllene oxyde									1476	1492	1476	1492
Thymol	1662	1662	1662	1662	1662	1662			1661	1662	1662	1662
Carvacrol	1689	1691	1690	1692					1689	1692	1690	1692

1, *Thymus vulgaris carvacroliferum*; 2, *Thymus vulgaris thymoliferum*; 3, *Thymus vulgaris linaloliferum*; 4, *Thymus vulgaris thujanoliferum*; 5, *Thymus vulgaris geranioliferum*; 6, *Thymus vulgaris paracymeniferum*.

indexation method allowed to assert that each compound could be identified by the last method. The fact that two indices corresponded to one compound increased the security of identification. A slight fluctuation of indices could be observed for a same compound when working at 6 or 10 °C/min. On the other hand, a compound had approximately similar retention indices whatever the essential oil for the two temperature gradient programs.

Some compounds were present in almost all essential oils even though others were represented in a few essential oils or in only one. Each essential oil was characterised by a typical chromatographic profile, this latter could play the role of marker for a rapid identification of a determined species, sub-species or chemotype.

4. Conclusions

The method of the double retention indices was proved to be very useful to identify rapidly some of the major constituents of essential oils without having recourse to mass spectrometry.

The qualitative comparative study of 10 essential oils of *Citrus*, 7 essential oils of *Mentha* and 6 essential oils of *Thymus vulgaris* allowed us to affirm that their composition, within a species, vary substantially highlighting the importance of plants origin. This illustrates clearly the difficulty to obtain an essential oil of strictly constant quality when so much factors (geographic, climatic, cultivation conditions, plant life cycle, ...) affect the intrinsic composition of the plant.

In the future, it would be interesting to establish retention indices data banks of numerous essential oils and essential oils compounds under specific conditions in order to facilitate their quick identification.

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