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Note

Separation of alkanes in *Citrus* essential oils by on-line coupled high-performance liquid chromatography–high-resolution gas chromatography

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The development of gas chromatography (GC) and especially high-resolution GC (HRGC) has made it possible to study essential oils in depth. The high resolving power of capillary columns has allowed separations of the many components present, but the great complexity of the matrices often requires the use of different stationary phases and the use of mass spectrometry (MS) in order to identify the components accurately. Sometimes not even MS is able to identify all the components because similar components give similar fractionation patterns, *e.g.*, as happens with sesquiterpenes[1].

The recently developed coupling of high-performance liquid chromatography (HPLC) with HRGC has been a great help towards improving our knowledge of the composition of essential oils[2,3], because it is possible to use simultaneously the high separating power of HPLC and HRGC to separate the numerous components which are present. The components are first separated by HPLC into relatively small volume of eluate which can be introduced on-line into the gas chromatograph, thus avoiding too much handling of the sample, which can lead to errors.

The alkanes in *Citrus* essential oils have not been studied much up to now[4–7]. On-line HPLC–HRGC with concurrent solvent evaporation[8–10] has been used in our research to study the alkane fraction of cold-pressed *Citrus* essential oils. The HPLC eluate is injected into the gas chromatograph by using the concurrent solvent evaporation technique. The temperature of the GC column is maintained at a higher value than boiling point of the HPLC eluent, taking into account both the composition of the eluent and the inlet pressure of carrier gas[11,12].

EXPERIMENTAL

Equipment

The liquid chromatograph was a Perkin-Elmer Series 4 with a Rheodyne 7125 S microinjection valve equipped with a 6-μl loop. The column was Hypersil Silica (5 μm) (10 $\times$ 0.21 cm I.D.). A Perkin-Elmer LC 55 S spectrophotometric detector was used.

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The gas chromatograph was a Carlo Erba Fractovap 2900 equipped with a flame ionization detector. An OV-1 glass capillary column (22 m × 0.32 m I.D.) with a 0.4-μm film thickness was used, with a fused-silica retention gap (4 m × 0.53 mm I.D.). A Perkin-Elmer LCI 100 computing integrator was used.

Other equipment consisted of Valco six-way and four-way valves ($\frac{1}{16}$ in.), a Valco T-piece ($\frac{1}{16}$ in.) and Carlo Erba needle valves.

Reagents

n-Hexane of HPLC grade was obtained from Omnia Res, methylene chloride of HPLC grade from Koch-Light, eneicosane, docosane, tricosane, tetracosane, pentacosane, hexacosane, heptacosane, octacosane, triacontane, dotriacontane and tritriacontane from Fluka and nonacosane from Aldrich.

Chromatographic analysis

The liquid and gas chromatographs were coupled using the interface shown in Fig. 1, which consists of a six-way sample valve (A), connected to the HPLC detector exit, equipped with a sample loop, and a four-way valve (B) connected to the flow regulator of the carrier gas. The two valves are connected by a steel capillary with two needle valves (C_1 and C_2) and by a T-piece. Valve A is connected to the T-piece by a 25-cm steel capillary and valve B is connected to the same T-piece by a fused-silica capillary (2 m × 0.32 mm I.D. × 0.45 mm O.D.) which passes through it, entering the precolumn to a distance of 1.5 cm (Fig. 1). The transfer steps are shown in Fig. 2.

During the GC analysis the loop with the eluent must be washed because it was

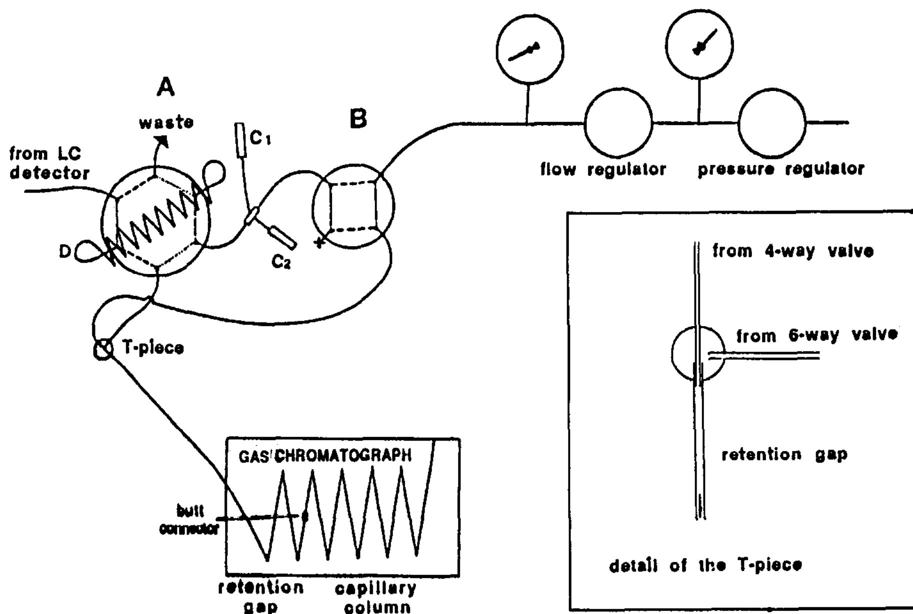

Fig. 1. HPLC–HRGC coupling interface. A = sampling valve; B = carrier gas valve; C_1 and C_2 = needle valves; D = sampling loop.

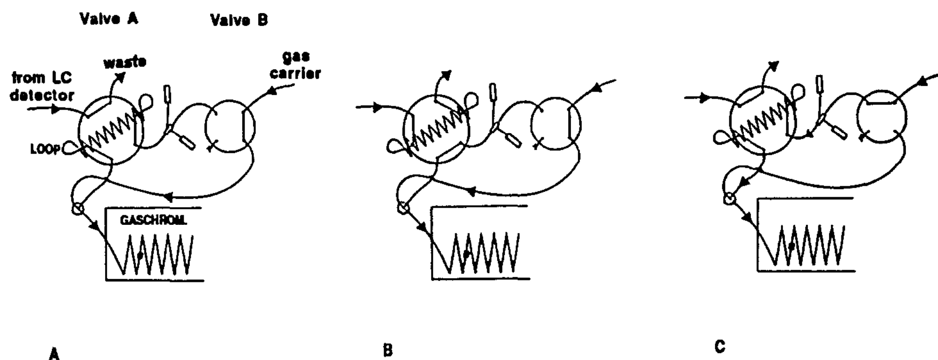

Fig. 2. Configuration of the interface during the transfer steps. (A) Basic working condition; (B) isolation of the fraction of interest in the loop; (C) transfer of the loop content into the capillary column.

observed that, owing to component carryover, washing the loop only with the carrier gas, as proposed by Grob and Stoll[9], proved to be insufficient. For this purpose, valve A is made to rotate so that the mobile phase flows through the loop; the interface appears as shown in Fig. 2B. At the end of the GC analysis, in the cooling phase of the GC oven, valve A is returned to the position in Fig. 2A. The eluent trapped in the loop is removed by opening the needle valve C_1 simultaneously.

The needle valve C_2, regulated at a flow-rate of 2 ml/min, controls any eventual increases in pressure of the system, making the evaporation of the solvent more regular.

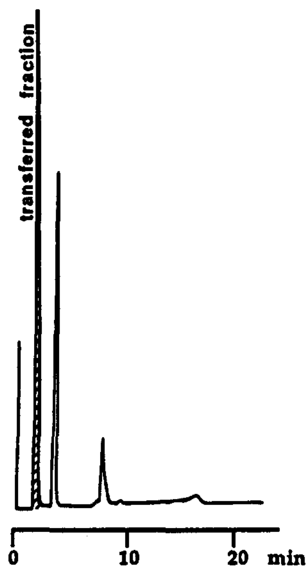

Fig. 3. HPLC of a mandarin essential oil.

The *Citrus* essential oils diluted in hexane (1:5) were injected into the liquid chromatograph through a Rheodyne valve with a 6-μl loop. A 10 × 0.21 cm I.D. Hypersil Silica 5-μm silica gel column was used with hexane at a flow-rate of 0.2 ml/min and UV detection at 220 nm. The UV absorption of terpenes was a marker for the non-absorbing hydrocarbons (Fig. 3). Alkanes were eluted in a single peak together with terpenes and sesquiterpenes. This fraction of 250-μl volume was directly transferred to the gas chromatograph.

For the GC analysis we used an OV-1 glass capillary column, connected by a butt-connector to an untreated fused-silica column (4 m × 0.53 mm I.D.) which comes out of the GC oven and is connected to the T-piece. We used hydrogen as the carrier gas at a column flow-rate of 4.5 ml/min, obtained by regulating the inlet pressure at 3 atm and the flow regulator at 1.15 atm. This regulation was performed at 124°C, which was the temperature of concurrent solvent evaporation. The temperature was programmed from 124°C (isothermal for 12 min) at 4°C/min to 180°C and rat 6°C/min to 285°C. Under these conditions, the alkanes in the sample were separated whereas the terpenes and the sesquiterpenes were eluted with the solvent in a single peak.

During the GC analysis, the LC column was washed for 15 min with methylene chloride in order to elute the oxygenated compounds left in the column; finally, it was reconditionated with hexane.

The chromatographic identification of the alkanes was carried out by using pure

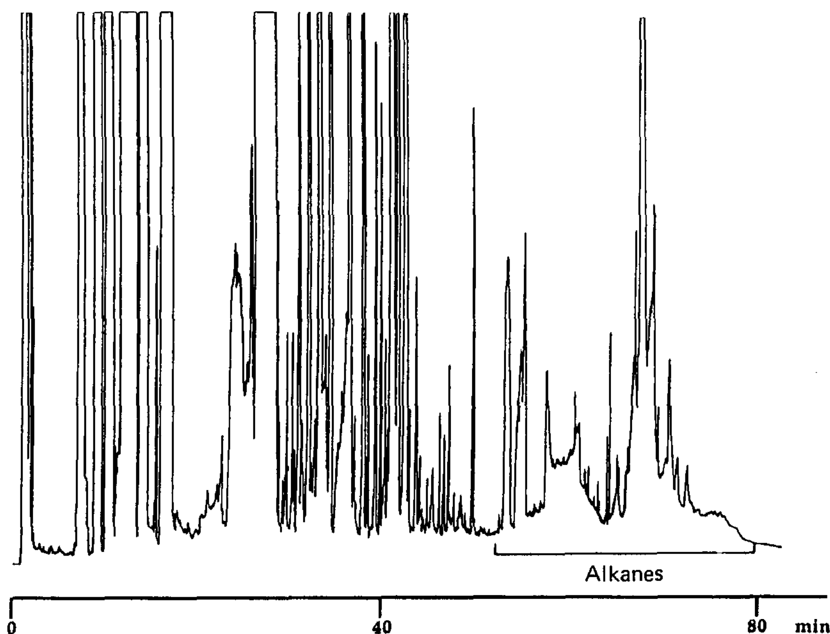

Fig. 4. Gas chromatogram of a bergamot essential oil. Injection "on-column" of 0.5 μl of essential oil solution diluted 1:5 in hexane. Temperature programme: 45°C for 2 min, increased at 2°C/min to 120°C, at 5°C/min to 230°C and at 10°C/min to 300°C. Carrier gas, hydrogen; flow-rate, 3.5 ml/min; detector temperature, 320°C.

standards; the isoalkanes were identified by using the few data available in the literature[4–7].

RESULTS AND DISCUSSION

The "on-column injection" GC analysis generally used to study *Citrus* essential oils does not give sufficient information on the alkanes because there are only small amounts present and also because of interferences from other compounds (Fig. 4). On-line HPLC–HRGC coupling allowed us to overcome this problem. A suitable amount of the sample can be injected for the accurate detection of the alkanes. The chromatograms thus obtained are less complex and the alkanes are separated without interferences, which makes their identification easier and more reliable.

Fig. 5 shows typical chromatograms of alkanes in lemon, bergamot, mandarin, sweet and bitter orange essential oils. For each essential oil ten different genuine samples were analysed.

The examination of the chromatograms obtained showed that, in the essential oils analysed all the alkanes in the range C_{21}–C_{33} and the isoalkanes in the range C_{23}–C_{31} are present. The concentrations of the various *n*-alkane components vary, sometimes greatly, from one essential oil to another. The highest concentration was found in sweet orange essential oil and the lowest in bitter orange. In all the essential oils analysed, the contents of alkanes with odd numbers of carbon atoms are generally higher than those with even numbers. In mandarin essential oil, the *n*-C_{24} concentration is comparable to that of the alkanes with an odd number of carbon atoms.

In lemon, bergamot and sweet orange essential oils, the *n*-alkanes present in the greatest concentrations are generally in sequence *n*-C_{29}, *n*-C_{31}, *n*-C_{27} and *n*-C_{25}.

We found the same composition in bitter orange essential oil. There were, however, small concentrations of other compounds, probably isomers of the various *n*-alkanes. In mandarin essential oil, the *n*-alkanes present in the greatest concentrations are generally *n*-C_{23}, *n*-C_{24}, *n*-C_{25}, *n*-C_{27} and *n*-C_{29}. In all the essential oils analysed we found the isoalkanes between *i*-C_{23} and *i*-C_{31}, which precede the corresponding *n*-alkanes in the chromatograms. As there are no pure standards available, their identification was carried out on the basis of the few data reported in the literature[4–7], according to which data the even-carbon isoalkanes are the 3-methylalkanes and the odd-carbon compounds are the 2-methylalkanes.

In all the essential oils analysed, the isoalkane found in the greatest amount is *i*-C_{25} (2-methyl-$C_{24}H_{49}$). The concentration of the other isoalkanes normally decreases as the number of carbon atoms increases, until they are scarcely detectable in the range *i*-C_{28} (3-methyl-$C_{27}H_{55}$) to *i*-C_{31} (2-methyl-$C_{30}H_{61}$).

In sweet orange and bergamot essential oil the isoalkanes have lower concentrations than the corresponding *n*-alkanes. Bitter orange, mandarin and lemon essential oils often had concentrations of *i*-C_{23}–*i*-C_{26} isoalkanes comparable with those of the *n*-alkanes.

The proposed combined technique is new and easier to perform than the traditional method, which requires isolation on an open alumina column of the various classes of compounds present in essential oils, followed by GC analysis of the fraction of interest after having adequately concentrated the sample[6,7,13]. The preseparation of the alkane fraction and its direct transfer to the gas chromatograph are carried out very

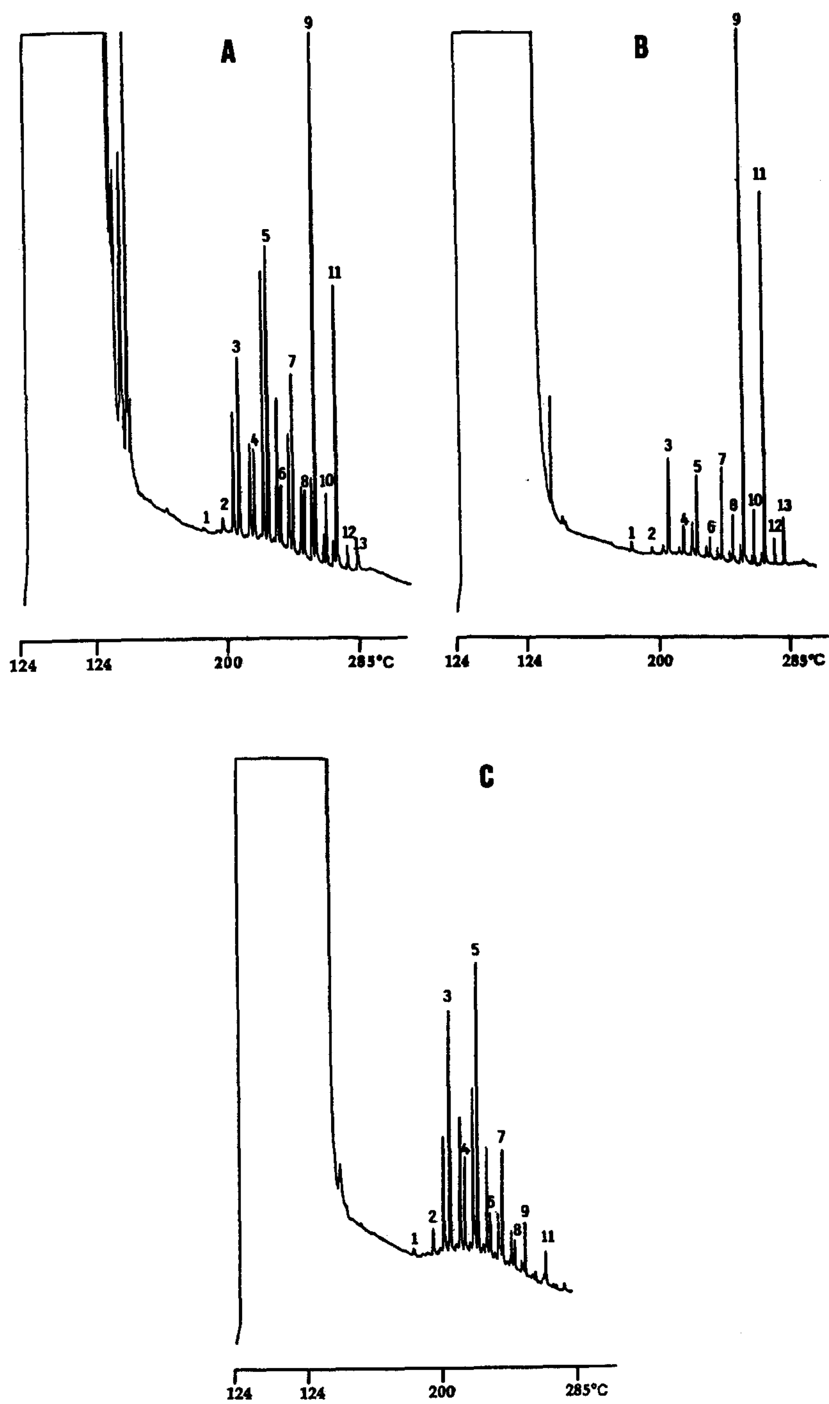

Fig. 5.

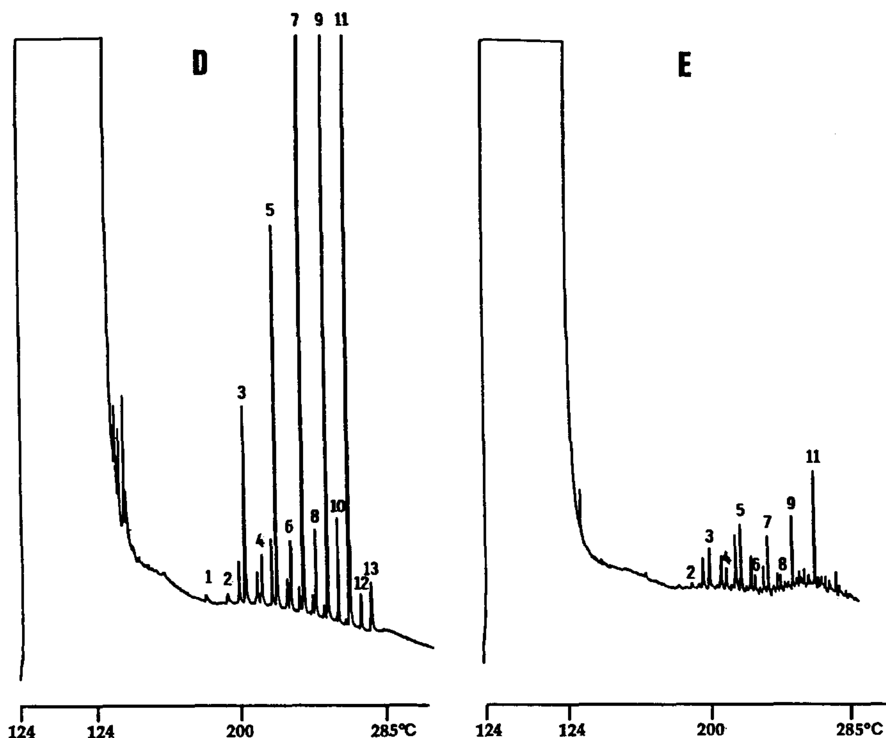

Fig. 5. Chromatograms obtained by HPLC–HRGC with flame ionization detection. (A) Lemon essential oil; (B) bergamot essential oil; (C) mandarin essential oil; (D) sweet orange essential oil; (E) bitter orange essential oil. Peaks: 1 = n-C_{21}; 2 = n-C_{22}; 3 = n-C_{23}, 4 = n-C_{24}; 5 = n-C_{25}; 6 = n-C_{26}; 7 = n-C_{27}; 8 = n-C_{28}; 9 = n-C_{29}; 10 = n-C_{30}; 11 = n-C_{31}; 12 = n-C_{32}; 13 = n-C_{33}. The peaks preceding the n-alkanes are the respective isoalkanes.

rapidly. The amount of sample required is small, and the analytical sample is obtained by simple dilution in an appropriate solvent, thus avoiding possible causes of errors, *e.g.*, due to isomerization on the alumina column. The analysis is carried out in a short time and peak identification is simplified and more certain.

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