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Determination of the threshold odor concentration of main odorants in essential oils using gas chromatography–olfactometry incremental dilution technique

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Abstract

An essential oil, obtained by steam distillation of *Clinopodium tomentosum* (Kunth) Govaerts (Lamiaceae), collected in Ecuador, was analyzed by gas chromatography–olfactometry (GC–O) and GC–MS techniques. To our knowledge, the composition of this essential oil is described here for the first time, both from the chemical and olfactometric viewpoints. A preliminary analysis by GC–MS and using Kovats' retention indexes, lead to characterize and quantify the oil constituents, while GC–O was then applied for the identification of the main odorants. By the incremental dilution method (AEDA, CHARM Analysis), applied to the GC–O technique, the flavor dilution (FD) chromatogram was obtained. In order to calculate the TOC values of the main odorants, the relationship between the odorant concentration at the sniffing port and that one in the injected solution was established. This relationship was calculated by comparing the injected amount with the TOC value of a reference compound (limonene), obtained by dynamic dilution olfactometry. A good agreement was found between calculated and measured TOC values of few odorants.

Keywords: GC–O; TOC; Essential oil; *Clinopodium tomentosum* (Kunth) Govaerts; Dynamic olfactometry; AEDA; GC–MS

1. Introduction

The threshold odor concentration (TOC) in air of a pure compound is the lowest concentration perceived by the 50% of the tested population; TOC values reported in the literature often show a great variability, in some cases as high as several decades [1–4]. In fact, TOC values are strongly affected by the measurement technique and by the number of candidates. Dynamic dilution olfactometry may be considered a suitable technique for an accurate individual TOC values determination. Olfactometry is usually employed in odor concentration measurement: the olfactometer is an air dilution device, where a gaseous sample in a bag is sucked and diluted with a known amount of purified air, filtered on active charcoal, and presented to several panelists at the sniffing port, starting from the highest dilution and then reducing it in step of two, alternatively with purified air as reference.

Each panelist will signal when he perceives a different odor in the sample air from the reference: his individual threshold will lie between the first perceived dilution and the last not perceived dilution; the average numerical value of the dilution ratio at the odor perception threshold represents the odor concentration of the sample expressed in OU_E/m^3 (European olfactometric units per cubic meter), according to EN 13725/2003, and may be obtained by the geometrical mean of several individual values [5].

If different dilutions of a standard gaseous mixture of a pure compound are presented at the sniffing ports, it is possible to calculate for each panelist the individual TOC as the standard concentration divided by the geometrical mean of the first perceived dilution and the last not perceived dilution; the population TOC value in air may be inferred as the median of a distribution of individual TOC values of a suitable number of candidates.

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Gas chromatography–olfactometry (GC–O) is an old and rediscovered technique devoted to the study of complex mixtures of odorous compounds [6]. It consists of a simple modification of a traditional gas chromatograph, where a split column divides the separated compounds between a detector and a sniffing port; this one allows the human nose to analyze the peaks at the same time they are revealed by the detector**.** Two techniques, Charm analysis and aroma extract dilution analysis (AEDA), developed by Acree and Barnard [7] and Grosch [8] respectively, represent powerful tools for the screening of the main odorants in food extracts; sample is diluted stepwise by a factor of two or three, and each dilution is analyzed by GC–O, until no odor is perceived at the sniffing port. Charm analysis builds a chromatogram, called aromagram, where peak areas are proportional to the odor concentration of the corresponding compounds; AEDA builds a bar graph, where each odorant is represented by a bar, and the height of each bar is the flavor dilution (FD) factor, the highest dilution ratio still allowing odor perception at the sniffing port for that odorant.

The main difference between these two techniques is that Charm analysis measures the dilution value during all the peak elution, whereas AEDA finds the maximum dilution value detected [9,10].

Due to the dependence of the AEDA bar graph on the concentration of the sample, we think it possible to calculate the threshold odor concentration (TOC) of every peak by smelling incremental dilutions of the sample, and then applying the typical calculations of the dynamic dilution olfactometry. An important difference between the latter and the GC–O techniques is the different physical state of the sample: actually in the dynamic dilution olfactometry the sample is incrementally concentrated from an air diluted volume; thus the result can be directly referred to the sample because its physical state does not change from the injection to the sniffing port. By contrast, in the GC–O technique applied to the TOC determination of mixtures, the sample, after having being dissolved and volatilized in the GC, is smelt by the analyst; so a different concentration between the injected liquid solution and the smelt gaseous compounds exists. For this reason, a correlation between the GC–O and the dynamic dilution olfactometry is necessary. To our knowledge, this technique has never been applied before in this way. Essential oils have been selected as useful samples to evaluate this experiment, due to their chromatographic complexity and the usually powerful odorous properties of their constituents. The essential oil of *Clinopodium tomentosum* (Kunth) Govaerts, a South American plant collected and hydrodistilled in Ecuador, has been studied in this work. This plant is known with some synonyms, reported in the International Plant Names Index (IPNI) [11]: *Gardoquia tomentosa* Kunth, *Gardoquia elegans* Kunth, *Gardoquia incana* Ruiz & Pav., *Satureja pavoniana* Briq., *Satureja tomentosa* (Kunth) Briq., *Satureja elegans* Briq., *Satureja kuntheii. C. tomentosum* is still used in the traditional medicine of Ecuador as oral antiseptic, with the indigenous name of Pumin. Curative properties of gastrointestinal and respiratory affections are also attributed to the plant. To our knowledge, its essential oil has never been studied before, neither under the name of *C. tomentosum* (Kunth) Govaerts nor under its various synonyms.

2. Experimental

2.1. Instruments

GC–O analysis was performed with a modified chromatograph HP 5890, equipped with a FID detector and a HP-5 (30 m × 0.53 mm I.D., 2.65 μm film thickness) column. Helium was employed as the carrier gas, with a constant pressure of 12 psi (a flow of 2.5 ml/min at 60 °C). The aromagrams were recorded with the oven temperature set at 60 °C for 1 min, than increased to 100 °C with a gradient rate of 10 °C/min, followed by an increase to 200 °C with a gradient rate of 5 °C/min. A third gradient rate of 30 °C/min increased the temperature to 250 °C, where it was hold for 10 min. The detector and injector temperatures were set at 200 °C and 250 °C, respectively. The injector was operated in the splitless mode and programmed to return to the split mode after 1 min from the beginning of the run. The sniffing port was a model ODO II, produced by SGE (Ringwood, Victoria, Australia). The GC–MS analysis was performed with an Agilent Technologies chromatograph model 6890 N, coupled with an Agilent Technologies MS detector model 5973 Network. The instrument was equipped with a HP-5 (30 m × 0.25 mm I.D., 0.25 μm film thickness) column. Helium (purity 99.999%) was employed as carrier gas, with a constant flow of 1.0 ml/min.

The injector was operated in the split mode (split ratio 15:1), at a constant temperature of 250 °C. The GC oven temperature program was 40 °C, hold 1 min, then it was increased to 250° with a gradient rate of 5 °C/min and hold at 250 °C for 10 min. The mass range was scanned in the full scan mode from 35.0 to 350.0 *m/z*. Experimental parameters for the ionization were: multiplier voltage, 1600 V; filament/multiplier delay, 2.70 min.

The dynamic olfactometry was performed with an ECOMA olfactometer model TO7, in a clean room equipped with an active charcoal air filtration system.

2.2. Solvents and standards

The solvent free essential oil, received from Ecuador, was diluted and injected with reagent grade dichloromethane, purchased from Carlo Erba Reagenti (Rodano, Milan, Italy), stabilized with 20 ppm of amylene. (*R*)-(+)-limonene and *n*-butanol, employed as standard compounds for olfactometry, were purchased from SIAD s.p.a. (Bergamo, Italy) in cylinders of compressed nitrogen solutions, with gaseous certified concentrations of 4.99 and 5.91 ppm, respectively. (*R*)-(+)-limonene (purity 97%), 1,8-cineole (purity 98%), 1-octen-3-ol (purity 98%), 6-methyl-5-hepten-2-one (purity 98%) and pulegone (purity 99%) were purchased from Sigma-Aldrich.

Ethanol (purity 99%) purchased from Carlo Erba Reagenti (Rodano, Milan, Italy) was used for single standard solution preparation.

2.3. Preparation of the essential oil of *C. tomentosum* (Kunth) Govaerts

The plant was collected near the river Chambo (Ecuador), on August 2001. The aerial parts (stems, leaves and flowers) of the

fresh plant were subjected to steam distillation in the same day of collection. The essential oil separated from water as upper phase, without any added extraction solvent; it was then dried on anhydrous sodium sulphate. The process produced an oil with a yield of 2.8%, referred to the dried plant.

2.4. GC–MS analysis

GC–MS analyses were performed by injecting 1.4 μl of a solution of 11.4 mg/ml of essential oil in CH_2Cl_2. The injection was made with and without adding a mixture of linear alkanes from C7 to C19, in order to obtain the Kovats' retention index of every quantified peak**;** linear retention indices according to Van den Dool algorithm were calculated [12]. Integration algorithm selected 92 peaks, of which 37 were identified and quantified. The identification was performed by comparing the EI MS spectra of the peaks with the spectra tabulated in the Adam's comprehensive work [13] for compounds with similar retention indexes. Only values of RI comprised in the range of ±5 units with respect to the tabulated ones were considered while, out of this range, they were discarded. In addition, the spectra were compared with the Wiley electronic database to confirm the identifications.

2.5. Dynamic dilution olfactometry

A dynamic olfactometer is an instrument devoted to the calculation of odor thresholds; it consists of an electronically controlled system of valves which, by the aid of a software, splits a flow of air among four volunteers called panelists. The sample air, furnished to the instrument in a plastic bag, is manually diluted in odorless medical air by an operator, called panel leader, according to the concentration indicated by the software. Every flow of sample reaching the panelists is twice concentrated with respect to the previous one, and the flows are alternated with odorless air. The panelists must indicate, pushing a button, the presence of an odor in the air flow they are smelling, avoiding to click on the odorless flows randomly introduced by the computer. When every panelist has correctly pushed the button on two subsequent odorous flows, the analysis is stopped; the threshold odor is then calculated as the geometric average value of the four most diluted perceived concentrations, each one multiplied by $\sqrt{2}$ [5].

2.6. GC–O analysis by incremental dilution technique (AEDA)

GC–O analysis was performed by injecting, at incremental dilutions, the solution of the essential oil in dichloromethane, while the four previously selected panelists signaled the perceived peaks [14–17]. In addition, the panelists were asked to describe the odor detected at the sniffing port by pushing a button, without seeing the chromatogram in progress. The electric signal corresponding to a perceived odor results in a square wave which, overlapped to the chromatogram, produces the aromagram. When a peak was no longer perceived due to its dilution, its threshold was obtained like in olfactometry, but the value calculated from the peak area was corrected by multiplying it by a conversion factor, obtained for (*R*)-(+)-limonene in olfactometry. The average value of these four thresholds is considered as the threshold odor concentration of the compound corresponding to a given peak, which had been previously characterized by GC–MS and identified in GC–O by comparing the linear retention indexes. The incremental dilution of the sample proceeded by a factor 2, beginning from 24.20 mg/ml in dichloromethane. Every panelist was asked to use one or more words to describe each odor.

3. Results and discussion

3.1. Phytochemistry

The essential oil of the plant *C. tomentosum* (Kunth) Govaerts is composed by many monoterpenes, among which the main constituents are menthane-derivatives. The results of the GC–MS analysis are reported in Table 1. A search in the literature about the biological activities of the constituents of this plant revealed that the essential oils of many *Mentha* species are, indeed, characterized by a moderate antibacterial activity. Interestingly, many constituents of these essential oils also occur in the essential oil of *C. tomentosum* (Kunth) Govaerts, strongly supporting the healing properties attributed to this plant in the traditional medicine of Ecuador. This does not exclude, of course, that other secondary metabolites, not present in the essential oil, can contribute to the biological activities of the plant. As the odorous properties of the essential oil are concerned, the chemical composition established in this work nicely justifies the total hedonic tone of the mixture, which is fresh and minty.

3.2. Retention indexes calculation

For the calculation of the retention indexes, all the homologue alkanes had to be injected in the range of retention times of interest, in order to avoid abnormal deviations from the correct values. In addition, only a linear interpolation of couples of alkanes, according to Van den Dool approach, permitted to calculate RI values in a range not exceeding the Adams' tabulated indexes by ±5 units.

The linear retention indexes were also used to identify the same peaks in the GC–O and GC–MS chromatograms.

3.3. GC–O analysis

It is quite impossible to calculate the real concentration of an odorous compound at the sniffing port, mainly because the concentration is not constant during the peak elution, but it is affected by the peak width and the auxiliary gas flow. We decided to circumvent this problem by measuring, for every panelist, the individual TOC of a reference compound (limonene). To this purpose, two independent techniques were used, namely dynamic dilution olfactometry, through a gaseous solution of limonene, and GC–O, through the injection of different solutions of limonene in ethanol, stepwise diluted by a factor of

Table 1
GC–MS analysis of the essential oil of *Clinopodium tomentosum* (Kunth) Govaerts

Calculated linear retention index	Retention index from Adams	Attribution	(%)
934	930	Alpha-tujene	0.06
940	927	Alpha-pinene	1.10
953	954	Camphene	0.04
970	975	Sabinene	0.32
974	979	Beta-pinene	0.74
977	979	1-Octen-3-ol	0.04
987	–	6-Methyl-5-hepten-2-one	0.03
990	991	Beta-myrcene	0.37
1016	1017	Alpha-terpinene	0.05
1024	1025	*p*-Cymene	0.08
1028	1029	Limonene	0.76
1030	1031	1,8-Cineole	0.80
1048	1050	Beta-(E)-ocimene	0.11
1058	1060	Gamma-terpinene	0.06
1112	1113	1-Octen-3-ol acetate	0.30
1124	1123	Octan-3-ol acetate	0.11
1155	1153	Menthone	6.60
1170	1163	Isomenthone	41.72
1173	1172	Menthol	0.10
1177	–	Isopulegone	1.97
1191	1189	Alpha-terpineol	0.28
1231	1226	Citronellol	0.33
1244	1237	Pulegone	29.94
1256	1254	*cis*-Piperitone epoxide	7.54
1261	1261	Methyl citronellate	0.17
1271	1267	Geranial	0.49
1287	1286	Isobornyl acetate	0.11
1291	1290	Thymol	0.10
1338	1342	*trans*-Carvyl acetate	0.02
1343	1343	Piperitenone	1.83
1353	1353	Citronellyl acetate	0.04
1364	1362	Neryl acetate	0.02
1368	1369	Piperitenone epoxide	0.98
1383	1381	Geranyl acetate	0.09
1387	1388	Beta-bourbonene	0.03
1423	1419	*trans*-Caryophyllene	0.26
1453	1455	Geranyl acetone	0.02
1484	1485	Germacrene D	0.11
1527	1530	Zonarene	0.03
1994	1998	Manoyl oxide	0.19

two. In this way we could compare the most diluted solution of limonene still perceived by every panelist, at the olfactometer port and at the GC–O sniffing port, respectively, and we calculated a conversion factor between this two values. The factor may be used for calculating the individual TOC of a mixture of compounds analyzed by GC–O. In fact, this correlation permits to calculate the gaseous concentration at the sniffing port, given a certain concentration of the injected solution.

The individual conversion factor is calculated as the ratio between the individual TOC from dynamic dilution olfactometry, expressed as mg/m^3, and the individual TOC from GC–O, expressed as μg injected. The geometrically averaged conversion factor for our group of panelists was 0.02342 (dm^3)$^{-1}$; this means that 1 μg of an injected compound generated a concentration of 23.42 μg/m^3 at the sniffing port. As the ratio between the concentration of a compound in the injected solution and the gaseous concentration of the same compound at the sniffing port can be considered constant, we could calculate the concentration at the sniffing port of every constituent of an unknown mixture through the chemical analysis of the sample.

When the essential oil of *C. tomentosum* was subjected to GC–O analysis, many different odors, not perceivable from the whole mixture, were described by the panelists in association with minor peaks. In particular, one must consider the presence of 1-octen-3-ol (KI = 977, 0.04%) and 6-methyl-5-hepten-2-one (KI = 987, 0.03%), which were well separated and identified by GC–MS, but not separated and individually perceived in GC–O. All the panelists described the odor of the mixture of these two peaks as "mushroom-like", with a TOC value of 0.223 μg/m^3 calculated in GC–O. This description nicely corresponds to the "mushroom odor" reported for a 50 wt.% mixture of 1-octen-3-one and 1-octen-3-ol in the SAFC^{TM} Flavors & Fragrances catalogue [18]. The other TOC values calculated with the same procedure and the hedonic tones perceived for the corresponding peaks are reported in Table 2.

These values were obtained from a panel of four persons, correlating their threshold perceptions of (*R*)-(+)-limonene (0.134, 0.134, 0.213, and 0.067 mg/m^3, respectively) to the amount of limonene injected in GC–O at the TOC value (5.402, 5.402, 10.819 and 2.701 μg, respectively). The application of the corresponding conversion factor to the other peaks of the mixture, as previously described, gave the reported values.

The AEDA graph, relative to the considered peaks, is reported in Fig. 1. The reported dilutions are the geometric average values of the critical dilution values of the four panelists.

From a practical point of view, it is important to consider the width of the peaks in the GC–O analysis to ensure the results reproducibility. Actually, given a certain amount, the wider is a peak, the longer is the corresponding substance retained inside a column and more diluted it exits from the sniffing port. Therefore, it is important to calculate the conversion factor by measuring the TOC value in GC–O, injecting the standard sample (limonene) with the same temperature program employed for the analysis. In our experiments, the peak widths at the base were in the range of 5–10 s, depending on the amount of the corresponding compounds in the sample. This time is long enough

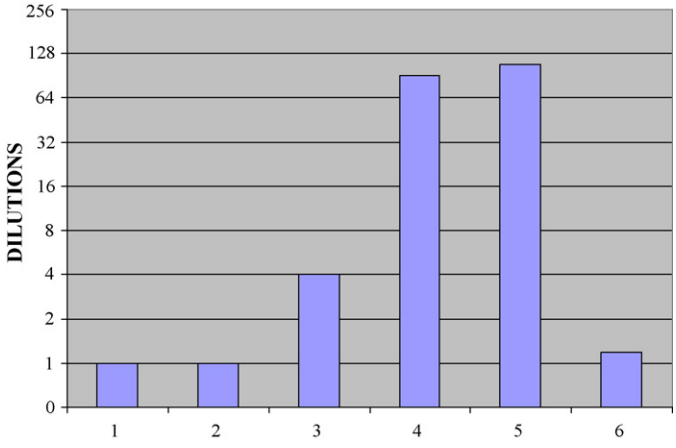

Fig. 1. AEDA graphic relative to the peaks in Table 2.

Table 2
TOC values calculated by AEDA method in GC–O technique and measured by dynamic dilution olfactometry

No.	Compound	Calculated TOC ($\mu g/m^3$)	Measured TOC ($\mu g/m^3$)	Descriptor
1	1-Octen-3-ol + 6-methyl-5-hepten-3-one	0.223	2.36 and 18.89	Mushrooms
2	1,8-Cineole	2.67	5.08	Balsamic
3	Isomenthone	40.451	n.d.	Wine bottle stopper
4	Isopulegone	0.076	n.d.	Minty
5	Pulegone	0.884	1.87	Minty
6	*cis*-Piperitone oxide	22.427	n.d.	Minty

to evaluate the odor quality and short enough to perceive the minor constituents.

3.4. TOC measurement

Selected main odorants found in the essential oil, i.e. 1-octen-3-ol, 6-methyl-5-hepten-2-one, 1,8-cineole and pulegone, and (*R*)-(+)-limonene were analyzed by dynamic dilution olfactometry for TOC determination. Gaseous standard solutions were prepared injecting 2 μl of a ethanolic standard solution of each compound through a septum into a 10 l Nalophan sampling bag and presented to four panelists; individual TOC measurements were averaged and results were compared with calculated values (Table 2).

(*R*)-(+)-limonene was analyzed for comparing the TOC value obtained from the standard cylinder (0.137 mg/m^3) with the homemade gaseous standard in a sampling bag (0.113 mg/m^3): the agreement between the two values is good.

1,8-cineole and pulegone TOC values measured by dynamic olfactometry were about twice the calculated values; the agreement between these values may be considered good, and this systematic difference seems related to difficulties in the odor perception at the threshold level in the most diluted solution. TOC values for 1-octen-3-ol and 6-methyl-5-hepten-2-one were measured separately, but they were present as unresolved peak in GC–O and perceived as 'odor-cluster' [19]; single TOC values found were much higher than the calculated value for the mixture: this discrepancy should be better evaluated improving chromatographic separation of the two compounds.

4. Conclusions

In this work two main goals have been achieved: a) the first analysis of the essential oil obtained from the Ecuadorian plant *C. tomentosum* (Kunth) Govaerts; b) the first application of the GC–O technique to the determination of TOC values with the AEDA method, through the use of dynamic olfactometry. The essential oil resulted rich in monoterpenes, among which isomenthone and pulegone were the most abundant components, constituting about 41.7 and 29.9%, respectively, of the entire mixture. We also found promising results coupling GC–O and AEDA techniques in the determination of TOC values of the constituents of complex mixtures. This required the preliminary calculation of a conversion factor to transform each peak concentration in GC–O to the corresponding aerial concentration at the panelist's nose. More work must be done in the near future for explaining some observed discrepancies; our approach verification will require many more results obtained with compounds having a broader range of TOC.

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