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Essential oils analysis. I. Evaluation of essential oils composition using both GC and MS fingerprints.

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Abstract

The chemical nature of essential oils makes them suitable for analysis using a gas chromatography-mass selective detector (GC-MSD). Mass spectra (MS) libraries can not be used as unique and absolute criteria for the identification of chromatogram peaks. The wide variety of MS of the libraries, recorded in different conditions, can lead us to erroneous results. In order to increase the reliability of the analytical results, we used as identity criteria, both GC fingerprints resulted from the relative retention indices (RRI) and the recorded MS of the separated compounds. The two criteria have been quantified by their correlation with the standards. A new parameter called global composition evaluation index (I_{GCMS}), which resulted from a well-balanced average of the two criteria, has been defined. Because the comparison of the results of the MS with databases is more accurate than the RRI, we considered that the ratio of the two criteria must be at least GC:MS 1:2. A database containing RRI of about 600 components, widely found in essential oils composition and separated on HP-5 column, was created. Two macros based on the Microsoft Excel spreadsheet were also created. The program offers the best 20 matches of each compound with the combined MS and RRI library. The composition of Romanian Acorus calamus L. essential oil was established and the results were compared with those obtained by 'classical' methods.

Keywords: Gas chromatography-mass selective detector; Relative retention indices; Essential oil analysis; Fingerprinting; Composition evaluation index

1. Introduction

The therapeutic and odoriferous properties of the essential oils are directly correlated with their qualitative and quantitative composition. The presence of some falsifications or artifacts often leads to the modification of the properties, especially of the therapeutic ones, which are a result of the synergetic effects of all the components. There are cases where the essential oils contain components with adverse properties that are under the control of the US Food and Drug Administration (FDA) or of the European Council Commission.

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Moreover, the essential oils are widely used in aromatherapy, so it appears obvious that it is absolutely necessary to know their exact composition.

The gas chromatographic method is used almost exclusively for the qualitative analysis of the volatiles. Retention times were utilised as primary criterion for the peaks identification. Without preliminary data regarding the compounds retention times, the identification is practically impossible. Even if the retention time for a certain compound is well known, it is possible that it can elute at the same time with other compounds in the sample.

The mass spectrometer used as chromatographic detector offers additional data for the identification of the separated compounds. As gas chromatography-mass spectrometry (GC-MS) availability has increased, a large number of scientists have turned to mass spectra (MS) for identification of peaks. The most frequent identification method is the comparison of the recorded spectra with an MS library. The computer decreases dramatically the time that is necessary for the comparison, even if the library contains hundreds of thousands of spectra. The wide variety of MS in the libraries, recorded in different conditions, can make it impossible to predict with sufficient precision, the identity of certain peaks of isomers or of similar substances, from the chemical structure point of view.

The essential oils are mixtures of terpene or phenylpropanic derivatives in which the chemical and structural differences between compounds are minimal. The MS of these compounds are very similar, the peaks identification being very difficult and sometimes impossible. In order to evaluate the composition by gas chromatography-mass selective detection (GC-MSD) as well as possible and for increasing the reliability of the analytical results, it is necessary to utilise both MS and relative retention indices (RRI) identities as identification criteria.

The retention times of the separated compounds cannot be utilised as such for the compounds identification owing to their dependence on many factors such as column, temperature, pressure, etc. In order to eliminate a part of the operation variables (pressure, temperature), instead of the retention times, relative retention times are used. For a standardisation of the chromatograms, van den Dool [1], suggested to plot the normalised peaks area related to the main peak area as function of the RRI. This plot is also indicated by the Analytical Methods Committee (AMC) [2]. Using this plot, the chromatograms of a certain essential oil are the same in different temperature or pressure conditions. They are dependent only on the utilised column. These standardised chromatograms represent the essential oil fingerprint and they can be used for the identification of the origin and of the falsification.

In order to improve the quality of the analytical results from the GC-MS analysis of some Romanian essential oils, we tried to correlate the standardised chromatograms with the correlation coefficient of the compounds MS. From this correlation results a new parameter that characterised the whole GC-MS method.

2. Materials and methods

2.1. Samples

The Acorus calamus L. essential oil was obtained by steam distillation of the fresh rhizome of the Romanian plant [3].

2.2. Reagents

All solvents (hexane) were of chromatographic grade and were purchased from Merck (Darmstadt, Germany). In order to compute the RRI, a mixture of n-alkanes from n-octane (C8) to eicosane (C20) was used.

2.3. Solutions

The sample solutions of essential oils for GC-MS were prepared dissolving 5 mg essential oil in 20 ml hexane.

2.4. Instrumentation

GC-MSD analyses were performed on a Hewlett-Packard 5890 series II-5972 MSD using a

HP-MS 5 column, 0.26 mm i.d. × m, 0.25 μm coating thickness. The GC was operated under the following conditions: manual injection, split 1:20; injector temperature, 250°C; carrier gas, He; flow, 1 ml min^{-1}; linear velocity, 36.4 cm s^{-1}; oven temperature programmed 60°C to 240°C at 3°C min^{-1}; detector temperature 280°C; and time run, 60 min. The MSD was operated under 70 eV, scan range 41–300 amu, scan-TIC. MSD were tuned before each injection using PFTBA (perfluorotributylamine) as tuning standard.

2.5. Computers and software

A HP Vectra Pentium 75 computer equipped with Hewlett-Packard ChemStation B.02.02. acquisition software was used. The peaks were integrated using both Chemstation integrator and RTE integrator. The integrators variables are initial threshold, initial peak width, initial area reject and shoulders.

The used MS library was Wiley275. The MS library search was performed using PBM (probability-based matching) algorithm that uses a reverse search.

In order to compute the RRI, and to develop an identification algorithm based on both GC and MS fingerprints, Microsoft Excel 97 and Visual-Basic for Applications were used.

3. Results and discussion

The difficulties in the components identification of some Romanian essential oils using GC-MSD technique forced us to find a fast, and as exact as possible screening method. We proposed a protocol for the utilization of MS as the primary identification criterion using correlation coefficient (CC_{MS}) computed by PBM algorithm. Then, each retention time is transformed in RRI using a series of n-alkanes from 8 to 20 carbon atoms. This RRI is compared with a homemade database containing around 600 compounds that are widely found in the composition of the essential oils. The results of the comparison are expressed as RRI correlation coefficient (CC_{RRI}). Correlating the two parameters, a new parameter, characterizing the whole method, the global composition evaluation index (I_{GCMS}) is calculated. Fig. 1 presents the main steps of the algorithm and the main aspects and the mathematical equations are discussed in the next paragraphs.

3.1. Parameters obtained using ChemStation software

The chromatograms and MS were recorded using Hewlett-Packard's ChemStation acquisition software. The peaks were integrated and retention times were obtained. There are two integrators (ChemStation and RTE) and four variables that can be changed (initial threshold, initial peak width, initial area reject and shoulders) in order to optimise the integration. The information obtained during integration consist of retention time (R_t) and peak area.

The recorded MS were compared with MS databases using PBM algorithm, a library-search routine that uses a reverse search to verify that peaks in the reference spectrum are present in the unknown spectrum. Extra peaks in the unknown are ignored, thus allowing the analysis of a spectrum resulting from a mixture of compounds. The PBM search results displays the list of the best 20 matches that resulted from the library search,

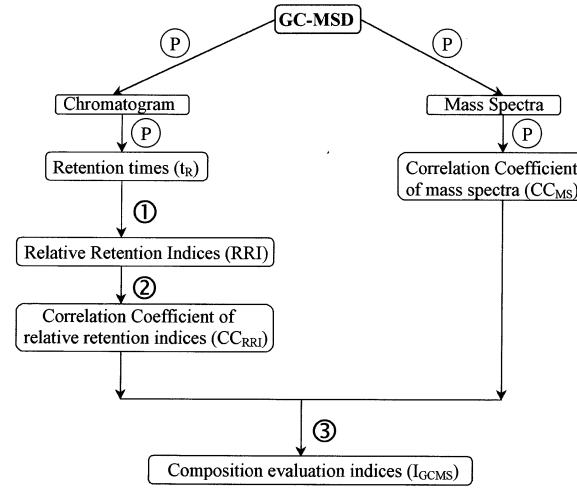

Fig. 1. Scheme of the algorithm steps for essential oils compounds identification. P, steps of ChemStation algorithm, 1, 2, 3, the main steps of the proposed algorithm.

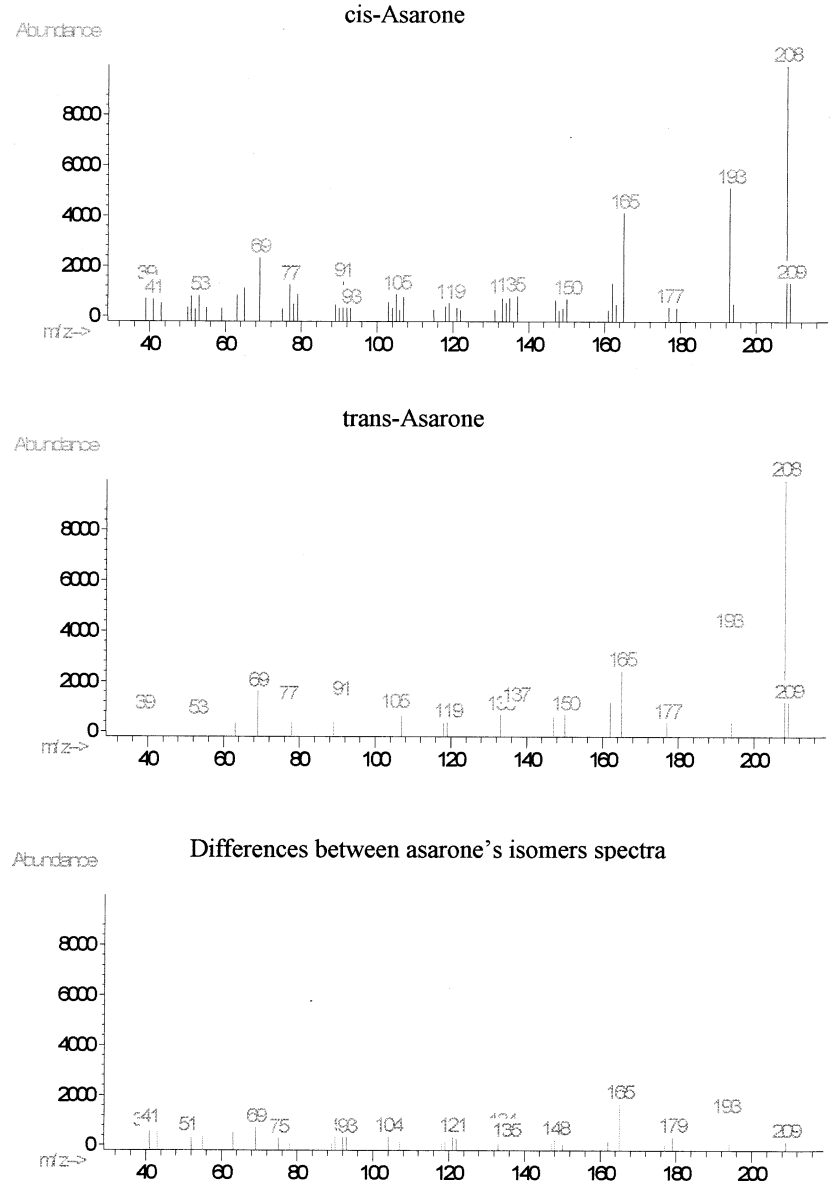

Fig. 2. MS of α- and β-asarone.

showing the name of each compound, the molecular weight (MW) and the CC_{MS}. We used this coefficient as the first identification criterion.

3.2. Correlation coefficient of relative retention indices

The retention times cannot be used as such because of their dependence on many separation parameters [4]. Instead of the retention times, it is useful to calculate the RRI for a certain column. These RRI are not influenced by the temperature or pressure program and they are calculated according to Kovats equation (Eq. (1)), using as reference compounds a series of n-alkanes (Fig. 1, step 1). AMC [2], suggest as recommended proce-

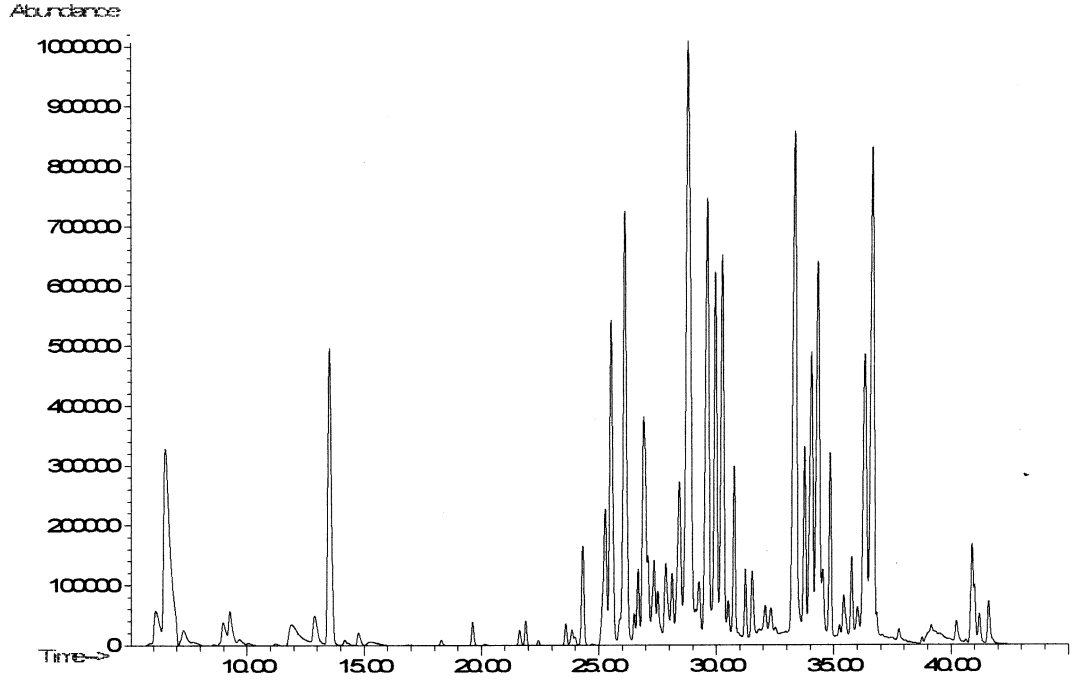

Fig. 3. GC-MSD chromatogram of Acorus calamus L.

dure the use of both even and odd numbered carbon n-alkanes and assumes linearity between them.

$$\mathrm{RRI} = \frac{[100(\mathrm{R}_{tC} - \mathrm{R}_{tn})]}{\mathrm{R}_{t(n+1)} - \mathrm{R}_{tn}} + 100n \quad (1)$$

where n, carbon number of the n-alkane eluting before the peak of the sample component; $n+1$, carbon number of the n-alkane eluting after the peak of the sample component; R_{tC}, retention time of the component; R_{tn}, retention time of the first hydrocarbon; $\mathrm{R}_{t(n+1)}$, retention time of the second hydrocarbon.

In order to identify the compounds we compared the obtained RRI with a homemade database that contained about 600 volatile compounds widely present in the composition of the essential oils (Fig. 1, step 2). The comparison was made according to Eq. (2):

$$\mathrm{CC}_{\mathrm{RRI}} = \left(1 - \frac{\sqrt{\mathrm{RRI}_{\mathrm{C}}^2 - \mathrm{RRI}_{\mathrm{DB}}^2}}{\mathrm{RRI}_{\mathrm{DB}}}\right) \cdot 100 \quad (2)$$

where $\mathrm{CC}_{\mathrm{RRI}}$, correlation coefficient of RRI; $\mathrm{RRI}_{\mathrm{C}}$, RRI of the component; $\mathrm{RRI}_{\mathrm{DB}}$, RRI of the compounds from database.

Some variation of RRI is normal. Because of this variation and because the magnitude of RRI values is between 5×10^2–4×10^3, we proposed the previous formula instead of the classical one for the percent calculation. Using the percent formula computation, the magnitudes of the $\mathrm{CC}_{\mathrm{RRI}}$ are very small and these are not statistically different. The $\mathrm{CC}_{\mathrm{RRI}}$ calculations are performed using a Microsoft Excel spreadsheet that displays the best 20 matches for each compound.

3.3. Composition evaluation indices

The previously calculated parameters ($\mathrm{CC}_{\mathrm{MS}}$ and $\mathrm{CC}_{\mathrm{RRI}}$) characterised separately the identity of the compounds. In order to quantify the $\mathrm{I}_{\mathrm{GCMS}}$ (Fig. 1, step 3), we proposed different formulas, depending on the $\mathrm{CC}_{\mathrm{MS}}$ because we considered that the MS results are more reliable than RRI and at the same time $\mathrm{CC}_{\mathrm{MS}} < 80$ could not be

Table 1
Identification of the Acorus calamus essential oils compounds by GC-MSD using both GC and MS fingerprints

Peak number	R_t	RI	Area (%)	CC_{MS}	Library/ID	CAS	RI	CC_{RRI}	I_{GCMS}
3	7.29	980	0.25	91	Piene ⟨beta-⟩	000127-91-3	976	91	91
				91	Sabinene	003387-41-5	971	86.5	89.5
				91	Piene ⟨alpha⟩	000080-56-8	933	69.4	84
10	19.63	1288	0.19	91	Bornyl acetate	000076-49-3	1284	92.1	91.4
				91	Fenchyl acetate ⟨alpha-⟩	004057-31-2	1721	11.4	65.3
13	23.59	1378	0.17	98	Ylangene ⟨alpha-⟩	014912-44-8	1371	89.9	96
				97	Copaene ⟨alpha-⟩	003856-25-5	1375	93.4	95.9
16	25.28	1418	1.79	93	Amorphene ⟨alpha-⟩	023515-88-0	1433	85.4	90.7
				94	Muurolene ⟨gamma-⟩	030021-74-0	1476	71.1	87.3
				90	Cadinene ⟨gamma-⟩	039029-41-9	1284	57.6	79.2
21	26.92	1457	3.82	89	Caryophyllene ⟨trans-⟩	000087-44-5	1418	77	84.9
				83	Farnesene ⟨trans-⟩	000502-61-4	1505	74.1	79.1
22	27.36	1467	0.95	83	Germacrene-D	023986-74-5	1479	87.2	84.8
				86	Cubebene ⟨beta-⟩	013744-15-5	1390	68	79.1
23	27.51	1471	0.54	95	Germacrene-B	015423-57-1	1494	82.2	91.3
				91	Acoradiene	090457-37-7	1462	89	90.4
25	28.12	1485	0.68	87	Elemene ⟨gamma-⟩	003242-08-8	1492	90.3	88.2
				70	Germacrene-D	023986-74-5	1479	91	80.5
				62	Muurolene ⟨alpha-⟩	010208-80-7	1499	86.2	74.1
				70	Copaene ⟨alpha-⟩	003856-25-5	1375	62.2	66.1
				70	Ylangene ⟨alpha-⟩	014912-44-8	1371	61.6	65.8
				64	Cubebene ⟨alpha-⟩	017699-14-8	1349	58.2	61.1
26	28.44	1493	2.23	99	Eremophilene	010219-75-7	1504	87.8	96.1
				93	Valencene	004630-07-3	1491	94.8	93.5
				93	Aromadendrene ⟨allo-⟩	025246-27-9	1460	79.1	88.8
28	29.26	1513	0.85	93	Valencene	004630-07-3	1491	83	90
				90	Ledene	021747-46-6	1493	83.8	87.9
				95	Gurjunene ⟨alpha-⟩	000489-40-7	1409	63.6	86
40	34.08	1635	3.52	99	Asarone ⟨beta-⟩	005273-86-9	1621	86.9	95.9
				99	Asarone ⟨alpha-⟩	002883-98-9	1678	76.9	93.3

considered as good matches with the MS libraries (Eqs. (3) and (4)):

If $CC_{MS} > 80$

$$I_{GCMS} = \frac{CC_{MS}\left(\frac{CC_{MS}}{10} - 7\right) + CC_{RRI}}{\frac{CC_{MS}}{10} - 6} \quad (3)$$

If $CC_{MS} \leq 80$ $\qquad I_{GCMS} = \frac{CC_{MS} + CC_{RRI}}{2}$ (4)

Eq. (3) provides a dynamic calibration in the 80–99.9 range of the CC_{MS}. The increase of the CC_{MS} coefficient leads to an increase in the accuracy results. Because the CC_{MS} is more reliable to the RRI, we considered that a quadratic equation fit the proposed model more precisely. If the CC_{MS} decreases under 80, the accuracy decreases too, and the model can be fitted by the average of the two correlation coefficients (CC_{RRI} and CC_{MS}, Eq. (4)).

The I_{GCMS} calculations are performed using a Microsoft Excel spreadsheet that displays the best 20 matches for each compound. A VisualBasic macro ordered the matches for each compound

and the best matches are display as the final result.

3.4. Application for the qualitative analysis of Romanian Acorus calamus L.

We choose this essential oil because it contains the asarone isomers [5]. The pharmacological studies on the essential oils extracted from Acorus calamus L. have put into evidence, besides their beneficial effects, their toxicity too. In 1974, the FDA banned the utilization of the Acorus calamus L. owing to the hepatic carcinomas that appeared in rats following the long time administration of volatile oil [6,7]. At the same time, the FDA proved that only β-asarone is susceptible to having carcinogenic action. It is obvious that it must be proved that the essential oil contains only one of the isomers or both of them. On the other hand, the MS of the two isomers look very similar (Fig. 2), and the PBM algorithm cannot differentiate them. It is necessary to utilise additional information in order to differentiate the two isomers.

We used the previously discussed algorithm and the calculation spreadsheets and macros in order to establish the most probable composition of the Romanian Acorus calamus essential oil and for the identification of asarone isomers. The chromatogram of the essential oil is presented in Fig. 3. As a rule, the identities of the compounds are similar using CC_{MS} and I_{GCMS} but there are some exceptions and one of them are the asarone isomers. The results tabulated in Table 1 show the separated compounds that cannot be identified with sufficient precision and their identities according to CC_{MS}, CC_{RRI} and I_{GCMS}.

The identified compounds are used to create the essential oil fingerprint on HP-5 column. These fingerprints can be used for the fast identification of the origin of the essential oils using a comparison algorithm with an essential oil fingerprints database. Such a database is under construction and it will contain about 2000 essential oils analyses from all over the world.

4. Conclusions

The proposed algorithm improved the analytical results of the GC-MSD analyses for the component identification. Even if the majority of the peaks identities are well resolved by mass spectrometry, there are some cases in which it is not enough to compare the MS. The identification of the geometric isomers and other compounds with similar MS is more precise and the differences between the comparison parameters are increased. Moreover, the used spreadsheets and macros reduce the identification time of the unknowns and the certitude of the final results is increased too.

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