Identification and resolving of trace and co-eluted components of Lamium amplexicaule essential oil using two chemometric methods-assisted GC-MS

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Abstract

Gas chromatography-mass spectrometry (GC/MS) due to high sensitivity for quantitying volatile compounds is one of the most practical methods for the analysis of essential oils. Accurate identification of trace components and complete separation of overlapped and embedded peaks are difficult to achieve even if precise conditions are imposed on the chromatographic separation process. In this study, the essential oil of *Lamium amplexicaule* L. (*L. amplexicaule*) after extraction by the Clevenger apparatus, was analyzed by GC/MS. This study focuses on the characterization of the trace and co-eluted components of essential oils in the mentioned species using chemometric methods. Advanced multivariate curve resolution (MCR) methods were used to overcome the problem of background, baseline offset and overlapping peaks, and recognition of the noises from the trace components in GC/MS. The analysis of GC/MS data without chemometric methods revealed that eighteen components exist in the *L. amplexicaule* essential oil. It is noteworthy that, by a combination of MCR with GC/MS method, this number was extended to more than twenty-five. Using chemometric tools and methods, components with a percent age higher than 0.01% were identified from noises, and other overlapped peaks were resolved for 85.56% of the total relative content of the *L. amplexicaule* essential oil. The most important volatile constituents were identified as hexahydrofarnesyl acetone, spathulenol, caryophyllene oxide, hexadecanoic acid and trans-phytol, respectively.

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1. Introduction

In recent years, a significant growth in scientific knowledge of the relationship between medicinal plants and health has been observed. Medicinal plants have an important role in therapy due to their less side effects in comparison to synthetic drugs (1, 2). The genus *Lamium* L. is one of the most popular medicinal plants and an important source of medicines and food aspects ,(belonging to the Labiatae (*Lamiaceae*) family), additives. The genus *Lamium comprises* more than fifteen species plus many other hybrid species throughout the world which all are native herbaceous plants in Asia, North Africa and Europe (3). In Iranian flora, there are 7-9 *Lamium* species with the Persian name of "Gazane-sa" (4, 5). *Lamium amplexicaule* L. is the most scattered annual species of the genus in Iran which is grown in different parts of the country.

The aerial parts of the plant have been used as an herbal medicine in Traditional Iranian Medicine against digestive disorders (6, 7). Also, the seed oil of the same species possessed strong antioxidant properties, so its use as a chemical food stabilizer has been proposed (8). Many parameters influence the yield and composition of

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the essential oils (EOs) (9). Therefore, the analysis and identification of the EOs composition of the plants is very important to ensure the plant quality for several applications.

The best technique for analyzing the volatile components of *L. amplexicaule* is gas chromatography–mass spectrometry (GC/MS). GC/MS technique due to possessing high sensitivity and ability to analyze and identify a great number of analytes by using mass spectra, is one of the most used analytical methods in many scientific fields (10, 11). But in the best experimental conditions, there are different types of noise, overlapped, embedded, retention time- shifted and low S/N ratio peaks in GC/MS data which make evaluation of GC/MS data difficult. Essential oils have complex matrixes with major and trace components. So, among these problems, trace signals and distinguishing them from noises and co-elution (overlapped and/or embedded peaks) are the most observed chromatographic difficulties. Scientists attempt to improve and resolve unresolved peaks by changing temperature, program, and stationary phase composition (generally classical chromatographic parameters). Besides, the overlapped peaks are decomposed to pure components using chemometric methods. However, changing instrumental conditions is not enough, and some overlapped peaks don't resolve after changing the method's condition. Also, the second approach depends on the pure chromatographic profiles (12-14).

Over the past few decades, different chemometric resolution methods have been applied to improve the sensitivity and resolve separation issues encountered in GC-MS analysis. The advantages of using multivariate curve resolution (MCR) for resolving the mixtures are achieving the mathematical resolution with less time, costs and chemical efforts. The multivariate curve resolution-alternating least square (MCR-ALS) technique is one of the most popular self-modeling curve resolution techniques that allows mathematical resolution of concentration and spectral profiles of pure chemical species in complex mixtures (15-17). MCR-ALS along with other chemometric methods, was applied to provide chemically meaningful quantitative and qualitative profiles of the pure constituents of EOs chromatographic data set (17-19). MCR-ALS has some advantages over other MCR such as the application of different constraints during the optimization process, simple generalization to high-complexity data sets and better understanding of the chromatographic process (11).

Recently, we have reported (18) perfect resolving and identification of volatile Co-eluting compounds of *Dracocephalum moldavica* L. essential oil by GC/MS with the help of MCR-ALS and MF-ICA methods for the first time. In the present study, we have used the optimized parameters for extraction and analysis of essential oil of *L. amplexicaule* by GC/MS, but still there were some peak clusters with severe co-elution problems in the total ion chromatograms (TICs), and so many short peaks that didn't identify as noises. Therefore, sophisticated data analysis techniques were needed for a complete separation and identification. In the present work, two MCR processes were applied and compared with each other. Finally, MCR-ALS was selected to resolve the strongly co-eluted and trace compounds, and improve the qualification, and quantification in GC/MS peaks. As far as we know, this is the first study that uses GC–MS combined with MCR techniques for the analysis of the essential oil of *L. amplexicaule*.

2. Material and methods
2.1. Plant Specifications
The aerial part of *L. amplexicaule* medicinal plant was collected in Apr/ 2016 from the South slopes of the Binaloud mountains of Neishabour city in Khorasan Razavi province (NE of Iran) with N: 36°14´80 " and E: 58°58´25" geographical coordinates.
Determination of collected plant species was done using valuable Flora of Country and then the voucher specimen (No: IMPH-7004) was deposited at the Herbarium of the Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran (IMPH).

2.2. Extraction of Essential Oils
The air-dried, powdered aerial parts (200 g flowers and leaves) of the plant were subjected to hydro-distillation in a Clevenger-type apparatus until there was no significant increase in the volume of the oil for 3 h. The oils were dehydrated by anhydrous sodium sulphate (Merck, Darmstat, Germany) and stored in sealed vials at 2 °C before analysis.

2.3. Analysis and identification by GC-MS

GC/MS analysis of *L. amplexicaule* volatile components was done by Agilent 6890 system (Agilent, Littleton, Colorado, USA) equipped with Agilent 5973 N mass selective detector and a BPX5 fused silica column (30 m×0.25 mm i.d., film thickness 0.25 μm). 5 min after injection, oven temperature was increased from 50 to 240 °C (3 °C min^{-1}) and then reached to 300 °C (15 °C min^{-1}) and hold 3 min in this temperature. Other operating conditions were as follows: carrier gas, He (99.999%), with a flow rate of 0.5 mL.min^{-1}; injector temperature of 250 °C; and split ratio of 1:35. Mass spectra were taken at 70 eV a scan time of 1 s and a mass range of 40-500 amu.

The essential oil components were characterized by comparing their resolved mass spectra by MCR-ALS methods with the computer library and obtained using authentic compounds. For confirmation of the results, identities of the compounds were confirmed by comparing their kovats retention indexes (KIs), either with the reference compounds or with data published in the literature (20, 21). The KIs were calculated for all resolved components using a homologous series of n-alkanes.

2.4. Software requirements

A PC interfaced to the GC, using the MSD ChemStation software package, was used for the collection and processing of GC/MS data. All chemometric resolution techniques were implemented using an MCR-ALS toolbox from the homepage of MCR under the Matlab R2009a environment, and also commercially available Unscrambler X 10.2 software for pre-processing, chemical rank determination, local rank analysis, MCR-ALS, and MF-ICA analysis. Identification of individual constituents in *L. amplexicaule* essential oil was made based on a comparison of retention indices, together with the mass spectral fragmentation pattern of resolved chemical constituents with Kovat's retention indices (KI) and mass spectra of standard components stored in the Wiley 7n.1 MS and National Institute of Standards, Technology (NIST05a) computer library and ADAMS reference book (21).

2.5. Curve resolution procedure

Curve resolution methods can identify the pure mass spectral and concentration profile of each component from the raw data matrix that is produced using GC/MS analysis software. In this work, at first, several peak clusters were obtained of the total ion chromatogram (TIC) and for each peak cluster, a primary data matrix was prepared. Then the following processes were applied to each data matrix:

2.5.1. Background correction

Three-way GC/MS data are affected by many instrumental conditions and tools (22). These conditions and tools can create major problems in resolving data into spectra and concentrations for pure chemical constituents that can be difficult to distinguish from minor chemical components. Background correction is a potent technique to remove the baseline drift and spectral background in the chromatographic data. Overlay, raw chromatographic data in the data matrix (matrix X) can be created from two sources: spectral background Xb, and component data Xc ($X_{total}= X_c + X_b$). During the chromatographic run, spectral background is approximately constant, and correction of background can correct the quality and quantity analyses because spectral background data is from initial raw data (matrix X). Without background correction, both identification of the trace components and resolution of overlapped peaks are difficult. In this work, the background correction was done based on the Kvalheim and Liang method (23, 24).

2.5.2. Denoising and smoothing

Usually produced signals by GC/MS contain heteroscedastic noise (i.e., proportional to the signal). This type of noise in GC-MS data can be misdiagnosed with the trace components. In this work, a smoothing filter was used to remove these noises. The noises from the data were removed using a morphological score to discriminate between the noises and the signals (25).

2.5.3. Chemical rank determination and local rank analysis

Determination of the number of chemical components (chemical rank) is a key level in the resolution of GC/MS overlapped peaks because the subsequent procedure is related to this step. Based
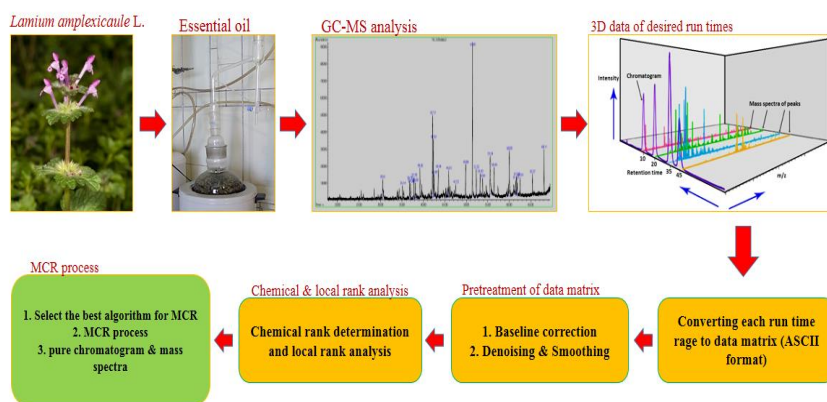
Figure 1. General steps of the MCR approach combined to the GC/MS for the analysis of the essential oil of *Lamium amplexicaule* L.

on the division for chemical rank determination method by Wasim and Brereton (26, 27), FSMW-EFA is a specialized rank-providing method. Also, this method can obtain the local rank map of co-eluted peaks. So at this step, FSMW-EFA was used for the determination of Chemical rank and local rank analysis.

2.5.4. Multivariate resolution

The final purpose of the analysis of GC/MS data is to obtain qualitative and quantitative profiles of the non-identified co-eluted chemical components. In the literature, multivariate resolution methods of MF-ICA and MCR-ALS were proposed for the resolution of noisy and overlapped peaks in chromatograms, respectively (20, 27, 28). In this study, there are some embedded, overlapped, and many numbers of noisy peaks. So, for the first time, we used the combination of two iterative chemometric methods of MF-ICA for the discernment of the small peaks from noise and MCR-ALS for the resolution of co-eluted and embedded peaks.

2.5.5. Qualitative and semi-quantitative analysis

Resolved mass spectra of the resolved peaks were used for matching with the reference mass spectra, and qualification and overall volume integration (OVI) was used for calculating the percent of the components in the essential oil (29).

3. Results

The essential oil of *L. amplexicaule* was identified by the MCR approaches for solving certain problems. Through this technique, spec-

tral background and noises, changes in the peak shapes and co-elution, were resolved more easily than is possible with other physicochemical techniques. Figure. 1 shows the overall framework of the MCR approach combined to the GC/MS analysis of *L. amplexicaule* EO (Figure 1).

3.1. Pretreatment of data

The three total ionic chromatograms (TICs) of noisy, noisy-overlapped, and embedded peaks of *L. amplexicaule* essential oil are shown in Figure 2a, b, and c, respectively. The identification of these peaks is difficult due to low similarity indices (SIs) obtained from direct searching with the MS database. Also, different mass spectra are possibly searched at the different points of the specific peak. In this study, as examples, three peak clusters (labeled by a, b, and c) are selected to show the performance resolution methods to identify low-signal and overlapping GC/MS data of essential oils (Figure 2).

At first, baseline correction, denoising, and smoothing on peak clusters were performed. These initial steps are necessary to obtain reliable results in the resolution process.

3.2. Determination the number of components

The next step was determining the number of independent components (ICs) and chemical ranks (CRs) that show the number of chemical components in the multi-component overlapped GC/MS data matrix. In this work, to avoid the accumulation of noise, a morphological score (30) was used to determine the number of ICs and CRs; and due to analyzing only key variables instead of the full rank matrix, reliable results were obtained.
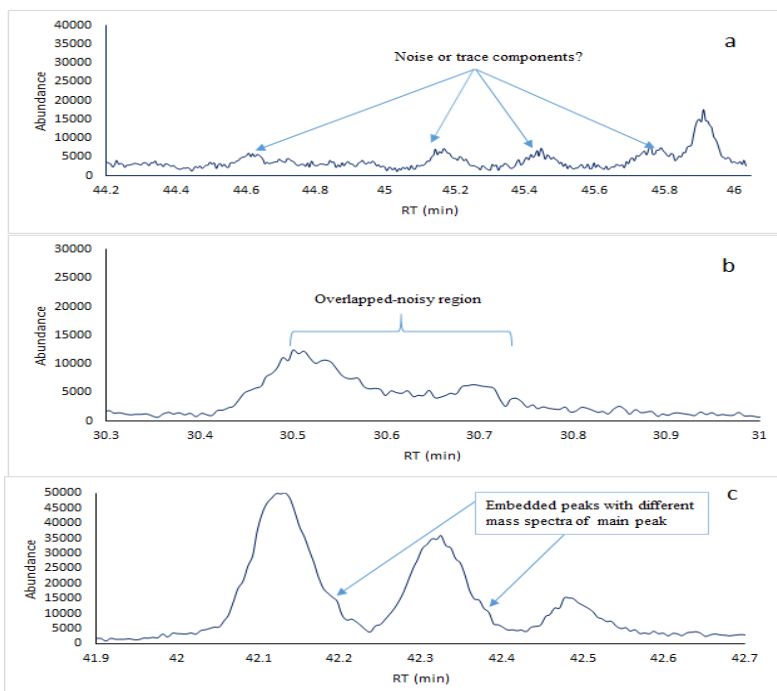
Figure 2. Total ionic chromatograms (TICs) for three regions of (a) the noisy, (b) noisy-overlapped and (c) the embedded peaks of *L. amplexicaule*.

In the present method, the level of noise in a specified probability was calculated and then the ICs and CRs numbers were reported by counting the number of ICs with morphological scores upper than that of the noise level. Figure 3(a), (b), and (c) shows the variation of morphological scores with the number of components for the peak clusters a, b, and c. It is clear, that the numbers of ICs and CRs in three peak clusters a, b, and c are 4, 2, and 5, respectively (Figure 3).
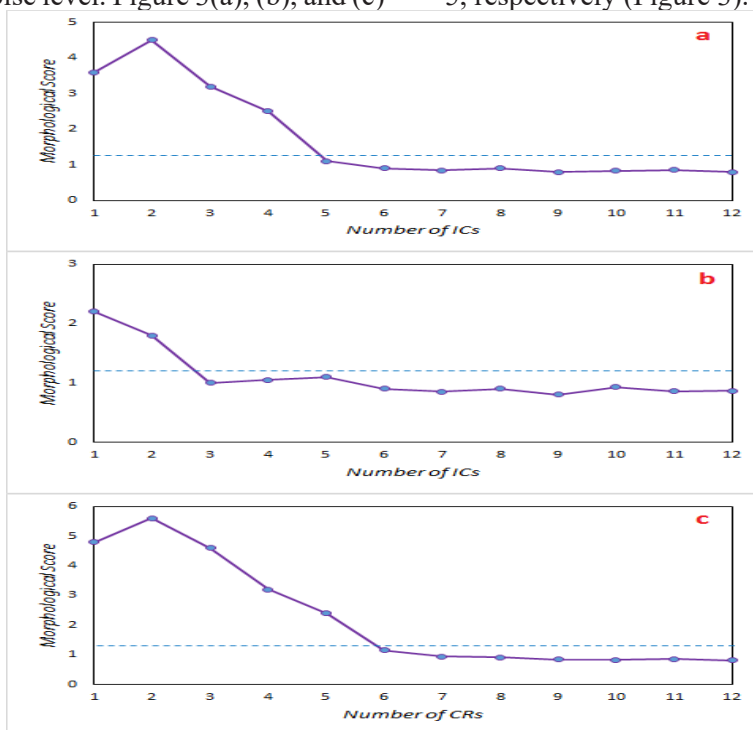

Figure 3. Morphological score plots for the determination of the number of the independent components for peak cluster a, b and c.

3.3. Extraction of mass spectra and concentration profiles

Using the obtained number of ICs, the number of chromatograms and mass spectra of the pure components in the overlapped peaks of the essential oil chromatogram can be recovered. Using initial estimates of mixing matrix (chromatograms) or sources (mass spectra) the concentration profile can be obtained. In this work, chromatograms were used for the estimation of the initial values. Peak cluster (c) was selected from the TIC of *L. amplexicaule*. In this cluster, the resolved ICs are due to five components with different degrees of overlapping. Figure 4 depicts the spectra of the five ICs extracted using OPA-MCR-ALS,

denoted as IC1–IC5, and Figure 5 shows related chromatographic profiles. A comparison of the two figures revealed that five chromatograms correspond to five ICs. Furthermore, the resolved IC1 with the NIST MS library was matched and demonstrated that this mass spectrum relates to spathulenol ($C_{15}H_{24}O$). For further verification of the presence of two compounds for the first and second peaks and the position of the other peaks in the TIC, a local rank map using evolving factor analysis (FSMW-EFA) (30) has been obtained. In FSMW-EFA plots, if the noise level is lower than the eigenvalues logarithm the presence of a new component is proven. For a system of one component, only one curve should be higher than the noise level in its FSMW-EFA plot. For example,
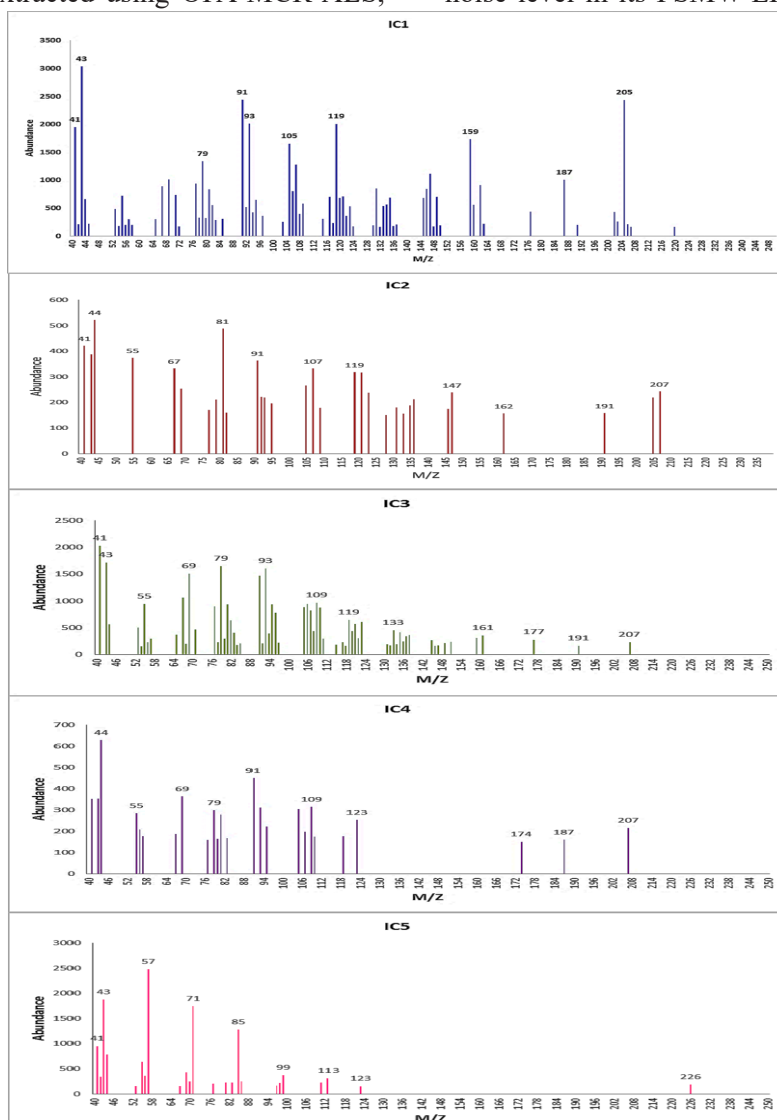

Figure 4. Estimated mass spectra for independent components from the peak cluster c using the OPA-MCR-ALS.
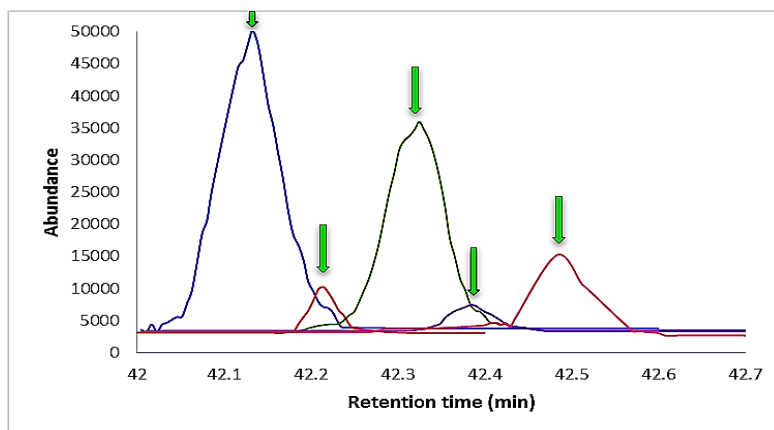
Figure 5. The resolved chromatographic profiles obtained by the OPA-MCR-ALS corresponding to the each estimated components.

if the local rank is two, two co-eluted components exist, and so on. The flat area in this plot shows the pure selective regions for one single component and the peak-shaped region represents the overlapping region containing at least two components. The local rank map showed a clear insight into the selected sequence. The number of compounds at every scan number and also the elution patterns for the concentration profiles would be determined. This map revealed the presence of the second peak for IC2 in the selected period. The five extracted ICs are matched and confirmed by the NIST MS library. IC2–IC5 were spathulenol, copaen-15-ol, caryophyllene oxide, viridiflorol and hexadecane, respectively. The complete information about all resolved components of tree peak clusters (a, b, and c) is shown in Table 1. For example, without curve resolution, only three components with low RMF values have been identified for the peak cluster c. The LOF and R2 values for OPA-MCR-ALS solutions of this peak cluster were 17.59% and 96.90%, respectively. Therefore, without applying curve resolution methods, it was impossible to obtain accurate qualitative and quantitative information for this peak cluster. The obtained results show the ability of the MCR-ALS method to extract more information from the GC-MS data (Figure 4, 5) (Table 1).

4. Discussion

In this work, GC/MS was applied as a method for identifying the volatile components of *L. amplexicaule* from the Neishaboor Mountains (situated in Khorasan Razavi). The separation and quantification of unresolved GC/MS signals were

achieved using MCR-ALS techniques. These chemometric approaches can help detect target overlapped compounds that were not detected before due to signal interference. In addition, using the MCR-ALS, setting a longer temperature program for GC/MS or more dilution of the sample to avoid creating overlapped peaks is not required. The main components of Iranian *L. amplexicaule* were hexahydrofarnesyl acetone (16.09%), spathulenol (9.53%), caryophyllene oxide (6.00%), hexadecanoic acid (7.01%) and trans-phytol (7.12%) that could be used as a fingerprint for Iranian L. amplexicaule.

Using the obtained pure chromatographic profile and mass spectrum for each component, the total two-way response for each component can be achieved from the outer product of its concentration and spectrum profile. On the other hand, the peak areas for the resolved elution profiles could be calculated by a semi-quantitative analysis, which is discussed in the following. After resolving all the peak clusters using the MCR-ALS, the number of identified compounds of the *L. amplexicaule* essential oil was increased from 18 to 27. It should be regarded as the relative amount of the identified compound expressed as a percentage of the obtained peak area relative to the total area of the TIC. The quantitative analysis identified 85.56% of the total content of *L. amplexicaule* essential oil. There are some reports on the analysis of the *L. amplexicaule* using GC and GC/MS that did not use any MCR techniques (31-33). For the sake of comparison, the number of identified components of *L. amplexicaule* is more than that of

Table 1. Identified components that resolved and separated from the *L. amplexicaule* essential oil with concentrations larger than 0.01%. (The identified peaks in each category are marked with different colors).

No	R.T.	%	Peak clusters	Components	Formula	RMF	KI	Type
				Gas chromatography–mass spectrometry (GC/MS) Analysis				
1	30.51	1.77	b	Thymol	$C_{10}H_{14}O$	946	1314	MO[a]
2	30.70	0.10		4-Acetylanisole	$C_9H_{10}O_2$	902	1318	Other[a]
3	35.14	1.55	c	3,4-dehydro-β-ionol	$C_{13}H_{20}O$	930	1419	MO
4	37.00	1.84		α-Humulene	$C_{15}H_{24}$	925	1464	SH
5	38.09	1.65		(E)-β-Ionone	$C_{13}H_{20}O$	912	1490	Other
6	39.19	3.15		2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	904	1517	Other
7	42.13	9.53		Spathulenol	$C_{15}H_{24}O$	947	1591	SO[a]
8	42.22	1.01		Copaen-15-ol	$C_{15}H_{24}O$	870	1594	SO[a]
9	42.33	6.00		Caryophyllene oxide	$C_{15}H_{24}O$	936	1596	SO[a]
10	42.37	0.66		Viridiflorol	$C_{15}H_{26}O$	920	1597	SO[a]
11	42.49	2.30		Hexadecane	$C_{16}H_{34}$	970	1600	Other[a]
12	43.44	3.32		Citronellyl pentanoate	$C_{15}H_{28}O_2$	914	1626	SO
13	44.60	0.10	a	α-Cadinol	$C_{15}H_{26}O$	901	1656	SO[a]
14	45.17	0.12		1-Methylene-2b-hydroxymethyl-3,3-dimethyl-4b-(3-methylbut-2-enyl)-cyclohexane	$C_{15}H_{26}O$	856	1672	SO[a]
15	45.45	0.09		X	-	-	1679	NI[b]
16	45.76	0.08		X	-	-	1688	NI
17	45.91	2.97		Heptadecane	$C_{17}H_{36}$	927	1692	Other[a]
18	51.40	16.09		Hexahydrofarnesyl acetone	$C_{18}H_{36}O$	948	1847	SO
19	52.32	3.37		Dibutyl phthalate	$C_{16}H_{22}O$	923	1874	Other
20	53.20	2.09		Nonadecane	$C_{19}H_{40}$	912	1900	Other
21	53.81	1.92		(E, E)-Farnesyl acetone	$C_{18}H_{28}O$	905	1919	SO
22	55.54	7.01		Hexadecanoic acid	$C_{16}H_{32}O$	940	1973	Other
23	56.44	3.39		Eicosane	$C_{20}H_{42}$	970	2000	Other
24	60.00	7.12		trans-Phytol	$C_{20}H_{40}O$	932	2059	DO
25	61.53	2.09		Methyl linoleate	$C_{19}H_{34}O_2$	906	2084	Other
26	62.53	1.94		Docosane	$C_{22}H_{46}$	948	2200	Other
27	68.11	4.30		Tetracosane	$C_{24}H_{50}$	939	2400	Other
		85.56		Total Identified				

Monoterpene Hydrocarbons MH   Oxygenated Monoterpenes MO
Sesquiterpene Hydrocarbons SH   Oxygenated Sesquiterpenes SO
Oxygenated Diterpenes DO
[a]This components were resolved and identified by MCR-ALS
[b]Not Identified

previous reports. This demonstrates the ability of the curve resolution methods, especially the MCR-ALS method in improving GC/MS quantitative and qualitative analysis of the complex samples.

Finally, the results of MCR-ALS for the three peak clusters a, b, and c were compared with the obtained results by the mean-field independent component analysis (MF-ICA). The results show that the statistical results of MF-ICA are similar to those of MCR-ALS. Also, the chromatographic peak area for the resolved peaks using MF-ICA was calculated and the results were comparable with those of MCR-ALS. It can be understood that both MF-ICA and MCR-ALS methods can be applied as proper methods for obtaining more information from the GC/MS data. In addition, MCR-ALS can recover the peaks of the components with similar mass spectra.

5. Conclusion

In this study, the number of identified components of *L. amplexicaule* is more than that of previous reports. In addition, based on RMFs, the obtained mass spectra by the MCR method were more matched and confirmed with reference mass spectra than those obtained by ChemStation. Finally, it can be concluded that the combination of hyphenated chromatographic techniques with MCR methods can open a new viewpoint for quick and accurate analysis of complex samples, such as *L. amplexicaule* organic extracts.

Conflict of Interest

The authors declare no conflict of interest.

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