Journal of Chromatography A 1710 (2023) 464409

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Chiral isotopic fractionation in lemon essential oil: A tool for authenticity assessment?

Lorenzo Cucinotta [a], Gemma De Grazia [a], Paola Donato [a], Monica Mondello [b], Danilo Sciarrone [a,*], Luigi Mondello [a,b]

[a] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina 98168, Italy
[b] Chromaleont s.r.l., c/o Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina 98168, Italy

ARTICLE INFO

Keywords:
Isotopic ratio mass spectrometry
Enantio-selective gas chromatography
Multidimensional gas chromatography
Lemon essential oil
Citrus
Isotopic fractionation

ABSTRACT

The present research aimed to retrieve key information about the genuineness of Sicilian lemon essential oils by evaluating simultaneously the chiral and isotopic data of target terpene components. With respect to previous literature references, where chiral recognition and isotope discrimination were performed by distinct gas chromatographic methods, this study aimed to develop a single analytical approach. To overcome limitations associated to monodimensional gas chromatographic approaches, an enantio-selective multidimensional gas chromatographic approach coupled to isotopic ratio mass spectrometry and to parallel single quadrupole detection (Es-MDGC-C-IRMS/qMS) was developed. Thanks to the features of this system, enantiomeric excesses and target $\delta^{13}C$ of the chiral and achiral components were evaluated in a single gas chromatographic run, allowing to reduce total time analysis, as well the consumption of electricity, solvents and samples. Moreover, due to the capability to baseline separate the enantiomeric couples, further considerations were done about the specific $\delta^{13}C$ value of the target separated enantiomers. Dealing with the genuine lemon oils analysed, a different $\delta^{13}C$ value was found between the enantiomers of the same chiral component, namely (-) and (+) of α and β-pinene, suggesting a different isotopic fractionation related to a specific biosynthetic pathway. This research aimed to evaluate the reasons behind this behaviour, paving the way to newer considerations in the field of authenticity assessment.

1. Introduction

Authenticity assessment of essential oils is an everlasting challenge for quality control checkers, continuously struggling with frauds. Because of the increasing consumers' request, the commercialization of cold-pressed *Citrus* products is spreading ever more worldwide. Amongst *Citrus* oils, lemon essential oil is one of the most valued, with a high economic impact in the flavour and fragrance industry. The oil, mainly obtained through cold-pressed extraction of the fruit peels, is widely employed in different fields, including renowned food and beverage applications, aromatherapy and newer pharmacological uses exploiting its antioxidant properties [1]. The oil volatile fraction mostly consists of olefinic monoterpenes, such as β-pinene, limonene, γ-terpinene, and minor amounts of aldehydes, such as citral (neral+geranial), which largely contribute to the flavour [2]. The oil chemical composition is usually investigated by means of gas chromatography coupled to flame ionization (GC-FID) and/or mass spectrometry detection (GC/MS) and may vary quantitatively upon the season [2]. A thorough evaluation of the oil components however requires more sophisticated analytical approaches.

Enantio-selective gas-chromatography (Es-GC) has been extensively described in the literature, for its capability to evaluate the genuineness and quality of essential oils [3]. Specific enantiomeric ratios are registered for key components of *Citrus* essential oils, as in the case of (+) limonene (> 95 %) and its levorotatory form [4]. Significant deviations from the genuine ranges allow the unveiling of fraudulent oil additions, by these means. However, key components with a pronounced enantiomeric excess may be selected by other sources, making authenticity assessment harder.

Complementarily to Es-GC, isotopic ratio mass spectrometry (GC-C-IRMS) is broadly recognized as a powerful tool for authenticity assessment. The different CO_2 uptake by the plant and the related biochemical pathways allow to distinguish amongst metabolites obtained from C_3, C_4 and CAM plants, as well as from synthetic sources [5–9]. Considering the

* Corresponding author.
E-mail address: dsciarrone@unime.it (D. Sciarrone).

https://doi.org/10.1016/j.chroma.2023.464409
Received 28 July 2023; Received in revised form 6 September 2023; Accepted 20 September 2023
Available online 21 September 2023

higher detection limits of IRMS compared to common MS instruments, it is crucial to introduce sufficient amounts of the analytes to meet the sensitivity level. This issue is associated to the approximately 100 times lower natural relative abundance of ^{13}C compared to that of ^{12}C. On the other hand, the sample amount cannot be increased over a certain level, to prevent column overloading. Different techniques have been developed, attempting to find the optimum on-column sample amount as a compromise between sensitivity and column capacity. Preparative layer chromatography was first exploited by Braunsdorf *et al.*, to obtain concentrated fractions to be injected into the GC-C-IRMS [10]. More recently, Schipilliti *et al.* developed three different GC-C-IRMS methods to evaluate the $\delta^{13}C$ values of the main terpenes in lemon essential oils. In some cases it was also possible to discern fraudulent additions, as well as to distinguish between different geographical origins [11]. Concerning the use of chiral chromatography, an Es-GC-C-IRMS approach was first reported by Mosandl and co-workers, for the differentiation of natural and synthetic products [12], and later by few more research groups [13,14].

The enantiomeric and carbon isotopic ratios of the target compounds could be determined simultaneously, within a single GC analysis. Despite the undeniable advantages of such approach, even in terms of analysis time, some drawbacks have seriously limited its widespread diffusion. Dealing with medium-high complexity samples, compounds with very similar physico-chemical properties may co-elute along a chromatogram. Such an issue may be even more pronounced in the case of chiral separations. Although the selection of a suitable cyclodextrin-based stationary phase can efficiently resolve the enantiomers of a chiral couple, overlapping with achiral components may still occur. A partial co-elution may not largely affect the enantiomeric ratio calculation, conversely it impairs the isotopic evaluation, severely. As already reported by our research group, the uneven distribution of carbon isotopes along an entire peak of CO_2 leads to unreliable (shifted) $\delta^{13}C$ values with respect to conditions where baseline separation is achieved [15,16]. To overcome these limitations, multidimensional gas chromatography (MDGC) may represent the best option, thanks to the higher peak resolution achieved by the employment of two columns with different selectivity.

Enantio-selective multidimensional gas chromatography (Es-MDGC), exploited in the heart-cut mode, was first reported by Schomburg *et al.* [17], selecting specific time windows in the first achiral dimension for a further chiral separation in the second one. Starting from this first study, several works have been carried out, also in the field of *Citrus* essential oils [18,19]. To this regard, Sciarrone *et al.* [18] demonstrated the capability of an Es-MDGC system to solve the co-elutions in conventional Es-GC analysis of mandarin essential oils. Later on, Hong *et al.* [19] employed an Es-MDGC system for the detection of four specific chiral couples in lemon essential oils. Conversely, very few works have dealt with the simultaneous determination of both the enantiomeric and the $\delta^{13}C$ evaluation for target compounds [20–25], due to the need for a baseline resolution before IRMS detection.

To this aim, an Es-MDGC-C-IRMS/qMS approach was developed in this research, employing an apolar and a chiral stationary phase in the first and second dimensions, respectively. By these means, the enantiomeric excess and the $\delta^{13}C$ of the enantiomers of the main chiral terpenes in lemon essential oils could be evaluated simultaneously, for the first time, together with the $\delta^{13}C$ of the main achiral compounds.

2. Materials and methods

2.1. Samples

Fourty genuine cold-pressed Sicilian lemon essential oils were provided by local producers in the season 2022/2023 and were stored at +4 °C. The samples were diluted 1:10 in *n*-hexane prior to GC analysis. A C_7-C_{30} *n*-alkane mix, kindly provided by Merck Life Science (Darmstadt, Germany), was used for the calculation of Linear Retention Index (LRI) values. For the calibration of the $\delta^{13}C$ with respect to VPDB scale, the CO_2 reference gas was calibrated using three certified alkanes from Indiana mix A7, namely hexadecane ($\delta^{13}C$ −26.15 ‰), octadecane ($\delta^{13}C$ −32.70 ‰) and eicosane ($\delta^{13}C$ −40.91 ‰) (Indiana University, Bloomington, IN). Isotopic ratio measurements of the samples of interest were made by the following formula:

$$\delta^{13}C = \frac{Rsample - Rstandard}{Rstandard}$$

where R represents the abundance ratio of the heavier carbon isotope against the lighter one ($^{13}C/^{12}C$).

2.2. Instrumental parameters

2.2.1. Monodimensional chiral GC–MS

GC–MS analyses were performed on a GC2010 Plus gas chromatographer equipped with an AOC-20i autosampler and coupled to a QP2010 Ultra quadrupole mass spectrometer (Shimadzu Europa, Duisburg, Germany). The split/splitless injector was maintained at 230 °C, at a split ratio of 30:1. The MEGA-DEX ASX 1 column, 25 *m* × 0.25 mm *I.D.* × 0.25 µm *f.t.* (MEGA, Milano, Italy), was used as chiral stationary phase at a column flow of 0.8 mL/min. The oven temperature was ramped from 50 to 220 °C at 2 °C/min. The qMS ion source and interface temperature were maintained at 200 and 220 °C, respectively; a mass range 40–400 *m/z* was monitored at an acquisition speed of 10 Hz. GCMS data were acquired by the GCMS solution software ver. 4 (Shimadzu Europa, Duisburg, Germany).

2.2.2. Multidimensional GC-C-IRMS/qMS conditions

The MDGC-C-IRMS/qMS prototype was already described in previous works [16,21]. Briefly, two GC-2010 Plus gas chromatographs (defined as GC1 and GC2) were connected by means of a heated transfer line (Shimadzu Europa, Duisburg, Germany). GC1 was equipped with a split/splitless injector, a flame ionization detector (FID) and a Deans-switch (DS) transfer device, connected to an advanced pressure control unit (APC), which supplied the same carrier gas (He) allowing to divert the first column eluent to the FID or to the second column in the GC2. The latter was hyphenated in parallel to a QP2010 Ultra quadrupole mass spectrometer (Shimadzu Europa, Duisburg, Germany) and to a VisION IRMS system by means of a GC V furnace system (Elementar Analysensysteme GmbH, Langenselbold, Germany) maintained at 830 °C.

For the current application, the split/splitless injector was maintained at 280 °C, split ratio 10:1, delivering to the ^1D column, an SLB-5 ms 30 *m* × 0.25 mm *I.D.* × 0.25 µm *f.t.* (Merck Life Science, Darmstadt, Germany), at a constant helium flow of 1.0 mL/min. A pressure program was used, from 185 kPa (5 min) to 261 kPa at 1.89 kPa/min, to 330 kPa at 7.28 kPa/min. The GC1 oven was ramped as follows: 50 °C (5 min) to 170 °C at 3 °C/min, finally to 250 °C (4 min) at 15 °C/min. The FID (330 °C; H_2 flow, 40.0 mL/ min; air flow rate, 400 mL/min; sampling rate, 80 ms equal to 12.5 Hz) was connected to the DS device via a 0.25 *m* × 0.18 mm *I.D.* stainless steel uncoated column and used to monitor the ^1D eluent. GC2 was equipped with a MEGA-DEX ASX 1 chiral column 25 *m* × 0.25 mm *I.D.* × 0.25 µm *f.t.* (MEGA, Milano, Italy), and the temperature was ramped as follows: 40 °C (10 min) to 82 °C at 2 °C/min, to 220 °C (4.9 min) at 7 °C/min. The ^2D column was connected at one side to the DS device and at the other side to a zero dead-volume tee-union (Valco).

The effluent was split on one side to the combustion chamber and to the IRMS system via a 0.85 *m* × 0.25 mm *I.D.* uncoated column while the other part was directed to the qMS via a 2 *m* × 0.1 mm *I.D.* uncoated column. A pressure program was applied to the APC device in order to maintain a constant carrier flow also in the ^2D column (≈1 mL/min): 140 kPa (10 min) to 189 kPa at 1.39 kPa/min, to a final pressure of 265 kPa at 8.04 kPa/min. The qMS ion source and interface temperature

were maintained at 200 °C; the mass range 40–400 *m/z* was monitored at an acquisition speed of 10 Hz. The target compounds were identified by searching their qMS spectra against the FFNSC 4.0 mass spectral library database (Shimadzu Europa, Duisburg, Germany), using a double filter based on spectral similarity and on a range of Linear Retention Index (LRI).

The VisION IRMS (Elementar Analysensysteme GmbH, Langenselbold, Germany) was a bench top 5 kV system equipped with an integrated monitoring gas delivery system. The combustion chamber was provided with a high performing silicon carbide tube furnace for the quantitative, fractionation-free conversion of the compounds to pure gases (CO_2 and H_2O). The CO_2 produced by pyrolysis of each component was transferred to the IRMS, while the water produced was removed through a Nafion membrane. The system was designed with reduced dead volumes to maintain the chromatographic integrity of the separated compounds, preserving the chromatographic resolution at the IRMS. An auxiliary He line (sample line He), automatically controlled through a second channel of the APC unit, was used in the furnace to allow a proper control over the open split conditions for the IRMS. The following settings were applied to the VisION system: acceleration voltage, 3795.007 V; trap current, 600.000 μA; magnet current, 3700.000 mA. The APC was operated in constant flow mode, to maintain the open split in a steady state. An electron-ionization (EI) gas source, a variable field, stigmatically focused electromagnet for beam separation

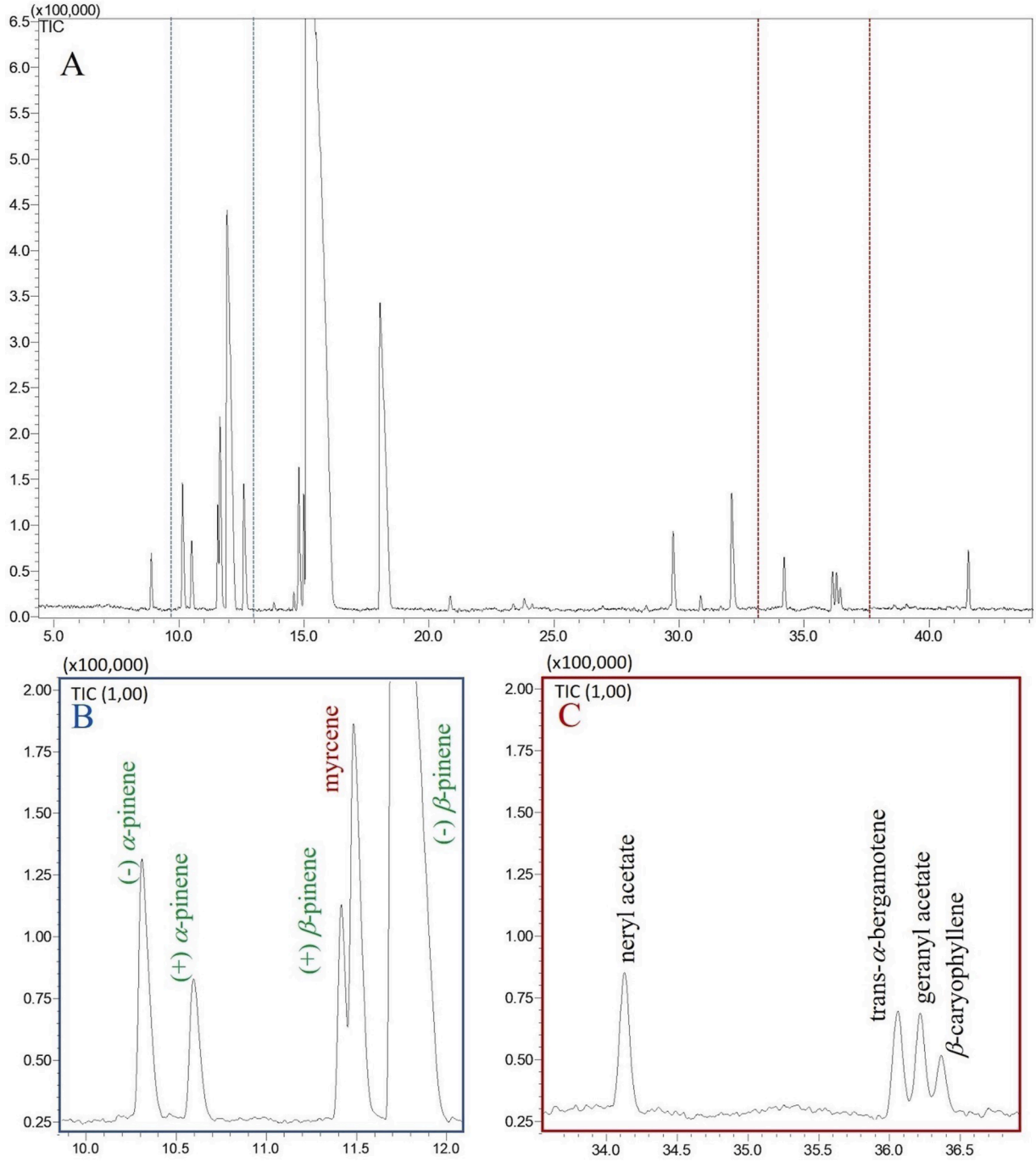

Fig. 1. (A) monodimensional Es-GC–MS profile of a lemon essential oil; (B) and (C) expanded retention windows showing some critical co-elutions amongst the sample components.

and multi-channel Faraday collectors for beam detection were used. IRMS data were collected by IonOS stable isotope data processing software ver. 3.0.0.5196 (Elementar Analysensysteme GmbH, Langenselbold, Germany); the ratio offset integration method was exploited to automatically find the correct starting and ending point of the peaks. All the analyses were led at least in triplicate and standard deviations for IRMS measurements were found to be < 0.5.

3. Results and discussion

3.1. Development of an Es-MDGC-C-IRMS/qMS approach

In a recent study, three different chromatographic runs were necessary to achieve reliable δ^{13}C measurement of high, medium and trace amount lemon essential oil components, respectively [11]. Three different sample amounts were injected on-column in separate mono-dimensional GC-C-IRMS runs, adjusting the sample concentration to allow measurement of the principal components, as well as the low concentrated ones and the trace components. Whilst such a method ensured a reliable evaluation of the target compounds, it was time-consuming and obviously, a further run was necessary to attain the chiral separation. As a result, this approach also involved an increased consumption of sample, helium used as carrier gas and last but not least, electricity.

To this concern, the current research aimed to reduce the total analysis time, cost, and electricity consumption by developing a greener analytical approach. The simultaneous evaluation of δ ^{13}C and enantiomeric ratios of the lemon oil sample components was obtained within a single analysis. Such accomplishment required the baseline separation of all the target components, including the chiral ones. The mono-dimensional Es-GC-MS analysis of a lemon essential oil is shown in Fig. 1A. While satisfactory resolution was obtained for all the enantiomers of the sample chiral terpenes, yet co-elutions occurred between single enantiomers and achiral sample compounds. In Fig. 1B, (+) β-pinene co-elutes with myrcene, hampering a reliable determination of the enantiomeric ratio of β-pinene, and even more of the δ^{13}C value of (+) β-pinene. Fig. 1C shows a series of partial co-elutions involving achiral sample components, namely *trans*-α-bergamotene, geranyl acetate and β-caryophyllene. Different from MS detection, where diagnostic fragments can still be monitored by means of extracted ion or single ion monitoring (SIM), this is not feasible in IRMS due to the conversion of all the organic components to CO_2 prior to detection. Moreover, the renowned chromatographic isotopic effect of CO_2 leads to unreliable δ^{13}C values whenever co-elutions occur [5,16].

An MDGC approach was then implemented, to increase the system peak capacity (Fig. 2). Additional separation was afforded by the coupling of an apolar capillary column in the first dimension (^{1}D), to the chiral stationary phase employed as second dimension (^{2}D). Since qualitative information could not be obtained by the ^{1}D detector (FID), each peak of interest was identified by GC–MS based on the elution order on the same apolar stationary phase. The experimental LRIs (LRI_{exp}) were calculated for each peak of interest after a ^{1}D stand-by (no heart-cuts) analysis and compared to the theoretical values (LRI_{theor}) in the FFNSC 4.0 GC–MS database. A positive identification with the aid of LRI as additional filter was assumed at ± 5 LRI units tolerance (Table 1) [21].

The transfer of entire peaks from ^{1}D to ^{2}D avoids any chromatographic fractionation, and to this regard the consistence of retention times in ^{1}D analysis is crucial. It is well known that different Deans switch devices can be affected by retention time shifts of the peaks after each heart-cut, and this is due to changes in ^{1}D column back-pressure during the transfer. Hereby, the peculiar configuration of the Deans switch device and the high repeatability typical of the apolar column ensured the repeatability of retention times. Thus, narrow heart-cut windows were selected, for the effective transfer from ^{1}D to ^{2}D [6]. Fig. 3 shows a data comparison between a *st-by* (black trace) and a *cut* (pink trace) analysis of a lemon essential oil. Eleven heart-cut windows were selected for the transfer of α-thujene, α-pinene, β-pinene, limonene, γ-terpinene, linalool, neral, geranial, neryl acetate, geranyl acetate, *trans*-α-bergamotene and β-bisabolene.

Fig. 4 shows the ^{2}D IRMS chromatogram obtained after the chiral separation. All the target components in the lemon oil sample were baseline resolved, allowing for determining the enantiomeric ratio in chiral terpenes and the δ^{13}C of all the target compounds, simultaneously. Twelve terpenes were investigated in the samples, including seven achiral (γ-terpinene, neral, geranial, neryl acetate, geranyl acetate, *trans*-α-bergamotene, β-bisabolene) and five chiral compounds (α-thujene, α-pinene, β-pinene, limonene, and linalool).

Amongst the latter, only the levorotatory form of α-thujene was observed, being (+) α-thujene below the detection limit. The

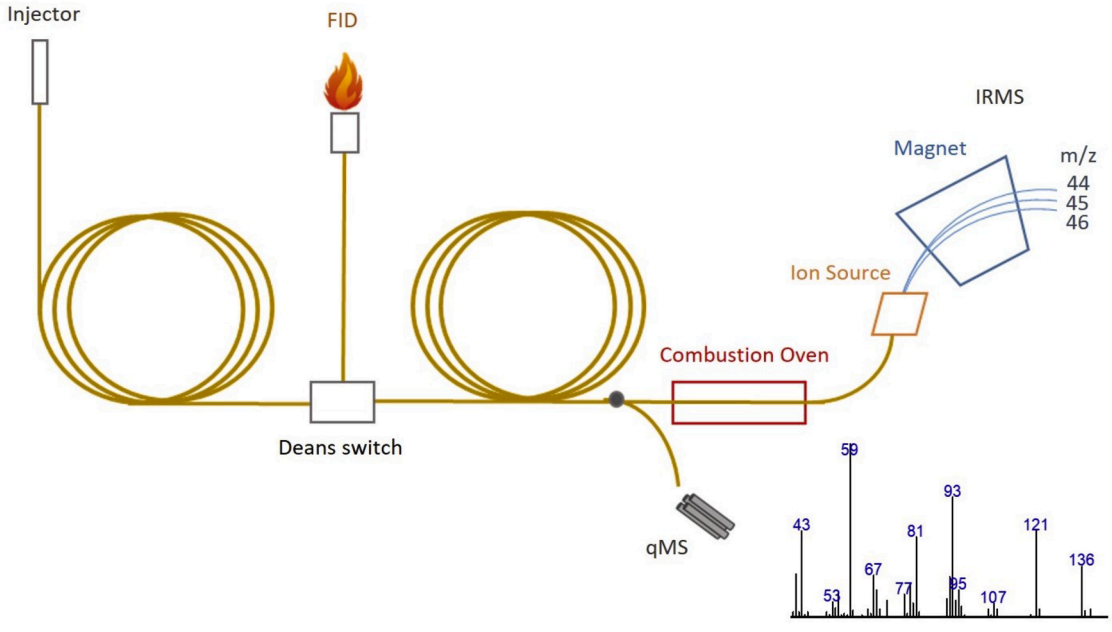

Fig. 2. Scheme of the Es-MDGC-C-IRMS/qMS system.

Table 1
Enantiomeric and isotopic data of the key terpenes investigated in the lemon essential oil samples, and their Linear Retention Indices (LRI). Experimental Linear Retention Indices (LRI_{exp}) were calculated on a SLB-5 ms column, while theoretical LRI (LRI_{theor}) are referred to the FFNSC 4.0 MS database, on the same stationary phase. *Tentatively assigned according to ref. [4]. nd: not determined.

Target compounds	LRI_{theor}	LRI_{exp}	Enantiomeric ratio range	$\delta^{13}C$ range
(+) α-thujene*	927	930	<1.00	nd
(−) α-thujene*			>99.00	−25.8
				−28.4
(−) α-pinene	933	935	64.10–71.64	−27.7
				−30.0
(+) α-pinene			35.90–28.36	−29.8
				−31.7
(+) β-pinene	978	980	4.96–8.67	−27.9
				−30.8
(−) β-pinene			95.04–91.33	−25.0
				−27.3
(−) Limonene	1030	1032	1.65–2.02	nd
(+) Limonene			98.35–97.98	−25.9
				−28.0
γ-terpinene	1058	1060	–	−27.8
				−30.9
(-) Linalool	1101	1103	48.00–75.66	nd
(+) Linalool			52.00–24.34	nd
Neral	1238	1242	–	−25.7
				−28.3
Geranial	1268	1272	–	−25.9
				−28.3
Neryl acetate	1361	1363	–	−29.2
				−32.5
Geranyl acetate	1380	1382	–	−29.6
				−32.5
trans-α-bergamotene	1432	1434	–	−28.8
				−31.8
β-bisabolene	1508	1511	–	−28.6
				−31.3

enantiomeric ratio of α-thujene was then obtained by means of the extracted ion chromatogram ($m/z = 93$) by qMS (Figure S1 in Supplementary Materials). With respect to previous research works [4], similar enantiomeric trends were found for all the compounds investigated, as shown in Fig. 5. It can be appreciated that an enantiomeric excess of the levorotatory forms was found amongst all the samples investigated, apart for limonene which showed an opposite trend.

In detail, an enantiomeric excess higher than 96% was observed for (+) limonene (see Table 1), and this data were in agreement with the study of Gionfriddo et al., employing the same MDGC approach [4]. Both (-) α-pinene and (-) β-pinene were in excess with respect to the dextrorotatory forms, in the sampling August 2022–April 2023, with the highest enantiomeric excess registered in the months of August and September. In the case of α-thujene, a constant enantiomeric excess (98 %) was registered for the levorotatory form, along the entire harvesting season. The data measured for linalool showed significant variations amongst the sampling period, as appreciable in Fig. 5. The max enantiomeric excess was observed for (-) linalool in the month of August and September, then it decreased reaching a racemic behaviour from December onwards.

For the determination of $\delta^{13}C$ data, only the signals > 0.5 nA were considered for (-) α-thujene, (-) and (+) α-pinene, (-) and (+) β-pinene, (+) limonene, γ-terpinene, terpinolene, neral, geranial, neryl acetate, geranyl acetate, *trans*-α-bergamotene and β-bisabolene. Notably, the $\delta^{13}C$ of γ-terpinene, representing one of the main lemon essential oil constituents, was determined herein for the first time. Otherwise, $\delta^{13}C$ values of (-) and (+) linalool were not taken into consideration, due to the very low signal intensities (< 0.5 nA). Also in the case of (+) α-thujene, as discussed earlier, the low concentration was below the IRMS detection limit, and this prevented $\delta^{13}C$ measurement. Last, $\delta^{13}C$ was not evaluated for (-) limonene, due to the co-elution still occurring in the 2D separation. Such an issue is well known in the literature, and could be addressed by transferring only a portion of the ^{1}D peak in the MDGC approach [25]. Unfortunately, in this case this would have resulted in isotopic fractionation of the peak, hampering the reliable determination of $\delta^{13}C$ data [16].

Overall, the results reported in Table 1 for the genuine lemon essential oils were in agreement with the literature, both in terms of enantiomeric [4] and isotopic ratios [11]. However, dealing with the $\delta^{13}C$ ratio of specific enantiomers, an unexpected isotopic data was found for the enantiomers of α and β-pinene, in all the samples

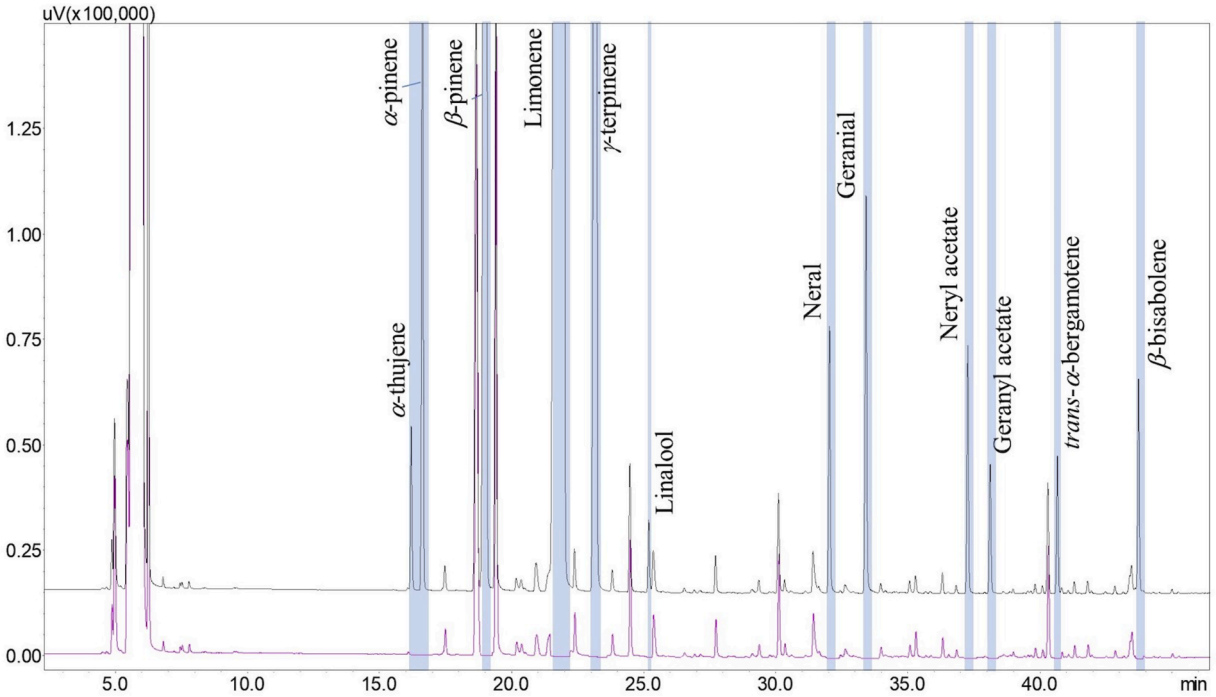

Fig. 3. Data comparison between ^{1}D *st-by* (black trace) and ^{1}D *cut* (pink trace) analysis in the MDGC configuration.

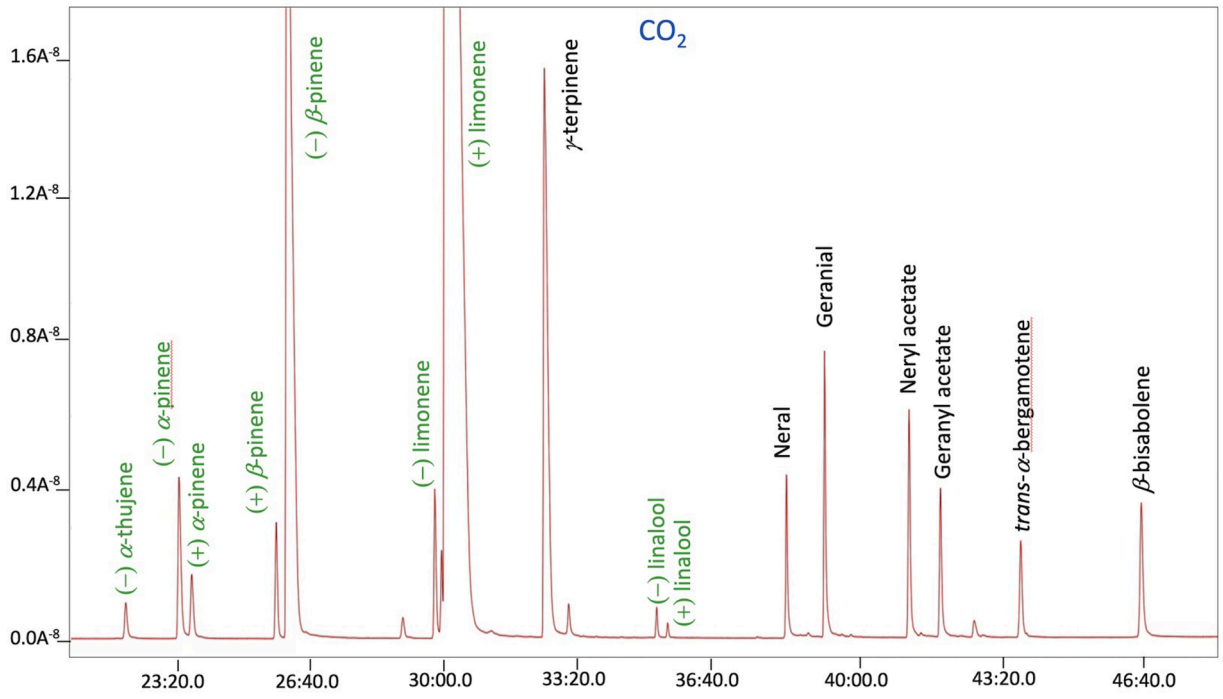

Fig. 4. ^{2}D IRMS profile of the selected terpenes components in a lemon essential oil. Green: enantiomeric terpenes, black: achiral terpenes.

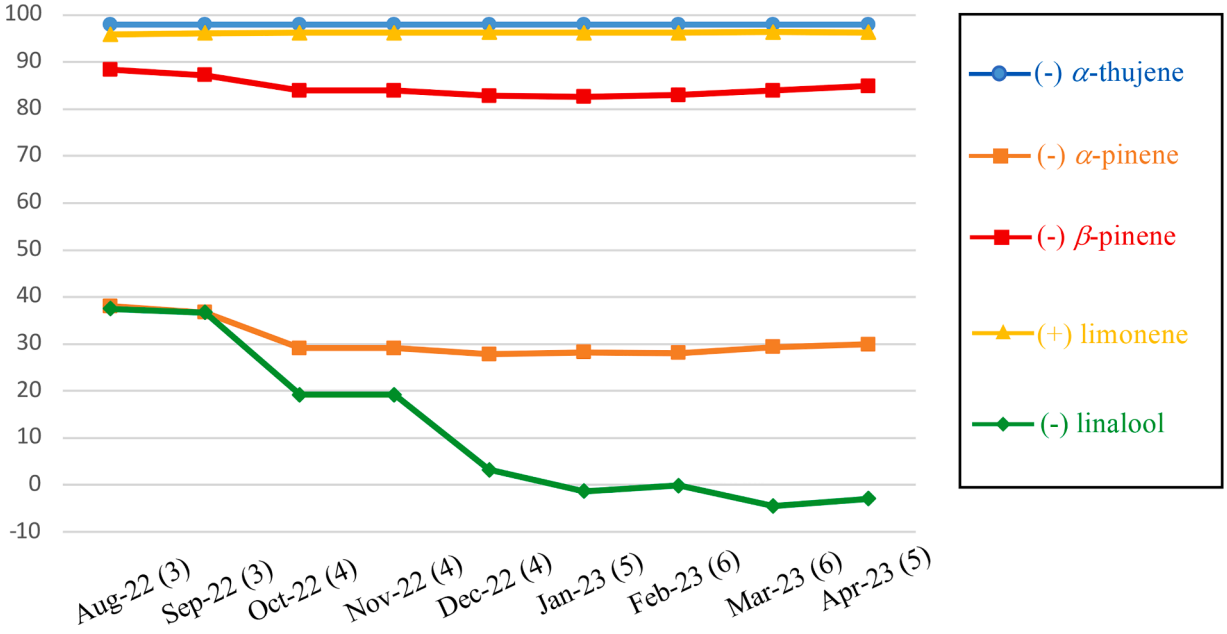

Fig. 5. Enantiomeric excess determined in lemon essential oil, showing the trends over the sampling period August 2022 - April 2023. The number of samples analysed for each period is reported in brackets.

investigated.

Fig. 6 shows a higher isotopic fractionation of the dextrorotatory forms with respect to the levorotatory ones, for both α and β-pinene enantiomers. In this sample, the difference between the two enantiomers was 1.7 ‰ for α-pinene and 2.4 ‰ β-pinene, significantly higher than the 0.5 ‰ (precision) tolerance [5]. A deep discussion about these results is provided in the next paragraph.

3.2. Isotopic fractionation of terpene enantiomers

The first hyphenation of enantio-selective GC to IRMS was demonstrated by Mosandl's research group [12], who has played a pioneering role in the field of flavour and fragrance analysis. Their studies focused on the analysis of key odorants, aiming to differentiate between genuine and adulterated samples by evaluation of the enantiomeric and isotopic ratios. Besides, the δ^{13}C of each enantiomer can be determined by Es-GC-C-IRMS, as discussed in the previous section. Most of the literature studies have reported consistent δ^{13}C values between enantiomers of a chiral compound, and to this regard the findings in Section 3.1 of this study were in disagreement [20–25]. Indeed, since two enantiomers originate from the same enzymatic pathway, identical ^{13}C/^{12}C ratios should be expected. Accordingly, authenticity studies

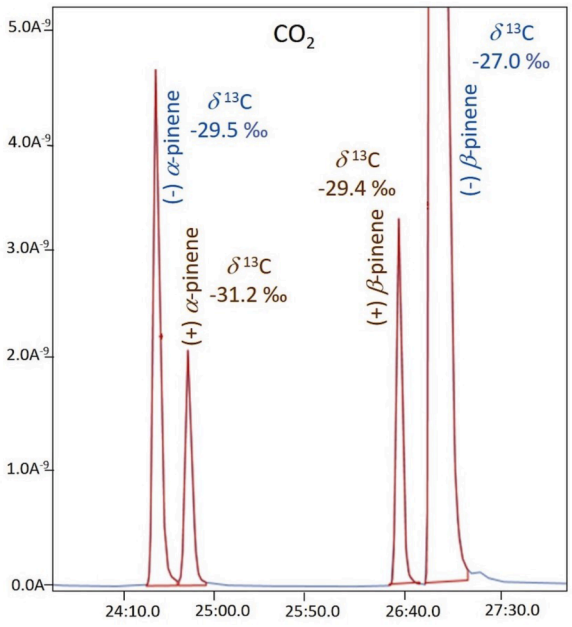

Fig. 6. 2D IRMS enlarged chromatogram relative to the separation of α and β-pinene enantiomers and the chiral isotopic fractionation observed.

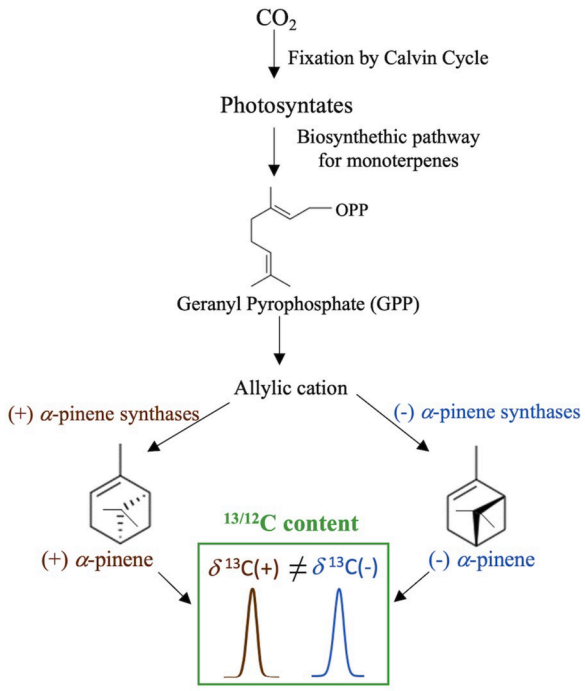

Fig. 7. Proposed chiral isotopic fractionation process in lemon essential oils for the dextrorotatory (brown) and levorotatory (blue) forms of α-pinene.

regard the detection of enantiomers with different $\delta^{13}C$ values as an indication of a reconstituted oil [23]. Only one report on genuine dill oil analysis has reported different isotopic data for the enantiomers of a key volatile, produced in a specific part of the plant [26]. In this concern, whilst the enantiomers of limonene produced in dill herbs had very similar $\delta^{13}C$ values, contrarily the enantiomers of limonene produced in dill buds were found to have an unexpected difference in terms of $\delta^{13}C$, *viz.* > 3 ‰. From a biochemical perspective, a review of Rodney Croteau [27] has illustrated the biosynthetic steps of the main terpene components, starting from the same achiral precursor (geranyl pyrophosphate, GPP). After the conversion of GPP to an allylic cation, the generation of a chiral centre leads to two parallel pathways in terpene biosynthesis. Starting from the two newly formed chiral reaction intermediates, different metabolic pathways are involved in the biosynthesis of the specific enantiomers, as in the case of (-) and (+) α-pinene [27,28]. Later on, Lucker *et al.* have provided interesting new insights about terpene biosynthesis in lemon essential oils [29]. Briefly, the authors demonstrated that four different terpene synthases of flavedo were responsible for the production of more than 90% of the characteristic terpenes in the oil, and that the final enantiomeric excess was the result of the different enzyme activities. More recently, the intercorrelation between terpene biosynthesis and carbon isotopic fractionation has been demonstrated [30]. Subsequent biosynthetic steps involve the formation and breakage of carbon-carbon bonds, resulting in kinetic carbon isotope effects. The final result will be a distinct isotopic ratio for each terpene compound, directly related to its biosynthetic pathways. Such findings provide a rational basis for interpretation of the isotopic differences observed between enantiomers of the same chiral compound. Based on such observations, a brief scheme of chiral isotopic fractionation occurring for α-pinene enantiomers is proposed in Fig. 7.

According to IRMS principles, CO_2 uptake by the plant results in a depletion of ^{13}C in the resulting metabolites, associated to the photosynthetic cycle. Following the discussion in Section 3.2, the different enzymatic pathways may be considered responsible for the chiral isotopic fractionation in pinene enantiomers.

In this study, a higher isotopic fractionation was evidenced for the levorotatory forms of α- and β-pinene in all the lemon samples analysed, as shown in Fig. 8. Apart from a certain variability along the production season, a constant trend was observed for all the samples, with higher isotopic fractionation (more negative $\delta^{13}C$ values) of the dextrorotatory forms.

Considering the different harvesting periods, the highest variation for α-pinene enantiomers was observed in the month of August, with differences in the $\delta^{13}C$ values > 2 ‰. In the other periods, the $\delta^{13}C$ values were between 1.5 ‰ (September, December, January, and February) and 2 ‰ (October and November) on average. The fractionation was more evident for the enantiomers of β-pinene: a maximum of 3.5 ‰ was observed in the middle (November) and at the end of the season (February) while in the other periods was between 2.5 ‰ on average (August, September, October, December, and January).

4. Conclusions

In the current study, the simultaneous measurement of both enantiomeric and isotopic data of different terpene compounds in lemon essential oil was achieved for the first time. With respect to existing IRMS-based methods, the total time analysis was significantly reduced since both measurements were obtained within a single GC analysis. An MDGC approach was implemented to attain baseline separation of the enantiomers of interest, for further evaluation of their distinct carbon isotopic ratios. The latter may also represent key information to depict the biosynthetic pathways within the plant of origin. Specifically, the differences observed for α- and β-pinene $\delta^{13}C$ enantiomers suggest a characteristic isotopic fractionation related to a specific biosynthetic pathway of the lemon plant. This finding paves the way for further developments in the field of authenticity assessment, since different essential oil types may present typical chiral isotopic fractionation also related to the geographical origin. Future studies will be devoted to the collection of a higher number of samples, aiming to expand this approach also to different *Citrus* fruits for authenticity studies.

CRediT authorship contribution statement

Lorenzo Cucinotta: Validation, Formal analysis, Investigation, Writing – original draft. **Gemma De Grazia:** Investigation, Formal analysis. **Paola Donato:** Writing – review & editing, Visualization.

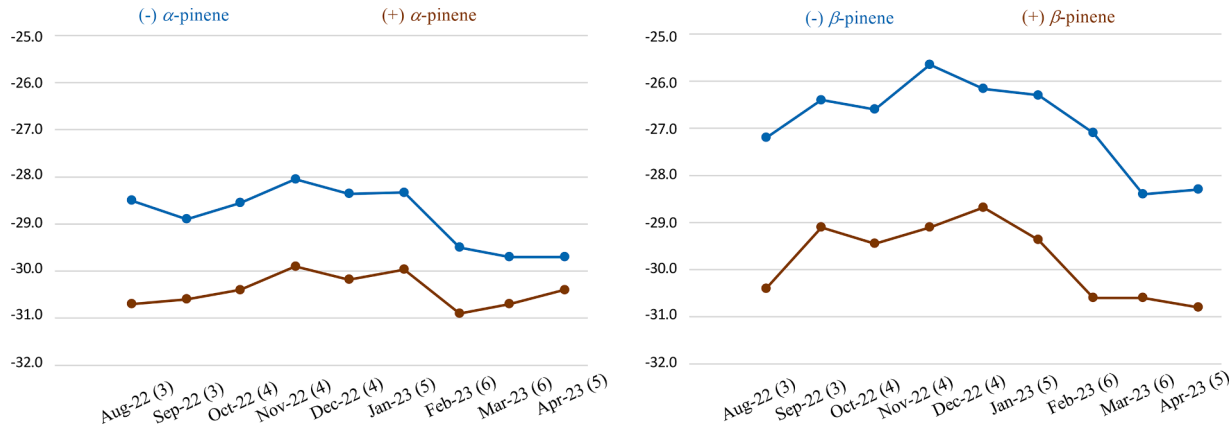

Fig. 8. $\delta^{13}C$ trends of α- and β-pinene enantiomers in the lemon essential oils analysed. The number of samples analysed for each period is reported in brackets.

Monica Mondello: Validation, Data curation. **Danilo Sciarrone:** Supervision, Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Luigi Mondello:** Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464409.

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