

Characterization of volatile compounds in *Mentha spicata* L. dried leaves

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Abstract: Gas chromatography-mass spectrometry (GC-MS) is one of the most common techniques used to measure and to characterize volatile organic compounds (VOCs). At the same time, the proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS) represents a recent innovative tool that allows the on-line monitoring of VOCs providing the whole mass spectra with short response time, high mass resolution and without sample preparation. We reported as major constituents of headspace in spearmint dried leaves the monoterpenes carvone, followed by dihydrocarvone, limonene and 1,8-cineole (eucalyptol) although other monoterpenes such as α -pinene, 3-carene, terpineol and neodihydrocarveol, alcohols (3-octanol, 1-octen-3-ol and 3-methyl-1-butanol), esters and ketones (3-octanone) were detected in low concentrations. In this study, the analytical GC-MS and PTR-ToF-MS techniques allowed to characterize the entire volatile profile of the sample bypassing the limitations of each tool.

1. Introduction

Recently, aromatic plants have received great attention in several fields such as agroalimentary, food, perfumes, pharmaceutical and natural cosmetic products (Baatour *et al.*, 2010). Among aromatic plants, mint is an herbaceous rhizomatous perennial plant belonging to the Lamiaceae family, widely used since ancient times for its characteristic flavor and aroma. Belonging to the *Mentha* genus, spearmint (*Mentha spicata* L.), cornmint (*M. arvensis* L.) and peppermint (*M. piperita* L.) are the species commonly used as spices in food preparation and nowadays represent one of the most important spices through-

out the world marketed fresh or dried. The drying process is used to increase shelf-life and to prevent some biochemical reactions that may alter the organoleptic characteristics. Moreover, mint essential oil is commonly used as flavoring food agent, perfumery, cosmetic and pharmaceutical products (Díaz-Maroto *et al.*, 2003). The chemical composition of spearmint essential oil depends on plant genetic structure, growth conditions and agronomic practices (Sangwan *et al.*, 2001; Figueiredo *et al.*, 2008). In the past, the spearmint essential oil has been intensively investigated using gas chromatography (GC) technique and the mainly compounds identified are carvone, monoterpene synthesized and stored in glandular thricomes (Gershenzon *et al.*, 1989), followed by limonene and 1,8 cineole (Díaz-Maroto *et al.*, 2003; Antal *et al.*, 2011; Orio *et al.*, 2012) while the monoterpene menthol is one of the main con-

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stituents of *M. piperita* essential oil (Orio *et al.*, 2012). Surprisingly, few studies have been carried out to assess the aroma composition of fresh or dried spearmint leaves (Díaz-Maroto *et al.*, 2003; Antal *et al.*, 2011; Silva and Câmara, 2013).

From several years the conventional sample technique used to analyze the aroma of plant material was the headspace solid phase microextraction (HS-SPME) (Arthur and Pawliszyn, 1990) coupled to GC-MS technique. This procedure has been introduced in early 90s, as a powerful solvent-free sample preparation technique where the volatile fraction is collected by exposing a fiber, coated with single or multiple polymers, directly to the headspace above the sample. Subsequently, the fiber is introduced into the injection chamber of a gas chromatograph and high temperatures allow the release of the analytes into the chromatographic column.

Recently, a new instrument has been developed such as proton transfer reaction mass spectrometry (PTR-ToF-MS), which allows the on-line monitoring of volatile compounds and the achievement of the whole mass spectra of complex volatile matrices with short response times, high mass resolution and without sample preparation (Vita *et al.*, 2015). From one side, the PTR-ToF-MS advantages include high sensitive with a rapid detection system (response time of about 100 ms) and very poor fragmentation of the volatile molecules (Taiti *et al.*, 2017). By the other side, the main disadvantage is represented by the difficulty to separate volatile compounds with same chemical formula, being the unique identification of the VOCs not always possible (Taiti *et al.*, 2017).

For the first time, in the present study two mass spectrometry techniques (HS-SPME/GC-MS and PTR-ToF-MS) are reported with the aim to evaluate and to characterize the aroma compounds of spearmint dried leaves.

2. Materials and Methods

Plant material

Mentha spicata seeds were germinated in fine peat in a growth chamber at 24°C, 68% humidity with a 10/14 h day/night cycle. Subsequently, seedlings were transplanted into pots (40 cm head diameter × 20 cm height), filled with sandy loam soil and transferred in greenhouse (University of Florence, DIS-PAA). After planting, plants were grown and maintained for 50 days in a greenhouse at day/night temperatures 26-30°C and 18-21°C, respectively and 80%

humidity. The plants were irrigated every 2 days and fertilized after 10-25-40 days of growth. Forty five days after planting, midlife leaves were collected from each experimental unit and dried at 45°C for 24 h using a common heater. Subsequently, the dried leaves were finely chopped with a grinder (Mulinex AR 11, Groupe SEB, France) with a particle size about 1.0 mm.

Determination of volatile composition

PTR-ToF-MS analysis. The volatile profile of spearmint dried leaves was assessed by PTR-ToF-MS (PTR TOF 8000 model, Ionicon Analytik GmbH Innsbruck, Austria) using H₃O⁺ as reagent ion for the proton transfer reaction. For a detailed explanation of the PTR-ToF-MS technology see Blake *et al.* (2009). For sample preparation and experimental setup has been followed the procedure previously used by Taiti *et al.* (2016). Briefly, one-gram aliquots of chopped spearmint dried leaves were transferred into a glass jar (2/3 l) suitable for volatile analysis and provided to screw caps with two holes respectively connected with Teflon pipes to zero air generator (Peak Scientific) and PTR-ToF-MS. The employed setup allows the formation of a dynamic headspace sampling system with a constant air flow of 0.3 l min⁻¹ and a constant humidity and temperature (75% UR and 24°C), which are critical parameters for VOCs determination (Mancuso *et al.*, 2015). Mass spectra were recorded in the mass-to-charge (m/z) range of m/z 20-220 with a time resolution of 1 s. The tool worked with the following parameters in the drift tube: pressure 2.3 mbar, voltage 594 V, temperature 110°C, extraction voltage at the end of the drift tube (U_{dx}) 35 V and was operated at an E/N value of 140 (1 Td = 10217 V cm²). The inlet line, consisted of a peek capillary tube (internal diameter 0.40 mm), was maintained at 110°C in order to minimize the adsorption of chemicals onto surface (Lanza *et al.*, 2015). Subsequently, the raw data and peak extraction were acquired and processed by the TofDaq software (Tofwerk AG, Switzerland), using a procedure previously reported by Taiti *et al.* (2016). In order to guarantee high mass accuracy for the post processing and for a precise conversion of time-of-flight into m/z values, the internal calibration was performed off-line, using together with known low mass ions (NO⁺ peak m/z = 29.997 and C₃H₇O⁺ peak m/z = 59.049) a known compound with high mass ion as the carvone (C₁₀H₁₅O⁺ peak m/z = 151.075). In this way the exact masses have been assigned and determined the sum formula of all VOCs recorded with the PTR-ToF-MS.

Finally, once determined the sum formula, each peak has been tentatively assigned to a specific compound, with the help of the results obtained by GC-MS analysis, and of the available literature on volatile compounds emitted by mint products. Raw data acquired by the PTR-ToF-MS were processed and analyzed using a TofDaq software (Tofwerk AG, Switzerland) and the methodology used for data processing is reported elsewhere (Taiti *et al.*, 2017).

HS-SPME/GC-MS procedure. As reported by Silva and Câmara (2013), the trivalent fiber divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) was the most effective SPME fiber able to isolate the volatile compounds from *M. piperita* and *M. spicata* fresh leaves. In our analysis, a 2 cm trivalent SPME fiber (DVB/CAR/PDMS) was used to extract the volatile fraction from the *M. spicata* dried leaves. SPME sampling device was manually inserted into the sealed vial and the fiber was exposed to headspace above the sample for 20 min at 25°C. After extraction the fiber was insert into the injector port of the GC-MS system, an Agilent 7890 a GC equipped with a 5975C MSD, where the volatile compounds where thermally desorbed at 280°C and then transferred to the analytical capillary column. The analytes separation was achieved with a Agilent DB InnoWAX, length 50 m, 0.20 µm id, 0.40 µm df column. Chromatographic conditions were: initial temperature 40°C for 0.5 min, then 6°C min⁻¹ up to 260°C. This temperature was maintained for 1 min. Only the compounds with higher intensity were identified in order to support the PTR-ToF-MS analysis; the identification was based on mass spectra matching with the standard NIST08/Wiley98 libraries.

3. Results and Discussion

The aim of our research was a comparison among PTR-ToF-MS and conventional HS-SPME/GC-MS techniques performed simultaneously on spearmint dried leaves. As reported in recent literature surveys, GC-MS can be considered the main method for the analysis of volatile fractions. The HS-SPME/GC-MS analysis led to the identification of the most representative compounds from a quantitative point of view, and the typical gas chromatogram obtained is reported in figure 1A. Consequently, over fifty volatile compounds were identified (Fig. 1A). Among these, the most abundant compounds were, in order of abundance: terpenes (with carvone, dihydro-carvone, D-limonene and eucalyptol or 1,8-cineole as the most represented); alcohol (mainly 1-octen-3-ol and 3-methyl-1-butanol); ketones (e.g. 3-octanone); esters (e.g. 2-methyl-ethyl ester-butanoic acid) (Fig. 2). The same compounds were reported by Silva and Câmara (2013) in the volatile fraction of spearmint fresh leaves analyzed using the same method.

The data obtained by PTR-ToF-MS analysis were filtered following the procedure previously described by Taiti *et al.* (2017); firstly all peaks imputable to water chemistry and to interfering ions (e.g. oxygen, nitrogen monoxide) and secondly the peaks whose average concentrations were lower than 1 ppbv (parts per billion by volume) were eliminated. After filtering, a total of 54 masses were revealed and could be tentatively attributed to compounds such as aldehydes, ketones, esters, terpenes and other unidentified compounds in the range of measured masses m/z from 20 to 220 (Fig. 1B, Table 1). As

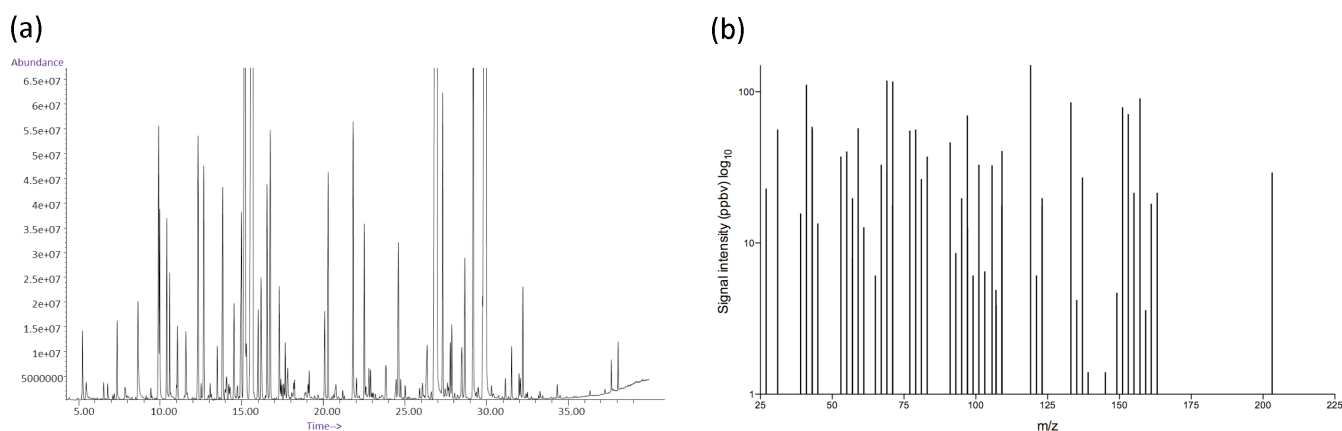


Fig. 1 - a) Typical gas chromatogram obtained by volatile fraction of *M. spicata* dried leaves obtained with HS-SPME/GC-MS using a DVB/CAR/PDMS fiber. b) Snapshot of the mass spectra of headspace of spearmint dried leaves obtained with PTR-ToF-MS.

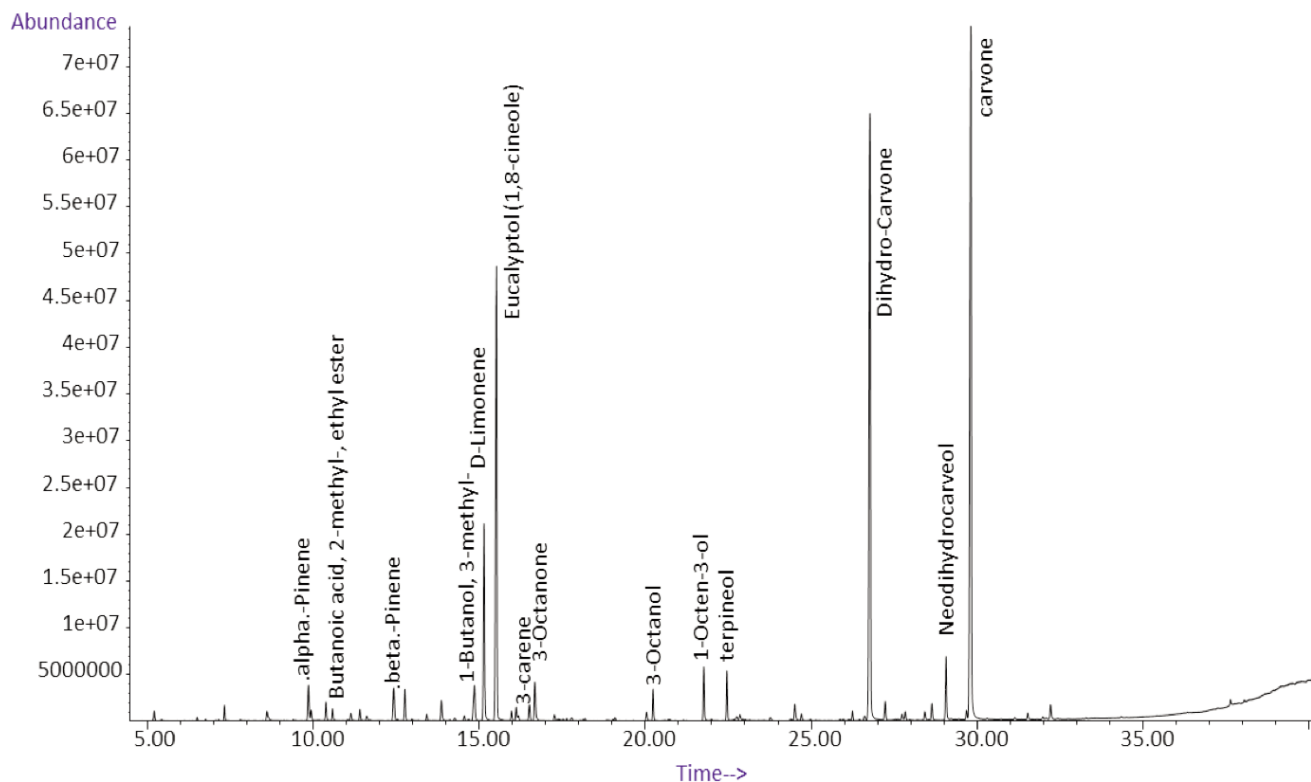


Fig. 2 - Mass spectra enlargement of widest class compounds identified by HS-SPME/GC-MS.

reported in Materials and Methods section, the detected VOCs were integrated with the fragmentation patterns of each compound (Maleknia *et al.*, 2007; Kim *et al.*, 2009; Brilli *et al.*, 2011) and with the results of the GC-MS-based profile. Indeed, previous researches performed on different matrices demonstrated the good agreements with gas-chromatographic peaks and PTR-MS mass spectral data (King *et al.*, 2010; Aprea *et al.*, 2015). Generally, the correlation between GC-MS and PTR-ToF-MS data allows the characterization of the sample entire volatile profile bypassing the limitations of each tool.

All compounds identified and their signal intensity are listed in Table 1, together with their m/z ratio, chemical name, molecular formula, percentage on the total, and additional reference for peak identification. The most abundant signals detected with PTR-ToF-MS (>3% of the total) were: alkyl fragment ($m/z = 41.038$), isoprene ($m/z = 69.069$), 3-methyl-1-butanol ($m/z = 71.085$), dimethyl-furan ($m/z = 97.065$), *p*-cymene fragments ($m/z = 119.085$), *p*-cymenene ($m/z = 133.101$), carvone ($m/z = 151.075$), dihydrocarvone ($m/z = 153.126$), neodihydrocarveol together with menthol ($m/z = 157.155$) (Table 1). Most terpenes volatile compounds were reported in

previous articles and identified as the main constituents of spearmint aroma (Kokkini *et al.*, 1995; Díaz-Maroto *et al.*, 2003; Orio *et al.*, 2012). Alcohols, esters and aldehydes were also detected in spearmint dried leaves even though in low content. Interestingly, 3-methyl-1-butanol ($m/z = 71.085$) was detected using both technique while some compounds such as 3-octanone, 1-octen-3-ol and 3-octanol were identified only with HS-SPME/GC-MS (Fig. 2, Table 1).

As reported by Kokkini *et al.* (1995) spearmint aroma is mainly formed by a complex mixture of various VOCs, mainly terpenes such as carvone, limonene, and 1,8-cineole that are the most important aroma constituents due to their high percentage in the volatile fraction. Indeed, terpenes (monoterpenes and sesquiterpenes) and terpenoids are the common constituents of flavor and fragrance of some plants and in particular of spearmint aroma (Telci *et al.*, 2010). Terpenes are hydrocarbons (carbon and hydrogen are the only elements present) whereas terpenoids have been denatured by oxidation and thus contain additional functional groups. Regarding monoterpenes ($m/z = 137$ and 135) and sesquiterpenes ($m/z = 205$) hydrocarbons, their

Table 1 - Volatile compounds identified via PTR-ToF-MS

m/z	Tentative identification	Protonated chemical formula	Signal intensity (ppbv)	Compound (% on the total)	References
27.022	Acetylene	C ₂ H ₃ ⁺	22.9 ± 6.4	1.11	Vita et al. (2015)
31.018	Formaldehyde	CH ₃ O ⁺	56.3 ± 5.2	2.79	Kushch et al. (2008)
39.022	Isoprene fragment	C ₃ H ₃ ⁺	15.7 ± 2.2	0.74	Maleknia et al. (2007)
41.038	Alkyl fragment	C ₃ H ₅ ⁺	111.3 ± 12.7	5.81	Vita et al. (2015)
43.018	Alkyl fragment	C ₂ H ₃ O ⁺	58.7 ± 21.5	2.9	Vita et al. (2015)
43.054	Alkyl fragment (propene)	C ₃ H ₇ ⁺	56.3 ± 5.2	2.66	Taiti et al. (2017)
45.033	Acetaldehyde	C ₂ H ₅ O ⁺	13.5 ± 6.8	0.77	Taiti et al. (2016)
53.04	Cyclobutadiene	C ₄ H ₅ ⁺	37.3 ± 0.1	1.83	Vita et al. (2015)
55.055	C ₄ aldehydes fragment	C ₄ H ₇ ⁺	40.3 ± 5.1	1.98	Taiti et al. (2017)
57.033	Alkyl Fragment (2-hexenal)	C ₃ H ₅ O ⁺	8.0 ± 2.4	0.38	Brilli et al. (2011)
57.07	Alkyl fragment (Hexanol/valeric acid)	C ₄ H ₉ ⁺	19.8 ± 0.2	0.97	Aprea et al. (2015)
59.049	Propanal, acetone	C ₃ H ₇ O ⁺	57.4 ± 17.2	2.7	Taiti et al. (2016)
61.028	Acetates	C ₂ H ₅ O ⁺	12.7 ± 4.6	0.62	Taiti et al. (2016)
65.039	Alkyl fragment	C ₅ H ₅ ⁺	6.1 ± 0.2	0.3	Goacher et al. (2010)
67.055	Terpene fragment	C ₅ H ₇ ⁺	33.0 ± 3.0	1.62	Yener et al. (2015)
69.033	Furan	C ₄ H ₅ O ⁺	29.9 ± 6.4	1.47	Taiti et al. (2017)
69.069	Isoprene	C ₅ H ₉ ⁺	118.8 ± 24.4	6.06	Taiti et al. (2015)
71.049	2-butenal	C ₄ H ₇ O ⁺	17.7 ± 2.2	0.87	Vita et al. (2015)
71.085	3-Methyl-1-butanol/Pentenal fragment	C ₅ H ₁₁ ⁺	117.3 ± 4.6	5.81	Aprea et al. (2015)
77.04	Alkyl fragment	C ₆ H ₅ ⁺	55.4 ± 36.8	2.7	Goacher et al. (2010)
79.054	Benzene	C ₆ H ₇ ⁺	56.3 ± 5.2	2.66	Vita et al. (2015)
81.069	1,3-cyclohexadiene/terpenes fragment	C ₆ H ₉ ⁺	26.5 ± 6.8	1.35	Taiti et al. (2016)
83.086	C ₆ compounds/hexenol fragment	C ₆ H ₁₁ ⁺	37.3 ± 0.1	1.83	Maleknia et al. (2007)
91.054	Alkyl fragment	C ₇ H ₇ ⁺	46.3 ± 5.1	2.27	Yener et al. (2015)
93.068	Terpene fragments	C ₇ H ₉ ⁺	8.6 ± 2.4	0.42	Maleknia et al. (2007)
95.085	Terpene fragments	C ₇ H ₁₁ ⁺	19.8 ± 0.2	0.97	Aprea et al. (2015)
97.065	Dimethyl-furan	C ₆ H ₉ O ⁺	69.8 ± 17.2	3.91	Yener et al. (2015)
97.101	Alkyl fragments	C ₇ H ₁₃ ⁺	12.7 ± 4.6	0.62	Aprea et al. (2015)
99.08	Cyclohexanone/hexenal	C ₆ H ₁₁ O ⁺	6.1 ± 0.2	0.3	Buhr et al. (2002)
101.096	3-hexen-1-ol	C ₆ H ₁₃ O ⁺	33.0 ± 3.0	1.7	Buhr et al. (2002)
103.101	2-hexanol	C ₆ H ₁₅ O ⁺	6.5 ± 1.0	0.32	Buhr et al. (2002)
105.069	Styrene	C ₈ H ₉ ⁺	32.7 ± 1.6	1.6	Yener et al. (2015)
107.049	Benzaldehyde	C ₇ H ₇ O ⁺	4.9 ± 1.2	0.24	Yener et al. (2015)
107.086	Ethylbenzene	C ₈ H ₁₁ ⁺	3.9 ± 0.7	0.19	De Gouw et al. (2003)
109.065	Phenylmethanol/benzenemethanol/p-cresol	C ₇ H ₉ O ⁺	17.6 ± 0.3	0.86	Leskinen et al. (2015)
109.101	Terpene fragments	C ₈ H ₁₃ ⁺	40.6 ± 0.6	1.99	Maleknia et al. (2007)
119.085	p-cymene fragments	C ₈ H ₁₁ ⁺	153.2 ± 10	7.68	Maleknia et al. (2007)
121.101	Sesquiterpene fragments	C ₉ H ₁₃ ⁺	6.1 ± 1.0	0.3	Demarcke et al. (2009)
123.08	4-ethylphenol	C ₈ H ₁₁ O ⁺	17.5 ± 2.7	0.86	Chatonnet et al. (2009)
123.116	Sesquiterpene fragments	C ₉ H ₁₃ ⁺	19.8 ± 2.1	0.97	Demarcke et al. (2009)
133.101	p-cymenene	C ₁₀ H ₁₃ ⁺	85.2 ± 10.7	4.18	Kus and Van Ruth (2015)
135.135	p-cymene	C ₁₀ H ₁₅ ⁺	4.2 ± 0.8	0.21	Maleknia et al. (2007)
137.137	Monoterpenes compounds (limonene, 3-carene)	C ₁₀ H ₁₇ ⁺	27.1 ± 9.3	1.18	Maleknia et al. (2007)
139.111	2-pentylfuran/nopinone	C ₉ H ₁₅ O ⁺	1.4 ± 0.2	0.07	Wisthaler et al. (2001)
145.135	Octanoic acid	C ₈ H ₁₇ O ₂ ⁺	1.4 ± 0.1	0.07	Aprea et al. (2015)
149.135	Sesquiterpene fragments	C ₁₁ H ₁₇ ⁺	4.7 ± 1.0	0.23	Kim et al. (2009)
151.075	Monoterpenes oxygenate (carvone, myrtenal, carvacrol)	C ₁₀ H ₁₅ O ⁺	79 ± 13.1	3.74	Kim et al. (2009)
153.126	Monoterpenes oxygenate (carveol, dihydrocarvone)	C ₁₀ H ₁₇ O ⁺	71.3 ± 1.3	3.45	Maleknia et al. (2007)
155.145	Alcohol monoterpenes (neodihydrocarveol/terpineol)	C ₁₀ H ₁₉ O ⁺	21.5 ± 8.8	3.07	Bourtsoukidis et al. (2014)
157.155	Decanol/Menthol	C ₁₀ H ₂₁ O ⁺	90.6 ± 26.8	4.55	Vita et al. (2015)
159.135	Nonanoic acid/C9 ester	C ₉ H ₁₉ O ₂ ⁺	3.6 ± 1.5	0.18	Aprea et al. (2015)
163.148	Sesquiterpene fragments	C ₁₂ H ₁₉ ⁺	18.2 ± 3.9	0.38	Demarcke et al. (2009)
203.18	Sesquiterpene compounds (trans-calamenene)	C ₁₅ H ₂₃ ⁺	21.5 ± 4.9	0.89	Taiti et al. (2017)
205.195	Sesquiterpenes compounds	C ₁₅ H ₂₅ ⁺	29.3 ± 7.9	1.15	Taiti et al. (2015)

Data are expressed in ppbv as mean ± deviation standard, n=3.

amount was similar and represented about 2.5% of total compounds detected by PTR-ToF-MS, while terpenoids (m/z 151, 153, 155, 157) were the most abundant (about 15%) (Table 1).

Finally, considering the entire dataset and following the odor descriptor (www.thegoodscentscompany.com), the spearmint aroma might be due to the interaction between compounds that give a different odors such as: minty (e.g. carveol, 1,8-cineole, carvone), citrus (e.g. limonene), spicy (e.g. carvacrol, menthol, p-cymene, terpineol), green or herbaceous (e.g. 3-hexen-1-ol, hexenal, hexenol), fruity (e.g. 3-methylbutanoate, ethyl 2-methyl butyrate) and floral notes (e.g. phenylmethanol).

4. Conclusions

PTR-ToF-MS has been shown to be a very useful tool to detect VOCs emitted by dried herbs. The results obtained in this study demonstrated that PTR-ToF-MS technique coupled with the GC-MS for the identifications of isomers, can be a usefully screening tool in order to (1) assess the volatile compounds of very complex samples such as aromatic herbs and (2) achieve a fast and simple fingerprinting. This technique is able to provide the whole mass spectra of complex trace gas mixtures with short response times, high mass resolution and without sample preparation. Moreover, this technique, when coupled with HS-SPME/GC-MS, allows the overcoming of the disadvantage related to the identification of VOCs. In summary, the spearmint aroma might be due to the interaction between compounds with minty (e.g. carveol, 1,8-cineole, carvone), citrus (e.g. limonene), spicy (e.g. carvacrol, menthol, p-cymene, terpineol), green or herbaceous (e.g. 3-hexen-1-ol, hexenal, hexenol), fruity (e.g. 3-methylbutanoate, ethyl 2-methyl butyrate) and floral notes (e.g. phenylmethanol).

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