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Short note

HPLC/DAD, GC/MS and GC/GC/TOF analysis of Lemon balm (*Melissa officinalis* L.) sample as standardized raw material for food and nutraceutical uses

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Key words: aromatic compounds, comprehensive two-dimensional (2D) gas chromatography, HS-SPME-GC×GC-TOF fingerprint analysis, hydroxycinnamic acids, lemon balm, phenols.

Abstract: *Melissa officinalis* L., commonly known as lemon balm, is a perennial herb belonging to Lamiaceae family. Traditionally administered in infusion form, it has therapeutic properties, such as sedative, carminative and antispasmodic, but also it is used for treatment of headache, rheumatism, indigestion and hypersensitivities. Lemon balm has a complex chemical composition. The aim of this work was the comprehensive characterization of secondary metabolites of a dried Lemon balm (*Melissa officinalis* L.) sample, through HPLC/DAD, GC/MS and GC/GC/TOF analysis, as raw material for the standardized phyto-complexes production useful for food and nutraceutical application. This sample contained rosmarinic acid (caffeic acid dimer) as the main compound of phenolic fraction (32.4 mg g^{-1}). Citronellal was the most abundant compound in the volatile fraction, followed by α-citral and β-caryophyllene. The total citral amount, in terms of sum of α- and β-citral, was 149.4 mg_{citral} kg^{-1}. Comprehensive two-dimensional GC fingerprint analysis of lemon balm produced rationalized peak patterns for up to 200 volatile compounds.

1. Introduction

The Lamiaceae are a promising source of natural antioxidants due to the large amount of phenolic acids found in many species of this family (Ziaková *et al*., 2003). *Melissa officinalis* L., commonly known as lemon balm, is a perennial herb belonging to Lamiaceae family. Lemon balm is used as aromatic, culinary and medicines and is also used by food industry to flavour different products owing to its particular taste (López *et al*., 2009). The raw plant samples originating from *M. Officinalis* L. species are also used in the traditional medicine for the treatment of headache, flatulence, colic, nausea, indigestion, anaemia, nervousness, vertigo, malaise, asthma, bronchitis, syncope, amenorrhea, cardiac failure, insomnia, epilepsy, depression, psychosis, hysteria, ulcers and wounds (WHO, 2004; Karasová and Lehotay, 2005; Dastmalchi *et al*., 2008). Therefore, its aqueous and alcoholic extracts are traditionally used for their spasmolytic, nervous sedative, antiviral and antioxidant activities (López, *et al*., 2009; Atanassova *et al*., 2011; de Carvalho *et al*., 2011; Lin *et al*., 2012). All of these properties of lemon balm have been related to the high levels of phenolic acids found in this species, mainly hydroxycinnamic acid derivatives such as rosmarinic acid (Fecka and Turek, 2007). Some studies already reported other phenolic compounds in lemon balm. Heitz *et al*. (2000), isolated luteolin 3-*O*-glucuronide as the major flavonoid presented in *M. officinalis* from France. In 2002, Patora and Klimek isolated and determined the structure of six major flavonoids (apigenin and luteolin derivatives) in lemon balm from Poland based on spectral data. Lemon balm has a complex chemical composi-

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tion. It contains hydroxycinnamic acids (up to 6% of rosmarinic acid, *p*-coumaric and caffeic acids) and up to 0.37% of an essential oil composed of monoterpenes (more than 40%) and sesquiterpenes (more than 35%). Among the most significant terpenoids there are citral, citronellal, geraniol, nerol, linalool, farnesyl acetate, humulene, caryophyllene and eremophilene (ESCOP, 2003; WHO, 2004). The essential oil is considered to be a therapeutic principle usually responsible for most of the biological activities, such as spasmolytic, antimicrobial, antitumour andantioxidant ones, but also plant polyphenols, especially rosmarinic acid, are involved as well (Sadraei *et al*., 2003; de Sousa *et al*., 2004).

Phenolic acids are secondary metabolites, which create a large group of naturally occurring compounds, showing a broad spectrum of biological activities. The phenolic acids are important bioactive constituents of *M. Officinalis* L.; among them rosmarinic, caffeic, chlorogenic and ferulic acids are especially interesting. A literature screening showed that rosmarinic acid exhibits anti-inflammatory, antibacterial, and antiviral activities, it reduces atopic dermatitis and prevents Alzheimer's disease (Huang *et al*., 2009; Fujimoto and Masuda, 2012). Ferulic acid has strong antioxidant, antimicrobial, anti-inflammatory, anti-thrombotic, and anti-cancer activities (Peng *et al*., 2012). Chlorogenic acid shows anti-inflammatory, anti-bacterial, and anti-obesity properties (Sun *et al*., 2013), whereas caffeic acid has anti-inflammatory, antioxidative and immunomodulatory effects (Anwar *et al*., 2012).

Chemical quality evaluation of a herbal medicine should consist of two aspects (Jin *et al*., 2008). The first is identification and quantitation of one or more constituents that occurred in high quantities. The other is development of chemical fingerprint, which has been introduced and accepted by the WHO and other authorities as a strategy for quality assessment of herbal medicines (Chang *et al*., 2008; Li *et al.,* 2010). Among the separation methods, e.g. HPLC, HPTLC, GC and CE, which have been recognized as a rapid and reliable means for the identification and quantitation of the herbal medicine constituents, HPLC is the most popular method and is widely used in the fingerprint analysis (Kong *et al*., 2009; Peng *et al*., 2011). Up to now, coherent data is missing about the evaluation of quality consistency of the medicinal raw plant samples of *M. Officinalis* L. obtained from different manufactures.

The aim of this work is the comprehensive characterization of secondary metabolites of a dried lemon balm (*Melissa officinalis* L.) sample, through HPLC/DAD, GC/MS and the innovative GCxGC/TOF analysis, as raw material for the standardized phytocomplexes production useful for food and nutraceutical application. Therefore, in this study a combinative method is developed based on the reference HPLC, GC and GCxGC fingerprint and quantitation of selected phenolic and volatile compounds to assess the quality consistency of lemon balm.

2. Materials and Methods

Plant material

The dried vegetal tissues of *Melissa officinalis* L. were collected by Officinali Agribioenergia factory (Medicina, Bologna, Italy). The dried sample were finely chopped with a grinder (Mulinex AR 11, Groupe SEB, France) with a particle size of about 1.0 mm.

Determination of volatile composition and polyphenolic compounds

GC-MS analysis. In the first step of GC-MS analysis, HS-SPME-GC-MS was selected as the most suitable technique to recover and analyze the highest number of Volatile Organic Compounds (VOCs) in *Melissa officinalis* L. samples. Consequently, a dried foliar sample was ground and a homogenous powder was obtained. Fifty mg of the powdered sample, together with 2 g of NaCl and 5 mL of deionized water were placed into a 20-ml screw cap vial fitted with PTFE/silicone septa. After 5 min of equilibration at 60°C, VOCs were absorbed exposing a 2-cm trivalent SPME fiber (DVB/CAR/PDMS by Supelco) for 10 min into the vial headspace under orbital shaking (500 rpm) and then immediately desorbed at 280°C in a gas chromatograph injection port operating in split less mode. The chromatographic analysis was performed in a GC system coupled to quadrupole mass spectrometry using an Agilent 7890a GC equipped with a 5975C MSD. The separation of analytes was achieved by an Agilent DB InnoWAX column (length 50 m, id 0.20 µm, df 0.40 µm). Chromatographic conditions were: initial temperature 40°C, then 10°C min^{-1} up to 260°C, hold for 6.6 min. Compounds were tentatively identified by comparing calculated Kovats retention index and mass spectra of each peak with those reported in mass spectral databases, namely the standard NIST08/Wiley98 libraries. Each sample was analyzed in triplicate.

In the second step of GC-MS analysis, solid-liquid extraction followed by liquid injection was selected to

quantify the main VOCs identified by HS-SPME-GC-MS, namely citronellal, α-citral (geranial), β-citral (neral) and β-caryophyllene. To this aim, 0.5 g of powdered sample was extracted with 3 ml of heptane; the extraction was performed for 15 min in an ultrasound bath and for 24 hours in a shaker at 1000 rpm and 24°C. The mixture was than centrifuged at 6.000 rpm for 30 min and the supernatant was recovered and used for the GC-MS analysis, which was performed by the same chromatographic system used for the HS-SPME-GC-MS analysis. Each sample was analyzed in triplicate.

Two five-level calibration curves were built using β-caryophyllene standard (range 0.625-40 ppm, R^2 0.9961) and citral standard (range 1-100 ppm, R^2 0.9995). Citronellal, α-citral and β-citral was expressed as mg_{citral} kg^{-1}, and β-caryophyllene was expressed as $mg_{caryoph}$ kg^{-1}.

GCxGC-MS analysis. VOCs were absorbed exposing a 2-cm trivalent SPME fiber as described in GC-MS analysis. An Agilent 7890a GC equipped with a 5975C MSD was used and comprehensive GC×GC analyses were carried out on an Agilent GC 7890B, with an Agilent flow modulator system, coupled to an TOF-DS Markes detector. The analytes separation was achieved with a HP-5MS UI column (0.18x0.18mm, 20 min) coupled with a InnoWAX column 0.23x0.32 mm, 5 min. A tentative compounds identification was performed by comparing mass spectra of each peak with those reported in mass spectral databases.

Extraction and HPLC/DAD analysis of phenolic compounds. An aqueous extract was prepared adding 50 ml to 2.5 g of dried material. The solution was heated at 75°C and kept at that temperature for 60 min, then, the mixture was centrifuged and analyzed by HPLC/DAD.

Standards and solvents. Authentic standards of kaempferol 3-*O*-glucoside and rosmarinic acid were purchased from Extrasynthèse S.A. (Lyon, France), β-caryophyllene and citral were purchased from Sigma-Aldrich. All solvents used were of HPLC grade purity.

HPLC/DAD analysis. Analyses of flavonols and phenolic acids were carried out using an HP 1200 liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA). Compounds were separated using a 250x4.6 mm i.d., 5 mm LUNA C18 column (Phenomenex, USA). UV/Vis spectra were recorded in the 190-600 nm range and the chromatograms were acquired at 250, 280, 330 and 350 nm. The samples were analyzed by gradient elution at a flow rate of 0.8 ml/min. The mobile phase was a multi-step linear solvent gradient system, starting from 95% H_2O (adjusted to pH 3.2 by HCOOH) up to 100% CH_3CN in 55 min.

Identification and quantification of individual phenolic compounds. The identity of phenols was ascertained using data from HPLC-DAD, by comparison with bibliographic data and combination of retention times and UV/Vis spectra with those of authentic standards. The quantification of individual phenolic compounds was performed directly by HPLC-DAD using a five-point regression curve (r≥0.998) in the range of 0-30 mg on the basis of authentic standards. In particular, flavonols were determined at 350 nm using kaempferol 3-*O*-glucoside as a reference compound, while the phenolic acid derivatives were determined at 330 nm using rosmarinic acid as reference compound. Each sample was analyzed in triplicate.

3. Results and Discussion

GC-MS is widely recognized as the most suitable analytical technique for the analysis of VOCs; in particular, the HS-SPME-GC-MS allows the identification of the most representative compounds.

We selected the DVB/CAR/PDMS fiber since it proved to be the most universal assembly for sufficient isolation of compounds with different physic-chemical properties (Cui *et al*., 2009).

Figure 1 shows the Total Ion Chromatogram (TIC)

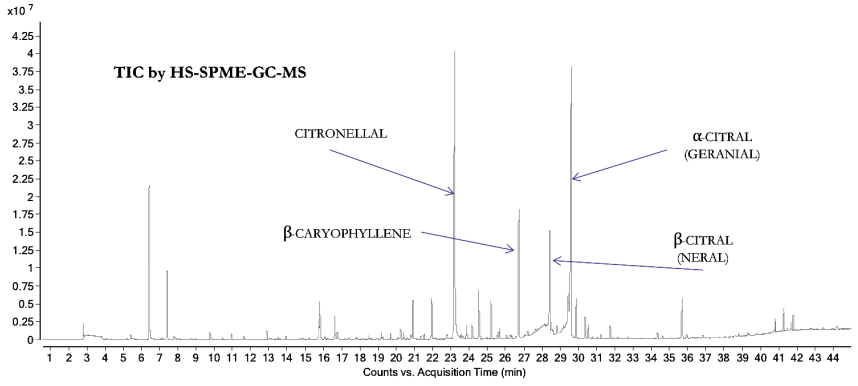

Fig. 1 - Total Ion Chromatogram (TIC) by HS-SPME-GC-MS analysis of *Melissa officinalis* L. foliar sample.

Table 1 - Relative abundance of the VOCs identified by HS-SPME-GC-MS analysis

rt (min)	Tentative identification	Abundance (% on the total)
23.2	citronellal	27.54
29.61	α-citrale (geranial)	25.00
26.73	β-caryophyllene	9.24
28.42	β-citrale (neral)	7.61
29.44	Germacrene D	3.00
24.55	linalol	2.82
35.7	caryophyllene oxide	2.70
15.81	(E)-2-hexenal	2.65
21.96	1-octen-3-ol	2.47
29.88	geranyl acetate	2.42
25.22	methyl citronellate	2.34
30.37	δ-cadinene	1.44
16.63	E-ocimene	1.35
30.55	γ-cadinene	0.91
23.87	copaene	0.88
31.75	geraniol	0.79
25.68	Isopulegol isomer B	0.64
20.24	(Z)-3-hexen-1-ol	0.57
16.77	3-octanone	0.52
34.34	β-ionone	0.48
25.58	Isopulegol isomer A	0.46
6.52	ethyl acetate	0.46
20.39	3-octanol	0.44
19.18	6-methyl-5-hepten-2-one	0.38
22.77	α-cubebene	0.36
19.7	2,6-dimethyl-5-heptenal	0.36
20.84	nonanal	0.33
11.61	hexanal	0.25
16.08	Z-ocimene	0.21
18.5	(E)-3-hexen-1-yl acetate	0.20
13.95	β-myrcene	0.17
37.78	nerol	0.15
17.86	methyl hex-2-enoate	0.10
17.81	octanal	0.10
10.44	2,5-diethyltetrahydro-furan	0.09
7.11	2-methyl-butanal	0.07
7.21	3-methyl-butanal	0.06
15.21	D-Limonene	0.05
9.85	1-penten-3-one	0.05
13.62	1-penten-3-ol	0.05
8.73	pentanal	0.05
4.40	dimethyl sulfide	0.04
15.02	3-methyl-1-butanol	0.04
18.34	(Z)-2-penten-1-ol	0.03
13.11	(E)-2-pentenal	0.03
13.37	5-methyl-hexanal	0.03
14.71	heptanal	0.03
16.41	(Z)-4-heptenal	0.02
5.22	2-methyl-propanal	0.02
8.6	2-pentanone	0.01

Data are expressed as area % on the total area of all the identified peaks.

obtained by HS-SPME-GC-MS analysis of the dried powdered foliar sample of *Melissa officinalis* L. Up to 50 Volatile Organic Compounds were identified and their abundance was reported as percentage on the total area of the identified peaks (Table 1). Terpenes were the most representative class of compounds, in terms of both number of molecules and relative abundance (15 monoterpenes, 71.91%; 8 sesquiterpenes, 19.01%). Among them, the most abundant compounds were citronellal (27.54%), α-citral (25.00%), β-caryophyllene (9.24%) and β-citral (7.61%). The other main classes of identified VOCs were 11 aldehydes (total 3.64%), 6 alcohols (3.60%), 4 ketones (0.96%) and 3 esters (0.76%).

Starting from these data, we selected the most representative VOCs from the HS-SPME-GC-MS analysis and quantified them in terms of mg kg^{-1} on dried foliar sample basis. To this aim, sample was extracted and analyzed by liquid injection GC-MS, as described in the experimental section. Table 2 summarizes the obtained results.

Table 2 - Content of the main VOCs by liquid injection GC-MS analysis

VOC	Amount
citronellal	119.2 (mg_{citral} kg^{-1})
α-citral (geranial)	109.7 (mg_{citral} kg^{-1})
β-citral (neral)	39.7 (mg_{citral} kg^{-1})
β-caryophyllene	74.3 ($mg_{caryoph}$ kg^{-1})

Data are the mean of three determinations (standard deviation <3%) expressed in mg kg^{-1} dry weight.

Citronellal was the most abundant compound in the heptanoic extract, followed by α-citral and β-caryophyllene. The total citral amount, in terms of sum of α- and β-citral, was 149.4 mg_{citral} kg^{-1}, that was more than citronellal amount.

HS-SPME and GC×GC-MS fingerprint analysis are ideal tools to analyze complex volatile matrices, and provide a sensitive method for the direct comparison and chemical visualization of plant volatile components.

GC×GC-MS is currently adopted as separation technique not only because of its high separation power and sensitivity but also for its ability to produce more widely distributed and rationalized peak patterns (Cordero *et al.*, 2008) for chemically correlated group of analytes.

HS-SPME GC×GC-TOF-MS analysis of the complex volatile fraction of lemon balm was submitted to

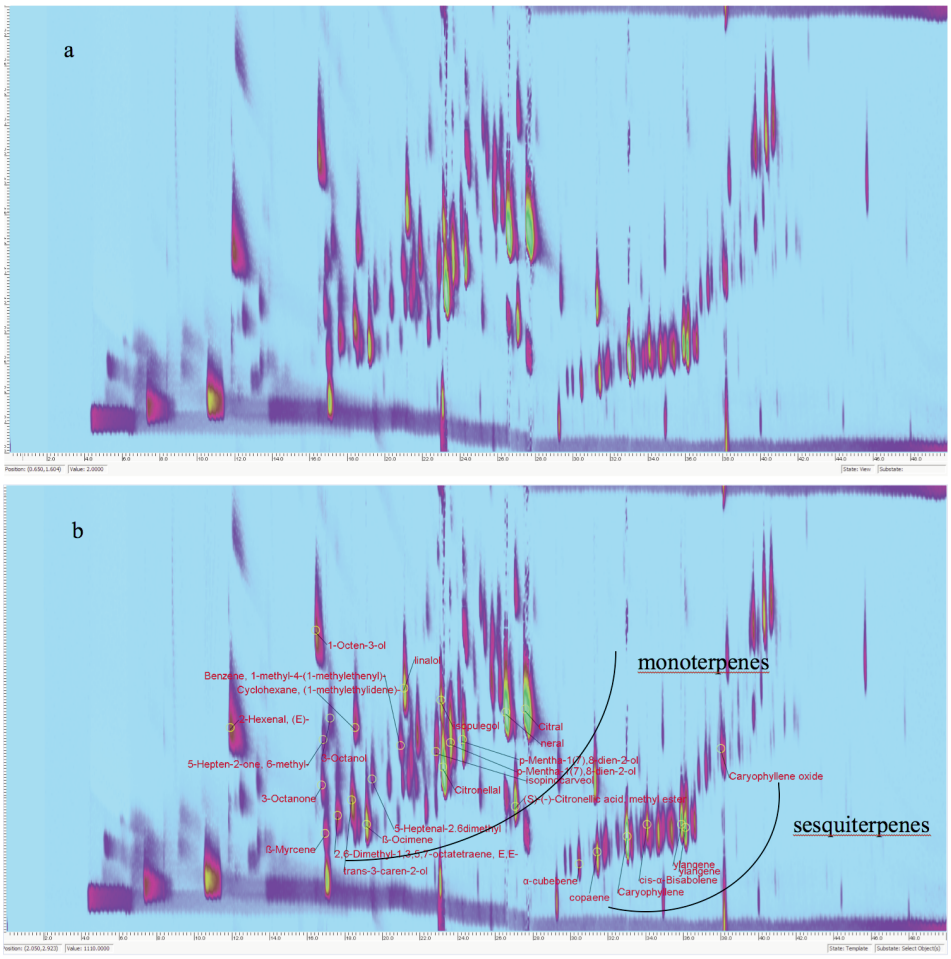

Fig. 2 - Contour plot from GC 2D-MS/TOF analysis of lemon balm. a: graphic view of the blobs/compound detected; b: sample template of the main compounds detected.

advanced fingerprinting analysis of 2D chromatographic data.

In figure 2a is reported a "contour plot" from GC 2D-MS/TOF analysis where the "blobs" correspond to a single volatile compound detected. 417 blobs were detected and, after subtracting base line blobs corresponding to fiber blending or background interferences, 203 blobs/compounds were identified.

An advanced, effective and reliable non-targeted analysis approach known as comprehensive template matching fingerprinting (Cordero *et al*., 2012) was adopted (Fig. 2b). This method considers, as comparative feature, each individual 2D peak together with its time coordinates, detector response and MS fragmentation pattern, and includes them in a sample template that is created by the analyst and can be used to compare plots from different samples directly and comprehensively. In this case a template was created for a comprehensive comparative analysis of 2D chromatographic data and to correctly interpret visual differences in further analysis.

The most intense blobs corresponded to citronellal, α-citral and β-citral as evidenced in the GC-MS analysis. Also the sesquiterpene β-caryophyllen was an intense blob. Up to 24 blobs/compounds belonging to the class of sesquiterpenes were distributed in a defined part of the contour plot (Fig. 2b). GC×GC-MS analysis produced distributed and rationalized peak patterns for sesquiterpenes, monoterpenes and oxigenated monoterpenes (Fig. 2b).

The analysis can be see also in the 3D view, showing the complex volatile fraction of lemon balm leaves (Fig. 3).

Data of the retention time and λ max in the visible region of specific standard (rosmarinic acid) obtained by HPLC-DAD analysis allowed the identification of phenolic compounds of the cultivated lemon balm sample. All these derivatives were calculated based on rosmarinic acid calibration curve (35.23 mg g^{-1}).

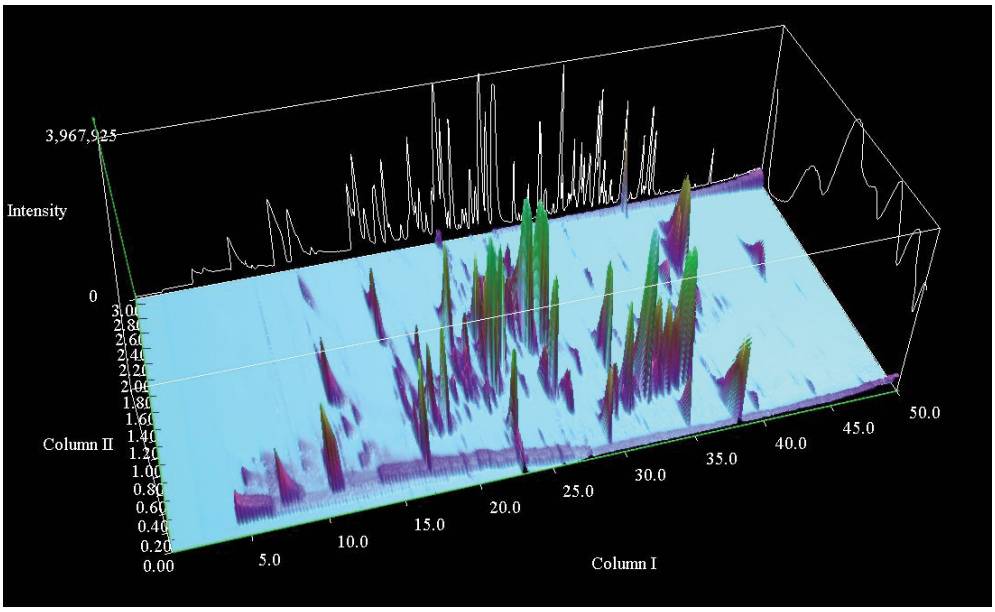

Fig. 3 - 3D view from GC 2D-MS/TOF analysis of lemon balm.

Data are the mean of three determinations (standard deviation <3%) expressed in mg g^{-1} dry weight. The lemon balm sample studied presented the rosmarinic acid (caffeic acid dimer) as the main compound. Few studies report the existence of rosmarinic acid as being the most abundant phenol in this species (Caniova and Brandsteterova, 2001; Ziaková *et al.*, 2003; Fecka and Turek, 2007; Lee, 2010).

The rosmarinic acid content was slightly high (32.4 mg g^{-1}) and in accordance with those reported by Fecka and Turek (2007) that presented values of rosmarinic acid in *M. officinalis* ranging from 32.6 to 5.1 mg g^{-1} of infusion.

It was also identified a flavone, the only flavonoid found in this sample (1.7 mg g^{-1}). This peak presented a UV spectra with λ max at 350 nm, and was tentatively identified as a luteolin derivative, described as the major flavonoid in *M. officinalis* by Heitz *et al*. (2000).

4. Conclusions

The results obtained, using integrated chromatographic techniques, allowed to evaluate the quali-quantitative content of secondary metabolites present in officinal species, such as *Melissa officinalis* L. Parameters evaluated, in particular the high-content of active ingredients, can be new values to be included in technical data sheets to define quality of raw materials for the production of standardized fraction in biomolecules content.

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