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Analysis of flavonoid diglycosides in leaves of *Mentha piperita* L by MALDI-MS/MS and LC-MS

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ABSTRACT

MALDI-MS/MS and LC-MS analyses were used for the investigation of flavonoid diglycosides in *Mentha piperita*. With eriocitrin, hesperidin, luteolin 7-o-rutinoside and diosmin identified in the butanolic extract in agreement with literature results, linarin was detected in peppermint for the first time in this work. The unambiguously identification of this compound was based on exact mass measurement, tandem mass spectrometry experiments and retention time data from LC analysis in comparison with its isomer fortunellin. Based on the use of available standards, low concentrations (mg g^{-1}) of flavonoid glycosides in the dry butanolic extract are measured: diosmin, 2.2 %, hesperidin, 0.5 % and linarin, 0.01 %. The low relative amount of linarin could maybe explain why this compound was hitherto not detected in peppermint.

KEYWORDS

Peppermint;
Flavonoids;
Linarin;
MALDI-MS/MS;
LC-MS.

INTRODUCTION

Flavonoids represent a large group of natural compounds with an impressive variety of biological activities, such as antioxidant[1], anti-inflammatory[2], antiallergic[3], antiplasmodial[4], anti-cancer[5], antimicrobial[6], antifungal[7], and neuroprotective[8] properties.

Many biological properties of *Mentha piperita* extracts are attributed to flavonoids. For instance, Sroka *et al*[9] reported that the strong antiradical and anti H_2O_2 activity of *Mentha piperita* from Poland are due to flavonoid diglycosides. Inoue *et al*[10] showed that luteolin-7-o-rutinoside is responsible of the antiallergic effect of *Mentha piperita*. From previous studies, it was demonstrated that the vast majority of flavonoids identified in polar extracts of peppermint (*Mentha piperita*) are diglycoside flavanones, such as eriocitrin, hesperidin and narirutin, and diglycoside flavones, such as luteolin-7-o-rutinoside, isorhoifilin and diosmin (Figure 1)[9,10]. As exemplified in Figure 1, these six flavonoid congeners are constituted by a 1-6 diglycoside moiety and then belong to the rutinose family. On the other hand, flavonoid diglycosides can also present a neohesperidoside form with a 1-2 diglucoside sequence.

Among all the analytical methods available for natural products analysis, mass spectrometry is now considered as the ideal tool given its intrinsic properties that are sensitivity, high resolution and specificity. Nowa-

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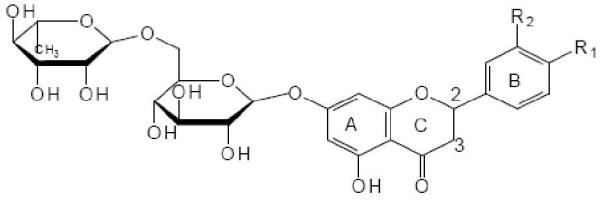
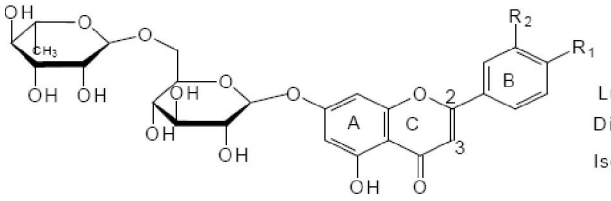
days, mass spectrometry offers a large panel of methodologies for the accurate mass measurements and the in-deep structural characterization of unknown molecules. In particular, tandem mass spectrometry (MS/MS) represents a powerful tool for structural characterization of ions (and molecules), and is mainly associated with the realization of collision-induced dissociation (CID) experiments[11]. Using the MS/MS procedure, flavonoids structural elucidations are nowadays well-defined[12,13a-13b].

The aim of the present study is to identify flavonoid diglycosides present in *Mentha piperita* from Burkina



Flavanone diglycosides:

	R^1	R^2	M.W.
Eriocitrin (1)	OH	OH	596
Hesperidin (2)	OCH_3	OH	610
Narirutin (3)	OH	H	580



Flavonoid diglycosides:

	R^1	R^2	M.W
Luteolin 7-O-rutinoside (4)	OH	OH	594
Diosmin (5)	OCH_3	OH	608
Isorhoifolin (6)	OH	H	578

Figure 1 : Structure of flavanone and flavone diglycosides previously identified in *Mentha piperita*

Faso by using MALDI-MS/MS (Matrix-assisted Laser Desorption/ionization Mass Spectrometry) and LC-MS (Liquid Chromatography Mass Spectrometry).

EXPERIMENTAL

Chemicals and plant material

Diosmin, hesperidin and eriocitrin (> 95%) were purchased from Sigma-Aldrich (Paris, France). Linarin and fortunellin were obtained from Extrasynthèse (Lyon, France). HPLC grade acetonitrile, methanol, formic acid and deionized water were used.

The aerial part of fresh peppermint was collected in 2010 March 26th in Ouagadougou (Burkina Faso) at the following GPS data: N 12°23'35, 8"; W 001°32'31,3".

Preparation of standards and calibration solutions

1 mg of each standard was firstly dissolved in 100 µL of dimethylsufoxide (DMSO) and then completed with 900 µL of CH_3CN/H_2O (50/50) acidified with formic acid (1%) to prepare stock solutions of 1 mg mL^{-1}.

For the establishment of the calibration curves, the stock solutions were dissolved with the same mixture of solvent (CH_3CN/H_2O/formic acid) to obtain solutions presenting concentrations in the range of 5 to 20 µg mL^{-1} for eriocitrin, 2.5 to 10 µg mL^{-1} for diosmin and hesperidin, and 0.1 to 0.5 µg mL^{-1} for linarin. The linearity of the calibration curves over the determined concentration range was checked for all the compounds.

Preparation of the extract

The plant material was washed with distilled water and then air dried at room temperature (72 hours) and powdered (size d" 1 mm). The powdered material (100 g) was extracted by refluxing in water (1 L, 3 hours). The filtrate was collected and filtered (Whattman N°1). The filtrate was then concentrated with a rotary evaporator to reach a final volume at about 500 mL. This solution was then extracted with hexane (3 x 300 mL). The resulting water layer was extracted successively with chloroform (3 x 300 mL) and *n*-butanol (3 x 300 mL). The *n*-butanolic extract was evaporated to dryness (3 g of dry extract were obtained). For LC analysis, 1 mg of the butanolic dried extract (BE) was prepared in the same conditions as the flavonoid standards.

Instrumentation

All mass spectrometry experiments were performed on a Waters QToF Premier mass spectrometer in positive ion mode, either using the MALDI or the Electrospray ionization (ESI) sources.

The MALDI source was constituted by a nitrogen

laser, operating at 337 nm with a maximum output of 500 mW delivered to the sample in 4 ns pulses at 20 Hz repeating rate. All samples were prepared using a 20 mg mL^{-1} solution of 2, 5- dihydroxybenzoic acid in acetone as the matrix. The matrix solution (1 µL) was spotted onto a stainless steel target and air dried. Then, 1 µL of each butanolic extract was applied onto the spots of matrix crystals, and air dried. Finally, 1 µL droplets of a solution of NaI (2 mg mL^{-1} in acetonitrile) was added to the spots on the target plate.

Typical ESI conditions were: capillary voltage, 3.1 kV; cone voltage, 15 V; MCP voltage 2300 V; source temperature, 100°C; desolvation temperature, 300°C. Dry nitrogen was used as the ESI gas. For the recording of the single-stage MALDI-MS or ESI/MS spectra, the quadrupole (rf-only mode) was set to pass ions between *m/z* 100 and 1000, and all ions were transmitted into the pusher region of the time-of-flight analyzer where they were mass-analyzed with a 1 s integration time.

For the CID experiments, the ions of interest were mass-selected by the quadrupole mass filter. The selected ions were then submitted to collision against argon in the T-wave collision cell (pressure estimated at 10^{-3} mbar) and the laboratory frame kinetic energy was selected to afford intense enough product ion signals. All the ions exiting the collision cell, either the product ions or the non-dissociated precursor ions, were finally mass measured with orthogonal acceleration time-of-flight (oa-ToF) analyzer. Time-of-flight mass analysis was performed in the reflectron mode at a resolution of about 10,000.

For the on-line LC-MS analyses, a Waters Alliance 2695 liquid chromatography apparatus was used. The LC device was coupled to the Waters QToF Premier mass spectrometer (ESI mode) and consisted of a vacuum degasser, a quaternary pump and an autosampler. Sample volumes of 20 µL were injected. Chromatographic separation was performed on a non polar column (Symmetry® C_{18}, 4.6× 75 mm, 3.5 µm column, Waters) at 30 °C. The mobile phase (1 mL min^{-1}) was a non linear gradient programmed from methanol (eluent A) and water (eluent B). The selected gradient was: 95% of eluent A at start, 0–15 min 95 to 40 % A and 15–18 min back to 95% eluent A. The mobile phase flow (1 mL min^{-1}) was split prior injection in the Electrospray ionization source (200 µL min^{-1}). Data were processed using Mass Lynx version 4.1 software (Micromass, Manchester, UK).

For the quantitative studies in LC-MS experiments, the mass-to-charge ratios of the ions corresponding to the protonated $[M+H]^+$ and the sodiated $[M+Na]^+$ flavonoids were extracted from the total ion current (TIC) chromatogram with a mass error of 0.01 Da. These measurements were realized on standard solutions of known concentrations (calibration) and on the solutions prepared from the dry extract. The area of both the $[M+H]^+$ and $[M+Na]^+$ extracted signals were then summed and used for the quantitative study.

RESULTS AND DISCUSSION

MALDI-MS analysis for flavonoids screening

MALDI-ToF was demonstrated at several occasions to represent an efficient tool for an initial screening of natural molecules from crude extracts[14]. Indeed, when compared to electrospray ionization, the MALDI ionization processes are less sensitive to the quality of the extracts and, in particular, MALDI is really tolerant to (i) the presence of salts and (ii) the coexistence of numerous molecules[15]. Indeed, at variance with electrospray ionization, the production of ions under MALDI is not limited by the maximum ion current phenomena[16]. Therefore, MALDI-ToF analysis was initially performed for the rapid screening of flavonoid compounds in the peppermint butanolic extract (BE). Upon MALDI ionization in the positive ion mode, the production of gas phase ions corresponding to flavonoids arises from two competitive processes: (i) protonation and (ii) sodium ion attachment. These two processes respectively generate protonated $[M+H]^+$ and sodiated [M+Na]+ molecules. Based on flavonoid glycosides in peppermint, reported in literature (Figure 1), a putative identification of flavonoid diglycoside compounds was done by measuring the mass-to-charge ratio of the detected ions (Figure 2). Indeed, on the MALDI-ToF mass spectrum of the peppermint BE, using 2,5-dihydroxybenzoic acid (2,5)-DHB as matrix, the presence of ions from at least six flavonoid diglycosides could then be envisaged.

As can be seen on Figure 2, the presence of ions relative to isorhoifilin, luteolin-7-o-rutinoside, eriocitrin, hesperidin, diosmin and an additional flavonoid diglycoside (linarin see below) at *m/z* 593 can be en-

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visaged. Definitive identification of the flavonoids was obtained by further examining the CID spectra of the protonated $[M+H]^+$ and sodiated $[M+Na]^+$ by MALDI-MS/MS analysis.

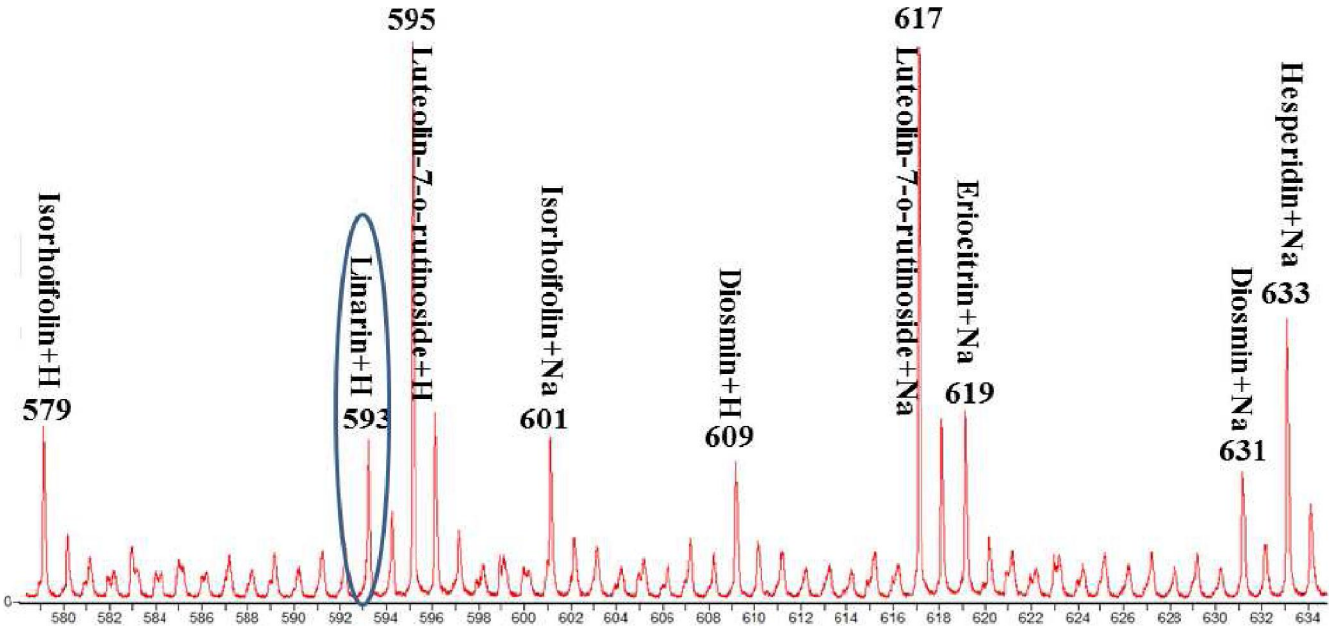

Figure 2 : Full-scan (+) MALDI-ToF mass spectrum analysis of the butanolic extract (BE) of peppermint

MALDI-MS/MS analysis

CID spectra for the available standards and literature data[16,18] were used to confirm the structure of the observed ions, see TABLE 1. Usually, the dominant fragmentation of protonated flavonoid diglycosides consists in the loss of 308 u that is assigned to the loss of the (full) rhamnoglucoside moiety. This formal loss of neutral rhamnoglucoside was also demonstrated to arise from an initial loss of rhamnose (146 u) followed by the loss of the glucose residue (162 u), see Figure 3[19]. At variance with flavone-based molecules, an additional fragmentation is observed with protonated flavanones and consists in a charge-driven decomposition leading to the loss of the inner glucose residue and eventually followed by the loss of the terminal rhamnose. Such a complex rearrangement was correlated to the favorable migration of the proton from the oxygen atom of the carbonyl group of the flavanone ring to the terminal rhamnose[17]. Interestingly, such a rearrangement is not observed when protonated flavones are subjected to CID experiment given the expected stronger proton affinity at the oxygen atom of the carbonyl group. Indeed, it is proposed that the presence of the C=C bond between C2 and C3 in the flavone ring will generate, by resonance, a stronger negative charge on the oxygen atom of the carbonyl group. Ma *et al*. showed by theoretical calculation of proton affinity (PA) that flavones present stronger proton affinities than flavanones[20]. On the other hand, when subjected to CID, sodiated flavonoid rhamnoglucosides do not expel the inner glucose, illustrating the requirement of the proton (migration) to induce the rearrangement. However, in the CID spectra of both sodiated flavanones and flavones, in addition to the saccharide loss, the aglycone moiety can also be competitively expelled from the decomposing ions yielding sodiated dissacharide as the CID products at m/z 331. Additional fragments involving the B-ring (Figure 1) were observed specifically for sodiated flavanones. All those considerations are gathered in TABLE 1 and were used to confirm the identification of eriocitrin, hesperidin, luteolin-7-o-rutinoside, diosmin and isorhoifolin. Unexpectedly, narirutin (figure 1) was previously mentioned in peppermint but was not identified in this study. Many factors like climate and soil induce the great qualitative and quantitative variety of flavonoids in plants[21,22].

Interestingly, from the MALDI mass spectrum (Figure 2) and from TABLE 2, the branching ratio between the $[M+H]^+$ and the $[M+Na]^+$ ions for a given flavonoid diglycoside seems to be highly dependent on the presence of the C=C double bond between C2 and C3 in the flavanone/flavone ring, see Figure 1. During the MALDI (also observed in ESI) process, the higher elec-

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TABLE 1 : MS/MS of flavonoid diglycoside ions detected in MALDI Full-scan (+) of the butanolic extract of peppermint

Identified Compounds	Protonated MS/MS data (*m/z*)				Sodiated MS/MS data (*m/z*)				
	(M+H)	(-R)	Y*	(-D)	($M+Na^+$)	(-R)	(-B)	(-D)	($D+Na^+$)
Eriocitrin	597	451	435	289	619	473	483	311	331
Hesperidin	611	465	449	303	633	487	483	325	331
Luteolin 7-o-rutinoside	595	449	×	287	617	471	×	309	331
Diosmin	609	463	×	301	631	485	×	323	331
Isorhoifolin	579	433	×	271	601	455	×	293	331
Linarin or fortunellin	593	447	×	285	615	469	×	307	331

Y*: loss of inner glucose by charge-induced rearrangement; (-R): loss of rhamnose; (-D): loss of diglycoside; (M+H): pseudo-molecular ion; ×: absence; (-B): loss of B-ring; ($M+Na^+$): sodiated ion

tron density on the basic site of the molecule will favor the protonation for the flavone-constituted molecules. As for an example, hesperidin is mainly observed as a sodium cationized species at *m/z* 633 (protonated molecule at *m/z* 611) whereas diosmin is clearly detected as a protonated molecule at *m/z* 609. Sodium cationized diosmin is also observed at *m/z* 631 but the protonation is clearly a competitive process for the flavone molecule.

As far as the m/z 593 cations are concerned, an accurate mass of m/z 593.1870 was obtained and is in really close agreement with a C28H33O14 composition (theoretical mass m/z 593.1873± 0.5 ppm). Based on the identification of different flavonoid 7-O-diglycosides in *Mentha piperita*, the presence of linarin or fortunellin could be envisaged upon CID experiments[9,10]. On the basis of the CID spectrum of the m/z 593 ions, the presence of a protonated flavone rhamnoglucoside was confirmed since, upon collisional activation, only consecutive losses of rhamnose and glucose were observed, as proposed in Figure 3. However, at this point of the work, it is not possible to make a clear-cut identification of the corresponding ions since both protonated linarin and fortunellin present the same decompositions upon CID experiments.

Nevertheless, it is already important to remind that, differently with the five other observed flavonoids (eriocitrin, hesperidin, luteolin-7-o-rutinoside, diosmin and isorhoifolin), linarin or fortunellin were never observed in peppermint extract [9,10]. Therefore, we decided to try to characterize the corresponding molecules by using LC-MS experiments.

TABLE 2 : Comparison of signal intensities for the $[M+H]^+$ and $[M+Na]^+$ ions of flavanones compared to flavones obtained in MALDI process

	Flavanones			Flavones		
Flavonoids	eriocitrin	hesperidin	luteolin-7-o-rutinoside	diosmin	isorhoifolin	linarin
$I_{[M+H]^+}/I_{[M+Na]^+}$	0.35	0.20	1.02	1.13	1.03	3.01

LC-MS analysis

To confirm by chromatographic method the presence of the different flavonoids previously identified by MALDI-MS/MS analyses and to try to assign the exact structure of the *m/z* 593 ions, LC-MS methodology was used. When possible, flavonoid retention times in extracted ion chromatogram were compared with those of available reference compounds, i.e. hesperidin, diosmin and eriocitrin. The retention times of flavonoids from the BE are then summarized in TABLE 3. LC-MS was then used to measure the retention times (RT) of the two standards: linarin and fortunellin. The RT's were measured at respectively 15.43 and 15.58 min, see Figure 4. The experimental value defining the retention time corresponding to the *m/z* 593 ion was measured from the BE at 15.44 min. We then propose that linarin was extracted by *n*-butanol from peppermint together with other flavonoids.

Quantitative analysis

Experiment quantification was carried out by the external standard method in a LC analysis. Due to the unavailability of standards, luteolin-7-o-rutinoside and isorhoifolin were not considered for the quantitative part of the work. For each studied compound, the total peak area value corresponds to the sum of the peak areas of the protonated and the sodium cationized molecules,

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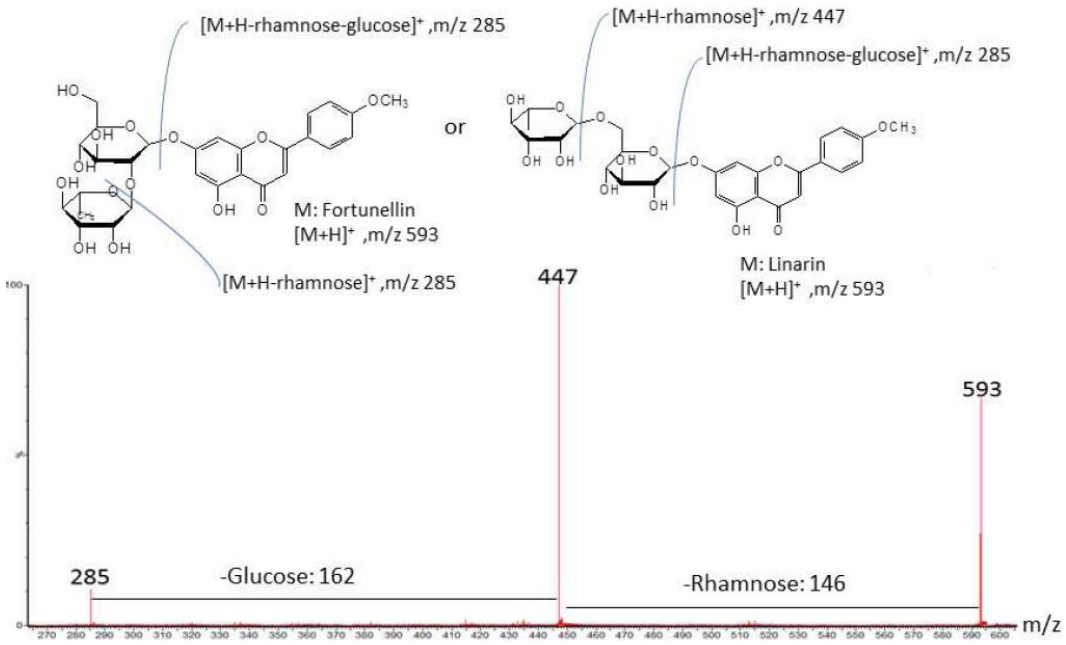

Figure 3 : Protonated fragmentation patterns of *m/z* 593 ion from butanolic extract

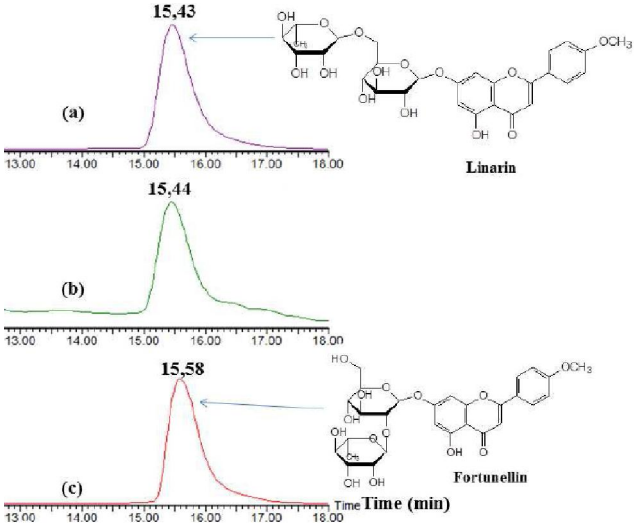

Figure 4 : LC-MS analysis of the butanolic extract of *Mentha piperita* and comparison with reference compounds: m/z 595 extracted ion chromatograms for (a) linarin; (b) flavone rhamnoglucoside in BE and (c) fortunellin

after extraction of the corresponding ion currents from the total ion current chromatogram. The studied compounds were diluted in order to fix them in a determined calibration range.

The results expressed in mg analyte g^{-1} of BE (n = 3; value = X ± SD) were 22.5 mg g^{-1} of diosmin, 5.7 mg g^{-1} of hesperidin and 0.1 mg g^{-1} of linarin in BE of *Mentha piperita*. Except diosmin, these values were relatively lower than those reported by Sroka *et al*[9]. The LC-MS data are summarized in TABLE 2. As additional information, one can note that a direct comparison between the peak intensities in the MALDI-ToF spectrum of Figure 2 and the quantitative data obtained upon LC-MS and gathered in TABLE 2 cannot be relevant. Indeed, although MALDI represents an efficient tool for a fast screening of the molecules contained in the extract, this analytical method is well-known to be hardly used to generate quantitative data. The quantification of the different flavonoids, is definitively not efficient for different reasons as: (i) it is not straight forward to obtain homogeneous solid samples on the target plate by the dry-droplet method, (ii) the desorption/ionization efficiencies are expected to be reasonably in the same order of magnitude for the different compounds but slight differences are expected between the different congeners of flavonoids and (iii) the ex-

TABLE 3 : LC-MS data for the flavonoid diglycosides identified in BE of peppermint from Burkina Faso

Identified Compound	Retention time (min)	Content (mg g^{-1} of BE)
Eriocitrin (1)	10.88	37.5± 1.5
Hesperidin (2)	12.53	5.7± 0.13
Luteolin 7-O-rutinoside (4)	12.00	-
Diosmin (5)	13.29	22.5± 0.05
isorhoifolin (6)	12.97	-
Linarin (7)	15.43	0.1± 0.00

(-): not quantified

traction method used for the present study is not efficient enough to generate pure mixture of flavonoids. This last point made the MALDI spectrum noisy as readily observed from Figure 2 and then no reliable quantitative data can be derived from such a spectrum.

CONCLUSIONS

This present paper illustrates the efficient use of MALDI-MS/MS and LC-MS to identify and quantify different flavonoid diglycosides in peppermint butanolic extract. Amongst flavonoid diglycosides expected in peppermint, only narirutin was not detected. Moreover, and for the first time, linarin was observed in peppermint butanolic extract in relative low concentration. The low relative amount of linarin (0.1 mg g^{-1}) could maybe explain why this compound was up till now not detected in peppermint.

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