1 **Short Communication**

2 **LC-MSⁿ characterization of saponins in mate (*Ilex paraguariensis*, St.**
3 **Hil) and their quantification by HPLC-DAD**

4
Raquel Mateos^{^*}, Gema Baeza[^], Sara Martínez-López, Beatriz Sarriá, Laura Bravo^{*}

Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition
(ICTAN), Spanish National Research Council (CSIC), Madrid, Spain.

5 [^] Raquel Mateos and Gema Baeza have contributed equally to this work.

6
7
8 **Running title:** Qualitative and quantitative LC-DAD-MSⁿ analysis of saponins in mate

9
10
11 ***Corresponding authors:**

12 Raquel Mateos (raquel.mateos@ictan.csic.es)

13 Laura Bravo (lbravo@ictan.csic.es)

14 Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National
15 Research Council (CSIC). C/Jose Antonio Novais 10, 28040 Madrid (Spain). Tel:
16 +34.915492300. Fax: +34 91 549 3627.

Abstract

Saponins are secondary plant metabolites constituted by a triterpenoid aglycone with one or more sugar chains. There is increasing interest in saponins due to their potential health effects. Yerba mate is an important source of saponins in many South American diets. The aim of this study was to develop a one-step procedure to isolate saponins from yerba mate, followed by their characterization by liquid chromatography/tandem high-resolution mass spectrometry (LC-ESI-MS) and quantification by high performance liquid chromatography-diode array (HPLC-DAD). Methanol extraction proved to be a highly effective method to extract yerba mate saponins. LC/MSⁿ analysis allowed identifying three free triterpenes and 16 saponins of which five were identified for the first time. HPLC-DAD results revealed that ursolic acid was the main free aglycone (1.557-1.807 mg/g vs. 0.229-0.294 mg/g oleanolic acid and 0.024-0.036 mg/g hydroxylated aglycone). Saponin contents varied from 4.4-5.5 mg/g in four commercial brands, being matesaponin D or J3a the most abundant saponin.

Keywords: Yerba mate, saponin composition, triterpenes, LC-MSⁿ analysis, HPLC-DAD quantification, ursolic acid, oleanolic acid, matesaponins.

Abbreviations: Ac, acetyl group; Agly, aglycone; Ara, α -L-arabinospyranosyl; Glu, β -D-glucopyranosyl; m.u., mass units; Ole, oleanolic acid; Rha, α -L-rhamnopyranosyl; Urs, ursolic acid; Xyl, D-xylopyranose.

1. Introduction

Yerba mate infusion is the most popular beverage in South America. Mate is prepared from the dried leaves of *Ilex paraguariensis* and indigenous populations have traditionally used it as a medicinal plant for the treatment of different pathologies. Nowadays, yerba mate is most wanted, better than coffee or tea, in countries such as Brazil, Argentina, Paraguay or Uruguay, with an estimated intake of more than 1 L/day/person. Moreover, the beneficial health properties of the beverage have favored its intake, which is spreading around the world (Bracesco, Sanchez, Contreras, Menini, & Gugliucci, 2011). Recently, numerous *in vitro* and *in vivo* studies have shown that mate presents antioxidant, hepatoprotective, hypocholesterolemic, anti-atherosclerotic, anti-inflammatory, anti-obesity and/or anti-carcinogenesis properties, among others (Arçari et al., 2009; Boaventura et al., 2012; Bravo et al., 2014; de Mejía, Song, Heck, & Ramírez-Mares, 2010; Gao, Liu, Wan, Qu, & Chen, 2013; Miranda et al., 2008; Mosimann, Wilhelm-Filho, & Da Silva, 2006). These beneficial health effects have been attributed to the high content of phytochemicals in yerba mate, particularly in phenolic compounds and methylxanthines, which have been associated with a wide range of medicinal properties (de Mejia & Ramirez-Mares, 2014; Khurana, Venkataraman, Hollingsworth, Piche, & Tai, 2013; Sarriá et al., 2015). However, other compounds in yerba mate may contribute to its health benefits, such as saponins, which have been associated to anti-inflammatory, hypocholesterolemic, anti-coagulant, neuroprotective or antioxidant effects (Güçlü-Üstündağ & Mazza, 2007; Osbourn, Goss, & Field, 2011; Rao & Gurfinkel, 2000). Indeed, saponins in yerba mate have been considered the most potent phytochemical to prevent inflammation and colon cancer by inhibiting cell proliferation *in vitro*, via the modulation of the inducible nitric oxide synthase (iNOS)/nitric oxide (NO) iNOS/NO and cyclooxygenase 2 pathways, as well as the inhibition of the NFκB nuclear translocation (Puangpraphant & de Mejia, 2009; Puangpraphant, Berhow & de Mejia, 2011). In addition, Sugimoto et al. (2009) pointed to anti-obesity effects partly due to yerba mate's inhibitory activity of porcine pancreatic lipase.

Saponins are high molecular weight molecules formed by an aglycone or sapogenin (triterpene, steroid or steroid alkaloid) linked to one or more sugar chains. According to the number of sugar chains attached, saponins are classified as i) mono-desmosidic, with a single sugar chain bonded by ether linkage at C-3, ii) bi-desmosidic, with two sugar chains bonded through an ether linkage at C-3 and an ester linkage at C-28 (like triterpene saponins) or an ether linkage at C-26 (like furastonol saponins), and iii) tri-desmosidic, with three sugar chains, although these compounds are rarely found

(Hostettmann & Marston, 2005). The most common monosaccharides in saponins are D-glucose, D-galactose, D-glucuronic acid, D-galacturonic acid, L-rhamnose, L-arabinose, D-xylose, and D-fructose (Hostettmann & Marston, 2005). Therefore, saponins show high variability and present a wide range of physical, chemical and biological properties based on the aglycone chemical structure, the number and type of monosaccharides, as well as the binding to the sapogenin.

Saponins from yerba mate are formed by a triterpene as aglycone; specifically ursolic or oleanolic acids in the leaves, and pomolic and rotundic acids in the fruits (De Souza et al., 2011; Gosmann, Guillaume, Taketa, & Schenkel, 1995; Martinet, Ndjoko, Terreux, Marston, Hostettmann, & Schutz, 2001; Taketa, Breitmaier, & Schenkel, 2004). Although fruits have a higher content in saponins than leaves (up 30 % vs. 4 %, w/w) (Borré et al., 2010; Pavei, Guzatto, Ros Petrovick, Gosmann, & González-Ortega, 2007), numerous studies have focused on the characterization of saponins in leaves as a source of the bioactive phytochemicals since yerba mate fruits are discarded. There is high variability in the saponin content reported in the few studies that have evaluated the presence of saponins in yerba mate leaves, in part due to the different analytical procedures followed (analytical technique and quantification) (Borré et al., 2010; Coelho, Gnoatto, Bassani, & Schenkel, 2010; Gnoatto, Schenkel, & Bassani, 2005; Puangpraphant & de Mejia, 2009). In addition, variation of the saponin content of yerba mate depends on the genetic origin, intra-species variation, the part of the plant being examined, environmental and agronomic factors, and post-harvest treatments such as storage and processing (Fenwick, Price, Tsukamoto, & Okubo, 1991; Nakamura, Donaduzzi, & Schuster, 2009; Scherer, Janssens, Marx, Urfer, & Schneider, 2007).

To date, complex extraction procedures with numerous steps have been used to isolate saponins from yerba mate leaves. The aim of the present study was to optimize a one-step procedure to isolate yerba mate saponins, deepening in the chemical characterization by LC/MS and quantification by HPLC-DAD of the saponin content in yerba mate.

2. Material and methods

2.1. Materials and reagents

Four different commercial brands of yerba mate (*Ilex paraguariensis* Saint Hilaire) were purchased in a local supermarket in Madrid (Spain). Oleanolic and ursolic acids were acquired from Sigma-Aldrich (Madrid, Spain). All other chemicals were of analytical or chromatographic grade.

2.2. Extraction of saponins from yerba mate

Saponins were extracted from 1.5 g of yerba mate leaves, in triplicate, using 10 mL of methanol for 1 h by constant shaking at room temperature. After centrifuging (10 min, 1250 g), the supernatants were made up to 10 mL, filtered using 0.45 µm pore filters and stored at 4 °C until its chromatographic analysis.

2.3. Identification and quantification of saponins

The characterization of saponins composition in yerba mate was carried out using a 1200 Liquid Chromatographic system with a Diode-Array Detector (LC-DAD) coupled to an Accurate-Mass Quadrupole Time-of-Flight (Q-ToF) detector with electrospray ionization (ESI)-Jet Stream Technology (Agilent Technologies, Waldrom, Germany). Saponin separation was performed using a Supherspher RP18 column (4 mm x 250 mm i.d., 4 µm particle sizes, Agilent Technologies) protected with an ODS RP18 guard column by using a binary gradient of 0.01 M ammonium formate at pH 2.8 (solvent A) and methanol (solvent B) at a flow rate of 0.8 mL/min at 35 °C. The gradient changed from 30 to 75 % solvent B over 20 min, 75 to 88 % solvent B over 15 min, followed by 15 min of maintenance and 88 to 100 % solvent B over 2 min with 2 min of maintenance. At last, the system returned to the initial conditions in 4 min which were maintained 4 min further. Chromatograms were acquired at 203, 210 and 245 nm. MS operating conditions were as follows: ESI source operated in negative ion mode programming temperature and gas flow at 350 °C and 10 L/min, respectively, sheath gas volume and temperature 11 L/min and 350 °C, respectively, and 45 psig of nebulizer pressure. Mass range was set between 100 and 2000 *m/z*. Capillary, fragmentor and nozzle voltages were 3500, 200 and 1000 V, respectively. Data were processed using Mass Hunter Workstation Software.

Quantification of the identified saponins in yerba mate was developed using an Agilent 1200 LC-DAD system. Similarly, separation of the compounds was performed on a Superspher RP18 column (4 mm x 250mm i.d., 4 µm; Agilent Technologies) protected with an ODS RP18 guard column, using the same solvents, elution gradient and conditions described above. Oleanolic acid was the standard used to quantify saponins. Limits of detection and quantification were 12.68 and 38.44 µM, respectively.

3. Results and Discussion

The development of an easy and simple analytical procedure to isolate saponins from yerba mate is of high interest considering the potential use of saponins as pharmaceutical or nutraceutical compounds, in addition to its soap-like foaming

properties (Scherer et al., 2007). It was an important challenge to quantify each component in the saponin fraction separately, considering the small differences in their lipophilic nature, which makes uneasy the chromatographic separation, and the chemical complexity of saponins. Bearing this in mind, in the present study a one-step isolation procedure of yerba mate saponins was developed and then saponins were quantified by HPLC-DAD, with enhanced chromatographic resolution. In addition, a complete chemical characterization of the saponin constituents was carried out by LC-MS-QToF. Moreover, the saponin composition of four commercial brands of yerba mate were comparatively studied.

3.1. Development of the method

Triterpene acids are insoluble in water and in non-polar solvents but are freely soluble in alcoholic solvents. However, these compounds are usually glycosylated, conferring hydrophilicity. Therefore, hot and cold extraction procedures which involve water, alcohol (methanol and ethanol) or water-alcohol mixtures, followed by an acid hydrolysis and/or extraction with butanol or chloroform, have been widely used for the extraction of saponins in plant matrices (Gnoatto et al., 2005; Gosmann, Schenkel, & Selignmann, 1989; Martinet et al., 2001; Morikawa, Nakamura, Kato, Muraoka, Matsuda, & Yoshikawa, 2007; Oleszek & Bialy, 2006; Yan et al., 2015). Nevertheless, these procedures require sequential steps that are time-consuming and involve potential losses of the most polar saponins during extraction (Güçlü-Üstündağ & Mazza, 2007; Oleszek & Bialy, 2006). Considering these drawbacks, and the absence of conclusive quantitative data, firstly, although the cold extraction with water:ethanol is the procedure most assayed in the literature (Oleszek & Bialy, 2006), three different solvents (methanol, ethanol and a water:ethanol mixture -4:6, v/v-) were compared at three shaking times (1, 2 and 3 h). Methanol extraction yielded the highest saponin content according to chromatography analysis, not showing differences due to shaking times tested. Afterwards, two cold extraction methods (shaking and sonicating) using methanol at 1 h were compared. Sonication leaded to increased free aglycone content, suggesting that this method promoted saponin hydrolysis (data not shown). To end, after comparing all conditions tested in triplicate, it was concluded that methanol extraction and shaking for 1 h was the best procedure for isolating the saponin fraction, showing the highest yield and lowest aglycone hydrolysis. Additionally, saponin extraction from yerba mate leaves with boiled water compared to cold extraction was evaluated and lower saponin recovery was observed with the later procedure.

HPLC analysis is widely used to determine compound contents in plants due to its robustness, reproducibility and sensitivity. However, polarity similitude of saponins in yerba mate and the lack of sensitive chromophores to UV light makes saponin identification complicated. Numerous chromatographic protocols have been tested involving different gradient elutions, mobile phases and/or detectors (UV-vis or refractive index), although the conditions most widely used involve reversed phase C18 or silica gel columns (Negi, Singh, Pant, & Rawat, 2011; Oleszek & Bialy, 2006). In the present study, different chromatographic methods for the characterization of saponins from yerba mate have been comparatively tested (Coelho et al., 2010; Gnoatto et al., 2005; Martinet et al., 2001; Puangraphant & de Mejia, 2009), and in certain cases, slightly modified to improve saponin peak resolution (data not shown). Initially, the mobile phases (0.01 M phosphate buffer (pH 2.8) and methanol) used in the chromatographic determination of triterpenic acids were similar to that used in Lee et al., (2009) in combination with the HPLC gradient elution described in Martinet et al. (2001). In addition to HPLC analysis, samples were analyzed on a 1200 LC-DAD system coupled to an Accurate-Mass Quadrupole Time-of-Flight (Q-ToF) to characterize their chemical structure. However, incompatibility of 0.01 M phosphate buffer (pH 2.8) with this sensitive equipment forced its replacement by 0.01 M ammonium formate (pH 2.8), a more friendly solvent for this system, which provided the same chromatographic profile than that obtained after HPLC-DAD analysis. Therefore ammonium formate was used in both systems (HPLC-DAD and HPLC-MS).

3.2. Saponin composition

The saponin compounds present in yerba mate from the four commercial brands tested were monitored by HPLC-DAD. UV spectra of the chromatographic peaks indicated that at least 19 glycoside triterpenes were present in yerba mate, with a single maximum at 206-210 nm. Figure 1 shows the chromatogram corresponding to a yerba mate extract at 210 nm. In addition, a methanol yerba mate extract was analyzed by high-resolution mass spectrometry using an ESI-QToF detector in the negative ion mode. Selected ion monitoring (SIM) identified three free triterpenes and 16 saponins. The structural identification of each saponin was carried out by subsequent LC-MSⁿ analysis by collision-induced dissociation mass spectrometry. The fragmentation pathway from glycoside cleavage can provide information on the aglycone mass, as well as the primary structure of oligosaccharide chains (Liu, Cui, Liu, Song, & Mo, 2004; Mroczek, Kapusta, Janda, & Janiszowska, 2012). However, different monosaccharide epimers cannot be distinguished by MS/MS analysis. In addition,

ionization in negative ion mode lacks enough energy to induce cross-ring binding fragmentation and subsequently does not provide information of linkages between sugar residues (Liu et al., 2004). Therefore, it was not possible to confirm the structure of each saponin in the present study. Table 1 shows the chemical characterization of the identified saponins and triterpenes, ordered according to peak elution: retention time, quasimolecular ion $[M-H]^-$, molecular formula, MS/MS fragment ions and tentative chemical structure. Chemical characterization of the identified saponins in yerba mate is described below.

Peaks **1** and **13** presented the same quasimolecular ion $[M-H]^-$ at m/z 911 and MS² base peak ion at m/z 749 due to the loss of a hexose moiety (162 mass units m.u.) corresponding to glucopyranosyl (Glu) or galactopyranosyl, probably linked at C-28 of the aglycone which is the most labile linkage and, therefore, the first to be cleaved (De Souza et al., 2011). Additionally, compound **13** yielded two fragment ions at m/z 587 and 455, derived from the loss of the hexose and a pentose like arabinopyranosyl (Ara) or xylopyranosyl (Xyl) (132 m.u.) moieties. The fragment ion at m/z 455 corresponded to ursolic or oleanolic acids, which were the aglycone (Agly) moiety of the molecule. The identical molecular formula (C₄₇H₇₆O₁₇) of peaks **1** and **13** inferred by Mass Hunter, based on their common $[M-H]^-$, indicated that these compounds were isomeric forms. According to the literature these compounds could be the well-known Matesaponin 1, defined by Gosmann et al. (1989) as ursolic acid-3-O- $\{\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -L-arabinospyranosyl)-(28 $\rightarrow$ 1)- β -D-glucopyranosyl ester, and its isomer oleanolic acid-3-O- $\{\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -L-arabinospyranosyl)-(28 $\rightarrow$ 1)- β -D-glucopyranosyl ester (Martinet et al., 2001). Based on the earlier chromatographic elution of oleanolic acid compared to ursolic acid standards, compounds **1** and **13** were tentatively assigned as the Matesaponin 1' and Metasaponin 1, having oleanolic and ursolic acids as aglycone moieties, respectively.

Chromatographic peaks **2** and **3** showed identical MS spectrum with a common $[M-H]^-$ at m/z 765 compatible with the molecular formula C₄₁H₆₆O₁₃. The MSⁿ spectrum of both compounds reported an intense fragment ion at m/z 603/585 originated from the loss of a hexose moiety linked at C-28 and a fragment ion at m/z 471 corresponding with the hydroxylated aglycone moiety after losing a pentose linked at C-3. Previous studies have identified hydroxylated aglycones in yerba mate, specifically hydroxyursolic acid, as the aglycone moiety of the saponin (De Souza et al., 2011; Sugimoto et al., 2009). The presence of two compounds (**2** and **3**) with hydroxylated aglycone moiety suggests they were derivatives of hydroxyoleanolic and hydroxyursolic acids, respectively, according to the elution order of their respective free aglycones

(oleanolic and ursolic acids). Thus, compound **3** was assigned as Matesaponin C (23-hydroxyursolic acid-3-O- α -L-arabinospyranosyl-(28 $\rightarrow$ 1)- β -D-glucopyranosyl ester) (Sugimoto et al. 2009), whereas peak **2** was tentatively identified as the oleanolic isomer (23-hydroxyoleanolic acid-3-O- α -L-arabinospyranosyl-(28 $\rightarrow$ 1)- β -D-glucopyranosyl ester), based on the same fragmentation pattern of both compounds (**2** and **3**). Compound **2** was named Mateglycoside C' to shorten the nomenclature, was tentatively identified in yerba mate for the first time.

The molecular formula $C_{65}H_{106}O_{31}$ was postulated for compound **4** based on its negative-ion spectrum, which displayed a $[M-H]^-$ at m/z 1381. The presence of m/z 895 and 485 fragments confirmed the linkage of an oligosaccharide composed of 3 hexose residues at C-28. According to the literature, this compound might be assigned as Matesaponin 5, ursolic acid-3-O- $\{\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)- $[\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)]- α -L-arabinospyranosyl)-(28 $\rightarrow$ 1)- β -D-glucopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyl ester (Kraemer, Taketa, Schenkel, Gosmann, & Guillaume, 1996; Martinet et al., 2001).

The MS spectrum of peak **5** exhibited a quasimolecular ion at m/z 1219 which well-matched molecular formula $C_{59}H_{96}O_{26}$. A MS^2 base peak ion at m/z 895 showed the loss of a disaccharide of hexoses linked to the aglycone by an ester linkage, while the secondary ion at m/z 733 suggested the loss of a third hexose moiety. The MS analysis was in agreement with the compound named Matesaponin 4, described by Gosmann et al., (1995), ursolic acid-3-O- $\{\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)- $[\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)]- α -L-arabinospyranosyl)-(28 $\rightarrow$ 1)- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyl ester, which allowed its tentative assignation. However, the corresponding isomeric glycoside, having oleanolic acid as aglycone, was not identified in contrast to other author studies (De Souza et al., 2011; Sugimoto et al., 2009).

Compound **6** showed a $[M-H]^-$ at m/z 1073 corresponding to molecular formula $C_{53}H_{86}O_{22}$. The fragmentation pattern gave a fragment ion at m/z 749 corresponding to $[M-H-hexose-hexose]^-$ and the fragment ion at m/z 587 was coincident with $[(M-H-2hexoses)-hexose]^-$ which allowed its tentative identification as the Matesaponin 3, ursolic acid-3-O- $\{\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -L-arabinospyranosyl)-(28 $\rightarrow$ 1)- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyl ester (Gosmann et al., 1995). Previous studies have described up to 5 different Matesaponin 3 isomers in yerba mate (De Souza et al., 2011).

One chromatographic peak (**7**) showed a MS spectrum with deprotonated parent ion at m/z 1409, compatible with the molecular formula $C_{66}H_{106}O_{32}$. The fragment ion at m/z 923 confirmed the presence of an oligosaccharide formed by 3 hexose residues linked at C-28. In agreement with the literature, this compound could have an acetyl

group (Ac) in their structure, being xylopyranose-(1→2)-glucopyranose-(1→3)-
(acetyl)arabinopyranose-(1→3)-aglycone-(28←1)-glucopyranose-(6←1)-
glucopyranose-(4←1)-glucopyranose (De Souza et al., 2011).

The MS spectra of peak **8** exhibited a quasimolecular ion at m/z 1277, equivalent to $C_{61}H_{98}O_{28}$. The secondary ions of MS^2 analysis allowed elucidating its chemical structure by the peaks at m/z 953 [M-H-hexose-hexose]⁻, 911/893 [(M-H-2hexoses)-Ac]⁻, 749 [(M-H-2hexoses-Ac)-hexose]⁻, 587 [(M-H-2hexoses-Ac-hexose)-hexose]⁻. Considering that the fragment ion at m/z 587 corresponded to the aglycone linked to a pentose residue, the structure of this compound was tentatively assigned to (acetyl)glucopyranose-(1→2 or 6)-glucopyranose-(1→3)-arabinopyranose-(1→3)-aglycone-(28←1)-glucopyranose-(6←1)-glucopyranose, in agreement with De Souza et al. (2011). Additionally, two different positional isomers had been previously described in yerba mate (De Souza et al., 2011) but were not observed here.

A molecular formula of $C_{60}H_{96}O_{27}$ was postulated for compound **9** according to its [M-H]⁻ ion at m/z 1247. The fragmentation pattern of this compound yielded the daughter ions at m/z 923, 881/863, 749 and 587 corresponding to [M-H-hexose-hexose]⁻, [(M-H-2hexoses)-Ac]⁻, [(M-H-2hexoses-Ac)-pentose]⁻, [(M-H-2hexoses-Ac-pentose)-hexose]⁻, respectively. The fragment ion at m/z 587 corresponding to the aglycone glycosylated with a pentose residue allowed its tentative identification as Ac-pentose-hexose-pentose-agly-hexose-hexose. De Souza et al. (2011) identified an isomer of this compound (Xyl-Glu-Ac-Ara-Agly-Glu-Glu) with a different fragmentation pattern; therefore, the proposed structure for compound **9** has been described for the first time in yerba mate. Complementary analysis using NMR is required to confirm its identity.

Compound **10** showed a molecular parent ion at m/z 1057 in negative ion mode. The molecular formula inferred by Mass Hunter software was $C_{53}H_{86}O_{21}$, coincident with Matesaponin 2 as identified by Gosmann et al., (1995). However, MS^2 analysis only offered the base peak at m/z 733 formed after the loss of a disaccharide, [M-H-hexose-hexose]⁻, attached to C-28 of the aglycone, which differs from the chemical structure of the mentioned Matesaponin 2 (ursolic acid-3-O-{β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]-α-L-arabinopyranosyl}-(28→1)-β-D-glucopyranosyl ester) (Gosmann et al., 1995; Martinet et al., 2001). Therefore, compound **10** was tentatively identified as rhamnopyranose-(1→2)-arabinopyranose-(1→3)-aglycone-(28←1)-glucopyranose-(6←1)-glucopyranose, which had been previously described in yerba mate, as well as two additional isomers (De Souza et al., 2011; Sugimoto et al., 2009).

MS spectrum of chromatographic peak **11** exhibited a quasimolecular ion at m/z 1115 compatible with $C_{55}H_{88}O_{23}$. The MS^2 fragmentation pattern showed a base peak

at m/z 749/731 corresponding to $[M-H-\text{hexose-hexose-Ac}]^-$ and the fragment ion at m/z 587 equivalent to the aglycone linked to a pentose residue, after losing a third hexose moiety, allowing its identification as hexose-pentose-agly-hexose-hexose-ac. Two positional isomers with the same molecular weight and different fragmentation pattern were previously described by De Souza et al. (2011), Ac-Glu-Ara-Agly-Glu-Glu and Glu-Glu-Ac-Ara-Agly-Glu, enabling the tentative identification of compound **11** as Glu-Ara-Agly-Glu-Glu-Ac, for the first time in yerba mate leaves, although complementary NMR analysis would be necessary to confirm its identity.

Two chromatographic peaks (**12** and **15**) showed identical mass spectrum in negative ion mode, with a $[M-H]^-$ at m/z 895 and the major fragment ion at m/z 733 formed after the loss of a hexose linked at C-28. The MS^2 analysis of compound **12** also showed two fragment ions at m/z 587 and 455, originated from the loss of the rhamnose and pentose moieties, respectively. Based on the well-known elution order of oleanolic and ursolic acids, compounds **12** and **15** were identified as the oleanolic and ursolic isomers, respectively, that have been previously identified as J3a or Matesaponin D (3-O- $\{\alpha\text{-L-rhamnopyranosyl-(1}\rightarrow\text{2)}\text{-}\alpha\text{-L-arabinospyranosyl}\}$ oleanolic acid (28 $\rightarrow$ 1)- $\beta\text{-D-glucopyranosyl}$ ester) and J3b (3-O- $\{\alpha\text{-L-rhamnopyranosyl-(1}\rightarrow\text{2)}\text{-}\alpha\text{-L-arabinospyranosyl}\}$ ursolic acid (28 $\rightarrow$ 1)- $\beta\text{-D-glucopyranosyl}$ ester), respectively (Schenkel, Montanha, & Gosmann, 1996; Sugimoto et al., 2009).

The MS spectra of peak **14** presented a $[M-H]^-$ at m/z 1085, compatible with the molecular formula $C_{54}H_{84}O_{22}$. The MS^n spectrum showed a base peak at m/z 923 after losing a hexose residue. The rest of secondary ions allowed deducing its chemical structure; m/z 881 $[(M-H-\text{hexose})-\text{Ac}]^-$, 749 $[(M-H-\text{hexose-Ac})-\text{pentose}]^-$, 587 $[(M-H-\text{hexose-Ac-pentose})-\text{hexose}]^-$ and 455 $[(M-H-\text{hexose-Ac-pentose-hexose})-\text{pentose}]^-$. This fragmentation pattern allowed identifying compound **14** as ac-pentose-hexose-pentose-agly-hexose, which was observed for the first time in yerba mate, although the identities of the aglycone and monosaccharides units were not provided. An isomer of this saponin was described by De Souza et al. (2011) as Xyl-Glu-Ac-Ara-Agly-Glu.

A molecular formula of $C_{49}H_{78}O_{18}$ was postulated for compound **16** from its $[M-H]^-$ at m/z 953. The MS^2 base peak at m/z 791 was compatible with the loss of a hexose residue linked at C-28. The rest of the fragment ions at m/z 749/731 and 587 corresponded to $[(M-H-\text{hexose})-\text{Ac}]^-$ and $[(M-H-\text{hexose-Ac})-\text{hexose}]^-$, respectively. Following the fragmentation pattern described for compound **16**, its structure may be tentatively assigned to ac-hexose-pentose-agly-hexose. An isomer (Glu-Ac-Ara-Agly-Glu or acetyl-matesaponin 1) had been previously described in the literature (De Souza et al., 2011; Martinet et al., 2001; Sugimoto et al., 2009) but with a different

fragmentation pattern, therefore compound **16** was another new compound described for the first time in yerba mate.

Compound **17** showed a quasimolecular ion $[M-H]^-$ at m/z 471 coincident with the molecular formula of $C_{30}H_{48}O_4$. According to Sugimoto et al., (2009) and Taketa et al., (2004), the compound was tentatively identified as 23-hydroxylated aglycone (oleanolic or ursolic acid), however its identity could not be determined by MS^2 analysis.

The last two chromatographic peaks (**18** and **19**) showed typical UV saponin spectrums and a deprotonated parent ion at m/z 455 compatible with $C_{30}H_{48}O_3$. The availability of standards allowed identifying both isomers as oleanolic and ursolic acids, respectively.

3.3. Saponin content

Although few studies have characterized saponins in yerba mate leaves (De Souza et al., 2011; Gosmann et al., 1995; Martinet et al., 2001; Sugimoto et al., 2009), only five main saponins, known as Matesaponins 1-5, had been quantified up to date (Coelho et al., 2010; Gnoatto et al., 2005). Using the chromatographic method here presented, the quantification of each of these saponins was obtained at 210 nm using HPLC-DAD analysis. The individual quantification of saponins is summarized in Table 2. The total saponin content in yerba mate leaves varied between 4.4 to 5.6 mg/g, in accordance with results reported by other authors (3.65-10.03 mg/g) (Coelho et al., 2010; De Moraes et al., 2009; Gnoatto et al., 2005). Compound **12** (Rha-Ara-Ole-Glu, J3a or Matesaponin D) was the most abundant (30-35 % of total saponins) in the four different commercial brands (A-D), followed by the traditionally named Matesaponin 1 (peak **13**), an isomer of Matesaponin 2, and Matesaponin 4 (peaks **10** and **5**). These results are in disagreement with those reported by Coelho et al., (2010), who pointed out that Matesaponin 2 was the most abundant saponin after individually quantifying Matesaponins 1-3. It is noteworthy that around 2 % of the total saponins are formed by an hydroxylated aglycone (compounds **2** and **3**) that had not been identified before in yerba mate leaves. Free ursolic acid content was significantly higher than that of oleanolic acid (over 7 times), greatly differing from the ratio described by De Souza et al., (2011): ursolic acid:oleanolic acid (1:0.8).

Saponin content varied among the different commercial brands, with Mate B showing the highest content of both saponins and free triterpenes. These differences might be due to genetic, environmental conditions and/or production factors, which affect the biosynthetic pathways of saponin formation (Fenwick et al., 1991; Nakamura et al., 2009; Scherer et al., 2007).

In summary, a simple, one-step procedure using methanol was developed to isolate saponins from yerba mate leaves, not requiring derivatization reactions. In addition, the chromatographic method here presented allows the quantification of 16 saponins and 3 free triterpenes in mate, of which saponins **2**, **9**, **11**, **14** and **16** were identified by the first time using LC-MS-QToF. Moreover, saponin quantification by HPLC-DAD revealed that ursolic acid was the main free aglycone. Total saponin content slightly varied among the four commercial brands of mate studied.

In conclusion, i) the extraction of saponin from yerba mate leaves should be carried out with cold methanol, shaking for 1 h; ii) up to 16 saponins and 3 free triterpenes may be determined chromatographically; iii) using the developed extraction method and considering all the resolved saponins and free triterpenes will drastically improve the saponin quantification in yerba mate.

4. Acknowledgments

This work was funded by the Spanish Ministry of Economy and Competitiveness (MINECO-FEDR), projects AGL2010-18269 and AGL2015-69986-R. G.B. is a FPI fellow (BES-2011-047476) funded by the Spanish Ministry of Science and Innovation and performed most experiments. S.M.-L. is a predoctoral fellow of the JAE Program (JAEP097) co-funded by CSIC and the European Social Fund. All authors revised and approved the final version of the manuscript. The technical assistance of ICTAN's Analytical Techniques Service (USTA) is acknowledged.

The authors declare no conflicts of interest.

References

Arçari, D.P., Bartchewsky, W., Santos, T.W., Oliveira, K.A., Funck, A., Pedrazzoli, J., de Souza, M.F., Saad, M.J., Bastos, D.H., Gambero, A., Carvalho, P. de O., Ribeiro M.L., 2009. Antiobesity Effects of yerba maté Extract (*Ilex paraguariensis*) in High-fat Diet-induced Obese Mice. *Obesity* 17, 2127-2133.

Boaventura, B.C.B., Di Pietro, P.F., Stefanuto, A., Klein, G.A., de Moraes, E.C., de Andrade, F., Wazlawik, E., da Silva, E.L., 2012. Association of mate tea (*Ilex paraguariensis*) intake and dietary intervention and effects on oxidative stress biomarkers of dyslipidemic subjects. *Nutrition* 28, 657-664.

Borré, G.L., Kaiser, S., Pavei, C., da Silva, F.A., Bassani, V.L., Ortega, G.G., 2010. Comparison of methylxanthine, phenolics and saponin contents in leaves, branches and unripe fruits from *Ilex paraguariensis* A. St.-Hil (mate). *J. Liq. Chromatogr. R. T.* 33, 362-374.

Bracesco, N., Sanchez, A., Contreras, V., Menini, T., Gugliucci, A., 2011. Recent advances on *Ilex paraguariensis* research: minireview. *J. Ethnopharmacol.* 136, 378-384.

Bravo, L., Mateos, R., Sarriá, B., Baeza, G., Lecumberri, E., Ramos, S., Goya, L., 2014. Hypocholesterolaemic and antioxidant effects of yerba mate (*Ilex paraguariensis*) in high-cholesterol fed rats. *Fitoterapia*, 92, 219-229.

Coelho, G.C., Gnoatto, S.B., Bassani, V.L., Schenkel, E.P., 2010. Quantification of saponins in extractive solution of mate leaves (*Ilex paraguariensis* A. St. Hil.). *J. Med. Food.* 13, 439-443.

de Mejía, E.G., Song, Y.S., Heck, C.I., Ramírez-Mares, M., 2010. Yerba mate tea (*Ilex paraguariensis*): Phenolics, antioxidant capacity and in vitro inhibition of colon cancer cell proliferation. *J. Funct. Foods.* 2, 23-34.

de Mejia, E.G., Ramirez-Mares, M.V., 2014. Impact of caffeine and coffee on our health. *Trends Endocrinol. Metab.* 25, 489-492.

De Moraes, E.C., Stefanuto, A., Klein, G.A., Boaventura, B.C., De Andrade, F., Wazlawik, E., Di Pietro, P.F., Maraschin, M., da Silva, E.L., 2009. Consumption of yerba mate (*Ilex paraguariensis*) improves serum lipid parameters in healthy dyslipidemic subjects and provides an additional LDL-cholesterol reduction in individuals on statin therapy. *J. Agric. Food Chem.* 57, 8316-8324.

De Souza, L.M., Dartora, N., Scoparo, C.T., Cipriani, T.R., Gorin, P.A., Lacomini, M., Sassaki, G.L., 2011. Comprehensive analysis of mate (*Ilex paraguariensis*) compounds: development of chemical strategies for matesaponin analysis by mass spectrometry. *J. Chromatogr. A* 1218, 7307-7315.

Fenwick, G., Price, K., Tsukamoto, C., Okubo, K., 1991. Saponins. *Tox. Subst. Crop Plant.* 285-327.

Gao, H., Liu, Z., Wan, W., Qu, X., Chen, M., 2013. Aqueous extract of yerba mate tea lowers atherosclerotic risk factors in a rat hyperlipidemia model. *Phytother. Res.* 27, 1225-1231.

475 Gnoatto, S.C., Schenkel, E.P., Bassani, V.L., 2005. HPLC method to assay total
 476 saponins in *Ilex paraguariensis* aqueous extract. J. Braz. Chem. Soc. 16, 723-725.

477 Gosmann, G., Schenkel, E.P., Selignmann, O., 1989. A new saponin from mate, *Ilex*
 478 *paraguariensis*. J. Nat. Prod. 52, 1367-1370.

479 Gosmann, G., Guillaume, D., Taketa, A.T.C., Schenkel, E.P., 1995. Triterpenoid
 480 saponins from *Ilex paraguariensis*. J. Nat. Prod. 58, 438-441.

481 Güçlü-Üstündağ, Ö., Mazza, G., 2007. Saponins: properties, applications and
 482 processing. Crit. Rev. Food Sci. Nutr. 47, 231-258.

483 Khurana, S., Venkataraman, K., Hollingsworth, A., Piche, M., Tai, T., 2013.
 484 Polyphenols: benefits to the cardiovascular system in health and in aging. Nutrients
 485 5, 3779-3827.

486 Hostettmann, K., Marston, A., 2005. *Saponins*: Cambridge University Press.

487 Kraemer, K.H., Taketa, A.T., Schenkel, E.P., Gosmann, G., Guillaume, D., 1996.
 488 Matesaponin 5, a highly polar saponin from *Ilex paraguariensis*. Phytochem. 42,
 489 1119-1122.

490 Lee, M.K., Ahn, Y.M., Lee, K.R., Jung, J.H., Jung, O.-S., Hong, J., 2009. Development
 491 of a validated liquid chromatographic method for the quality control of *Prunellae*
 492 *Spica*: determination of triterpenic acids. Anal. Chim. Acta 633, 271-277.

493 Liu, S., Cui, M., Liu, Z., Song, F., Mo, W., 2004. Structural analysis of saponins from
 494 medicinal herbs using electrospray ionization tandem mass spectrometry. J. Am.
 495 Soc. Mass Spectrom. 15, 133-141.

496 Martinet, A., Ndjoko, K., Terreaux, C., Marston, A., Hostettmann, K., Schutz, Y., 2001.
 497 NMR and LC-MSn characterisation of two minor saponins from *Ilex paraguariensis*.
 498 Phytochem. Anal. 12, 48-52.

499 Miranda, D.D., Arçari, D.P., Pedrazzoli, J., Carvalho, P.d.O., Cerutti, S.M., Bastos, D.
 500 H., Ribeiro, M.L., 2008. Protective effects of mate tea (*Ilex paraguariensis*) on H₂O₂-
 501 induced DNA damage and DNA repair in mice. Mutagenesis 23, 261-265.

502 Morikawa, T., Nakamura, S., Kato, Y., Muraoka, O., Matsuda, H., Yoshikawa, M., 2007.
 503 Bioactive saponins and glycosides. XXVIII. New triterpene saponins,
 504 foliatheasaponins I, II, III, IV, and V, from Tencha (the leaves of *Camellia sinensis*).
 505 Chem Pharm Bull (Tokyo). 55, 293-298.

506 Mosimann, A.L.P., Wilhelm-Filho, D., Da Silva, E.L., 2006. Aqueous extract of *Ilex*
 507 *paraguariensis* attenuates the progression of atherosclerosis in cholesterol-fed
 508 rabbits. Biofactors 26, 59-70.

509 Mroczek, A., Kapusta, I., Janda, B., Janiszowska, W., 2012. Triterpene saponin content
 510 in the roots of red beet (*Beta vulgaris* L.) cultivars. J. Agric. Food Chem. 60, 12397-
 511 12402.

Nakamura, K.L., Donaduzzi, E., Schuster, C., 2009. Genetic variation of phytochemical compounds in progenies of *Ilex paraguariensis* St. Hil. *Crop Breed. Appl. Biot.* 9, 116-123.

Negi, J.S., Singh, P., Pant, G.J.N., Rawat, M., 2011. High-performance liquid chromatography analysis of plant saponins: An update 2005-2010. *Pharmacogn. Rev.* 5, 155.

Oleszek, W., Bialy, Z., 2006. Chromatographic determination of plant saponins—an update (2002–2005). *J. Chromatogr. A* 1112, 78-91.

Osborn, A., Goss, R.J., Field, R.A., 2011. The saponins—polar isoprenoids with important and diverse biological activities. *Nat. Prod. Rep.* 28, 1261-1268.

Pavei, C., Guzzato, P., Ros Petrovick, P., Gosmann, G., González-Ortega, G., 2007. Development and Validation of an HPLC Method for the Characterization and Assay of the Saponins from *Ilex paraguariensis* A. St.-Hil (Mate) Fruits. *J. Liq. Chromatogr. R. T.* 30, 87-95.

Puangpraphant, S., de Mejia, E.G., 2009. Saponins in yerba mate tea (*Ilex paraguariensis* A. St.-Hil) and quercetin synergistically inhibit iNOS and COX-2 in lipopolysaccharide-induced macrophages through NFκB pathways. *J. Agric. Food Chem.* 57, 8873-8883.

Puangpraphant, S., Berhow, M.A., de Mejia, E.G., 2011. Mate (*Ilex paraguariensis* St. Hilaire) saponins induce caspase-3-dependent apoptosis in human colon cancer cells in vitro. *Food Chem.* 125, 1171-1178.

Rao, A., Gurfinkel, D., 2000. The bioactivity of saponins: triterpenoid and steroidal glycosides. *Drug Metabol. Drug Interact.* 17, 211-236.

Sarriá, B., Martínez-López, S., Sierra-Cinos, J.L., Garcia-Diz, L., Goya, L., Mateos, R., Bravo, L., 2015. Effects of bioactive constituents in functional cocoa products on cardiovascular health in humans. *Food Chem.* 174, 214-218.

Schenkel, E.P., Montanha, J.A., Gosmann, G., 1996. Triterpene saponins from maté, *Ilex paraguariensis*. *Saponins Used in Food and Agriculture*, 47-56. Springer US.

Scherer, R., Janssens, M., Marx, F., Urfer, P., Schneider, E., 2007. Saponin Content and Quality-Related Traits of Mass-Selected Yerba Maté (*Ilex paraguariensis* A. St.-Hil.) Trees. *J. Herbs Spices Med. Plants*, 12, 73-85.

Sugimoto, S., Nakamura, S., Yamamoto, S., Yamashita, C., Oda, Y., Matsuda, H., Yoshikawa, M., 2009. Brazilian natural medicines. III. Structures of triterpene oligoglycosides and lipase inhibitors from mate, leaves of *Ilex paraguariensis*. *Chem. Pharm. Bull. (Tokyo)*, 57, 257-261.

Taketa, A.T., Breitmaier, E., Schenkel, E.P., 2004. Triterpenes and triterpenoidal glycosides from the fruits of *Ilex paraguariensis* (Maté). J. Braz. Chem. Soc. 15, 205-211.

Yan, J., Duan, J., Wu, X., Guo, C., Yin, Y., Zhu, Y., Hu, T., Wei, G., Wen, A., Xi, M., 2015. Total saponins from *Aralia taibaiensis* protect against myocardial ischemia/reperfusion injury through AMPK pathway. Int. J. Mol. Med. 36, 1538-1546.

Figure captions

Figure 1. High Performance Liquid Chromatography-Diode Array (HPLC-DAD) chromatograms at 210 nm (a) and (b) enlarged of yerba mate extracts in red, and standards oleanolic (Ole) acid in blue and ursolic (Urs) acid in green.

Figure 1.

Table 1. Chemical characterization and negative ion tandem mass spectrometry (MS/MS) fragmentation of saponin compounds in yerba mate.

No	RT (min)	[M-H] ⁻ m/z	Molecular formula	MS/MS ions m/z					Tentative structures
				Base peak	Secondary peak				
1	22.99	911.5034	C ₄₇ H ₇₆ O ₁₇	749					Glu-Ara-Ole-Glu (Matesaponin 1')
2	23.21	765.4444	C ₄₁ H ₆₆ O ₁₃	603	585	471			Ara-Ole-OH-Glu (Matesaponin C')
3	25.95	765.4444	C ₄₁ H ₆₆ O ₁₃	603	471				Ara-Urs-OH-Glu (Matesaponin C)
4	26.17	1381.6677	C ₆₅ H ₁₀₆ O ₃₁	895	485				Glu-Rha-Ara-Urs-Glu-Glu-Glu (Matesaponin 5)
5	26.89	1219.6151	C ₅₉ H ₉₆ O ₂₆	895	733				Glu-Rha-Ara-Urs-Glu-Glu (Matesaponin 4)
6	27.27	1073.5536	C ₅₃ H ₈₆ O ₂₂	749	587				Glu-Ara-Urs-Glu-Glu (Matesaponin 3)
7	27.44	1409.6575	C ₆₆ H ₁₀₆ O ₃₂	923	485				Xyl-Glu-Ac-Ara-Agly-Glu-Glu-Glu
8	27.62	1277.6159	C ₆₁ H ₉₈ O ₂₈	953	911	893	749	587	Ac-Glu-Glu-Ara-Agly-Glu-Glu
9	28.05	1247.6114	C ₆₀ H ₉₆ O ₂₇	923	881	863	749	587	Ac-Pentose-Hexose-Pentose-Agly-Hexose-Hexose
10	28.56	1057.5627	C ₅₃ H ₈₆ O ₂₁	733					Rha-Ara-Agly-Glu-Glu (isomer of Matesaponin 2)
11	28.91	1115.5692	C ₅₅ H ₈₈ O ₂₃	749	731	587	323		Glu-Ara-Agly-Glu-Glu-Ac
12	29.64	895.5095	C ₄₇ H ₇₆ O ₁₆	733	587	455			Rha-Ara-Ole-Glu (J3a or Matesaponin D)
13	30.25	911.5049	C ₄₇ H ₇₆ O ₁₇	749	587	455			Glu-Ara-Urs-Glu (Matesaponin 1)
14	30.83	1085.5571	C ₅₄ H ₈₄ O ₂₂	923	881	749	587	455	Ac-Pentose-Hexose-Pentose-Agly-Hexose
15	31.63	895.5135	C ₄₇ H ₇₆ O ₁₆	733					Rha-Ara-Urs-Glu (J3b)
16	31.92	953.5135	C ₄₉ H ₇₈ O ₁₈	791	749	731	587		Ac-Hexose-Pentose-Agly-Hexose
17	35.80	471.3489	C ₃₀ H ₄₈ O ₄						Agly-OH
18	46.29	455.3506	C ₃₀ H ₄₈ O ₃						Ole
19	46.84	455.3549	C ₃₀ H ₄₈ O ₃						Urs

Abbreviations: Ac, acetyl group; Ara, α-L-arabinospyranosyl; Glu, β-D-glucopyranosyl; Ole, Oleanolic acid; Rha, α-L-rhamnopyranosyl; RT, retention time; Urs, Ursolic acid; Xyl, D-xylopyranose.

637

638 **Table 2.** Content of saponins and free triterpenes in methanolic extracts from different
 639 commercial brands (A, B, C and D) of yerba mate. Values represent means $\pm$ standard
 640 deviation (SD) expressed in mg/g (n = 3 analysed in triplicate).

641

	Peak	Mate A	Mate B	Mate C	Mate D
Saponins	1	0.092 $\pm$ 0.002	0.084 $\pm$ 0.002	0.092 $\pm$ 0.003	0.080 $\pm$ 0.003
	2	0.029 $\pm$ 0.001	0.023 $\pm$ 0.001	0.025 $\pm$ 0.002	0.033 $\pm$ 0.004
	3	0.026 $\pm$ 0.002	0.028 $\pm$ 0.002	0.022 $\pm$ 0.002	0.025 $\pm$ 0.002
	4	0.026 $\pm$ 0.002	0.029 $\pm$ 0.001	0.023 $\pm$ 0.001	0.022 $\pm$ 0.001
	5	0.300 $\pm$ 0.007	0.368 $\pm$ 0.007	0.257 $\pm$ 0.015	0.302 $\pm$ 0.026
	6	0.015 $\pm$ 0.001	0.013 $\pm$ 0.001	0.010 $\pm$ 0.000	0.012 $\pm$ 0.001
	7	0.009 $\pm$ 0.001	0.015 $\pm$ 0.001	0.014 $\pm$ 0.001	0.008 $\pm$ 0.000
	8	0.014 $\pm$ 0.001	0.0115 $\pm$ 0.002	0.011 $\pm$ 0.001	0.014 $\pm$ 0.001
	9	0.058 $\pm$ 0.004	0.048 $\pm$ 0.003	0.032 $\pm$ 0.003	0.090 $\pm$ 0.011
	10	0.390 $\pm$ 0.013	0.404 $\pm$ 0.013	0.357 $\pm$ 0.013	0.360 $\pm$ 0.013
	11	0.094 $\pm$ 0.006	0.097 $\pm$ 0.004	0.084 $\pm$ 0.002	0.086 $\pm$ 0.005
	12	1.064 $\pm$ 0.017	1.192 $\pm$ 0.030	0.720 $\pm$ 0.028	0.887 $\pm$ 0.034
	13	0.638 $\pm$ 0.009	0.769 $\pm$ 0.022	0.494 $\pm$ 0.022	0.588 $\pm$ 0.044
	14	0.011 $\pm$ 0.001	0.020 $\pm$ 0.001	0.013 $\pm$ 0.001	0.015 $\pm$ 0.001
	15	0.178 $\pm$ 0.007	0.183 $\pm$ 0.005	0.112 $\pm$ 0.006	0.163 $\pm$ 0.015
	16	0.119 $\pm$ 0.004	0.145 $\pm$ 0.004	0.093 $\pm$ 0.003	0.128 $\pm$ 0.005
TOTAL		3.064 $\pm$ 0.027	3.433 $\pm$ 0.041	2.359 $\pm$ 0.041	2.813 $\pm$ 0.066
Free triterpenes	17	0.025 $\pm$ 0.002	0.029 $\pm$ 0.002	0.036 $\pm$ 0.002	0.024 $\pm$ 0.002
	18	0.266 $\pm$ 0.005	0.294 $\pm$ 0.003	0.288 $\pm$ 0.016	0.229 $\pm$ 0.023
	19	1.649 $\pm$ 0.008	1.807 $\pm$ 0.023	1.751 $\pm$ 0.090	1.557 $\pm$ 0.161
TOTAL FREE TRITERPENES		1.940 $\pm$ 0.010	2.130 $\pm$ 0.023	2.075 $\pm$ 0.091	1.810 $\pm$ 0.162
TOTAL SAPONINS		5.005 $\pm$ 0.001	5.563 $\pm$ 0.002	4.434 $\pm$ 0.009	4.623 $\pm$ 0.027

642

643

644

645

646

647