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Short communication

LC–MS^n characterization of saponins in mate (*Ilex paraguariens,* St. Hil) and their quantification by HPLC-DAD

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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/MS^n 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 to 5.5 mg/g in four commercial brands, being matesaponin D or J3a the most abundant.

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 et al., 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-carcinogenic properties, among others (Arçari et al., 2009; Boaventura et al., 2012; Bravo et al., 2014; de Mejía et al., 2010; Gao et al., 2013; Miranda et al., 2008; Mosimann et al., 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 and Ramirez-Mares, 2014; Khurana et al., 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 and Mazza, 2007; Osbourn et al., 2011; Rao and 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) and cyclooxigenase 2 pathways, as well as the inhibition of the nuclear factor NFκB nuclear translocation (Puangpraphant and de Mejia, 2009; Puangpraphant et al., 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 and Marston, 2005). The most common monosaccharides in saponins are D-glucose, D-galactose, D-glucuronic acid, D-galacturonic acid, L-

Abbrevations: 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

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rhamnose, L-arabinose, D-xylose, and D-fructose (Hostettmann and 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 monossacharides, 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 et al., 1995; Martinet et al., 2001; Taketa et al., 2004). Although fruits have a higher content in saponins than leaves (up 30% *vs.* 4%, w/w) (Borré et al., 2010; Pavei et al., 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 et al., 2010; Gnoatto et al., 2005; Puangpraphant and de Mejia, 2009). In addition, variation of the saponin content of yerba mate depends on the genetic origin, intraspecies variation, the part of the plant being examined, environmental and agronomic factors, and post-harvest treatments such as storage and processing (Fenwick et al., 1991; Nakamura et al., 2009; Scherer et al., 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 Suphersphere RP18 column (4 mm × 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 psi 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 × 250 mm 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 their 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 et al., 1989; Martinet et al., 2001; Morikawa et al., 2007; Oleszek and 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 and Mazza, 2007; Oleszek and Bialy, 2006). Considering these drawbacks and the absence of conclusive quantitative data, although the extraction with cold water:ethanol is the procedure most assayed in the literature (Oleszek and Bialy, 2006), three different solvents (methanol, ethanol and a water:ethanol mixture −4:6, v/v−) were compared at three shacking times (1, 2 and 3 h). Methanol extraction yielded the highest saponin content according to chromatographic analysis, not showing differences due to the shaking times tested. Afterwards, two cold extraction methods (shaking and sonicating) using methanol for 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 former 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.