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Identification and characterization of chlorogenic acids, chlorogenic acid glycosides and flavonoids from *Lonicera henryi* L. (Caprifoliaceae) leaves by LC–MS^n

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ABSTRACT

The chlorogenic acids, chlorogenic acid glycosides and flavonoids of the leaves of *Lonicera henryi* L. (Caprifoliaceae) were investigated qualitatively by liquid chromatography tandem mass spectrometry. Thirty-one chlorogenic acids and their glycosides were detected and characterized to their regioisomeric level on the basis of their unique fragmentation pattern in the negative ion mode tandem MS spectra. All of them were extracted for the first time from this source and thirteen of them were not reported previously in nature. For the positive identification of chlorogenic acid glycosides by LC–MS^n, multiple reaction monitoring and targeted MS^n experiments were performed. We have developed an LC–MS^n method for the systematic identification of chlorogenic acid glycosides and were also able to discriminate between chlorogenic acids and their isobaric glycosides. It was also possible to discriminate between 5-*O*-(3′-*O*-caffeoyl glucosyl)quinic acid and 5-*O*-(4′-*O*-caffeoyl glucosyl)quinic acid by LC–MS^n. This method can be applied for the rapid and positive identification of chlorogenic acids and their glycosides in plant materials, food and beverages.

1. Introduction

Classically, chlorogenic acids (CGAs) are a family of esters formed between quinic acid and certain *trans*-hydroxycinnamic acids, most commonly caffeic, ferulic, *p*-coumaric acids and sometimes dimethoxycinnamic, trimethoxycinnamic and sinapic acids (Clifford, 1999, 2000; Clifford et al., 2010; Jaiswal and Kuhnert, 2010, 2011a,b,c; Jaiswal et al., 2010a). Some *cis* isomers are also known but seem to be restricted to plant tissues in which the corresponding *trans* isomers have been exposed to relatively strong UV–irradiation thus inducing *cis–trans* isomerisation (Clifford et al., 2008; Karakoese et al., 2011; Jaiswal et al., 2010b; Jaiswal et al., 2011a,b).

Several pharmacological activities including antioxidant activity (Slanina et al., 1999; Thuong et al., 2009), antispasmodic activity (Trute et al., 1997), ability to increase hepatic glucose utilization (Shearer et al., 2003), inhibition of the HIV-1 integrase (McDougall et al., 1998; Robinson et al., 1996), inhibition of DNA methyl transferase (Rajavelu et al., 2011) and inhibition of the mutagenicity of carcinogenic compounds (Stich et al., 1982) have been revealed by *in vitro*, *in vivo* and human intervention studies so far.

Lonicera henryi is an evergreen ornamental plant belonging to the genus *Lonicera* of the Caprifoliaceae family. The flowers, leaves and stem of *L. henryi* are edible (Facciola, 1990; Tanaka, 1976) and used in traditional Chinese medicines (Duke and Ayensu, 1985). There is only little known about the phytochemical profile of *L. henryi*. Leaveau et al. (1977) reported the presence of alkaloids and saponins in the fruits of *L. henryi*. The genus *Lonicera* is a source of phenolic acids (Jurikova et al., 2012), flavonoids (Sun et al., 2012; Luo et al., 2011), flavonoid glycosides, saponins (Chen et al., 2012a,b), chlorogenic acids (Zhang et al., 2013; Li et al., 2012), chlorogenic acid glycosides (Zhang et al., 2013) and saponin chlorogenates (Chen et al., 2012b). *Lonicera* plants are used in Flos Lonicearae, a traditional Chinese herbal medicine. On several occasions CGA glycosides were reported in food materials and plants but no information was given about their regiochemistry unless they were isolated and studied by spectroscopic methods (Clifford et al., 2007; Parveen et al., 2011; Zhang et al., 2013; Wang and Clifford, 2008; Kashiwada et al., 2012; Nikhat et al., 2008; McNamara et al., 2004; Ma et al., 2010, 2011). CGA glycosides are an interesting challenge from an analytical perspective since their pseudomolecular ions are isobaric to caffeoylquinic acids,

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caffeoyl-*p*-coumaroylquinic acids and caffeoyl-feruloylquinic acids (Alakolanga et al., 2014). Additionally, they provide tandem MS fragment spectra very similar to CGAs since a loss of the glucosyl (162 Da) or a rhamnosyl (146 Da) moiety leads to a fragment ion at *m/z* 353, 337, 367 and 515 (negative ion mode). To unambiguously identify these derivatives and discriminate them from CGAs high resolution and tandem MS are required since molecular formulas of CGA glycosides and CGAs are different.

In this study we have shown that the CGA glycosides can be easily identified and characterized to their regioisomeric level by liquid chromatography tandem mass spectrometry without a tedious isolation of the individual CGA glycosides.

2. Results and discussion

All the retention times and MS data were collected using a C18 amide column. For the LC–MS measurements negative ion mode was used to obtain better tandem mass spectra and high resolution mass spectra. The phenolics were positively identified by their typical UV–absorptions at 254, 280 and 320 nm. All the CGAs showed loss of the cinnamoyl/cinnamic acid part while CGAs glycosides showed loss of the glycosyl/cinnamoyl/cinnamic acid part in the negative ion mode MS spectra. For all the compounds the high resolution mass data was in good agreement with the theoretical molecular formulas, all displaying a mass error of below 5 ppm thus confirming their elemental composition. In general, peak identities were consistent both within and between analyses. Fragment ions with intensities <5% of the base peak were reported only when they were needed for comparison. All CGAs glycosides showed a typical fragmentation pattern and UV absorbance of chlorogenic acids. All CGAs glycosides were more hydrophilic than their isobaric chlorogenic acids. For instance, mono and di-acyl CGAs glycosides eluted before di- and tri-acyl CGAs, respectively. These glycosides showed fragments which originated from the cinnamoyl glycoside part *e.g.*, *m/z* 341 (*m/z* 341.0864, $C_{15}H_{17}O_9$) and 323 (*m/z* 341.0765, $C_{15}H_{15}O_8$) for caffeoyl-glucoside; *m/z* 355 (*m/z* 355.1025, $C_{16}H_{19}O_9$) and 337 (*m/z* 337.0915, $C_{16}H_{17}O_8$) for feruloyl-glucoside; *m/z* 325 (*m/z* 325.0918, $C_{15}H_{17}O_8$) and 307 (*m/z* 307.08124, $C_{15}H_{15}O_7$) for *p*-coumaroyl-glucoside. In this study we only observed flavonoid *O*-glycosides which was confirmed by the absence of the higher intensity or base peak MS^n ions ($[M-H^+-120]^-$) and ($[M-H^+-90]^-$) (March et al., 2006).

Acid hydrolysis followed by LC–MS showed the presence of arabinose, rhamnose and glucose as the glycone units of chlorogenic acid glycosides and flavonoid glycosides (Jaiswal et al., 2014b).

2.1. Characterization of chlorogenic acids

Four caffeoylquinic acids (**1–4**), four *p*-coumaroylquinic acids (**5–8**), two feruloylquinic acid (**9** and **10**), three dicaffeoylquinic acids (**13–15**), two caffeoyl-feruloylquinic acid (**16** and **17**), one caffeoyl-*p*-coumaroylquinic acid (**18**) and one tricaffeoylquinic acid (**19**) were easily located in the extracted ion chromatograms and assigned using the hierarchical keys previously developed as the well-known 3-*O*-caffeoylquinic acid **1**, *cis*-3-*O*-caffeoylquinic acid **2**, 5-*O*-caffeoylquinic acid **3**, *cis*-5-*O*-caffeoylquinic acid **4**, 3-*p*-coumaroylquinic acid **5**, *cis*-3-*p*-coumaroylquinic acid **6**, 5-*p*-coumaroylquinic acid **7**, *cis*-5-*p*-coumaroylquinic acid **8**, 5-*O*-feruloylquinic acid **9**, *cis*-5-*O*-feruloylquinic acid **10**, 3,4-di-*O*-caffeoylquinic acid **13**, 3,5-di-*O*-caffeoylquinic acid **14**, 4,5-di-*O*-caffeoylquinic acid **15**, 4-*O*-feruloyl-5-*O*-caffeoylquinic acid **16**, 4-*O*-caffeoyl-5-*O*-feruloylquinic acid **17**, 4-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid **18** and 3,4,5-tri-*O*-caffeoylquinic acid **19** (Table 1) (Jaiswal et al., 2011b; Jaiswal and Kuhnert, 2010; Clifford et al., 2003, 2005). The *cis*/*trans* ratio of mono acyl CGAs was as following: 3-*O*-caffeoylquinic acid 0.07, 5-*O*-caffeoylquinic acid 0.45, 3-*O*-*p*-coumaroylquinic acid 0.03, 5-*O*-*p*-coumaroylquinic acid 0.02 and 5-*O*-feruloylquinic acid 0.13. It is interesting to notice that 4-acylated CGAs are present only as diacyl and triacyl derivatives and mono 4-acylated derivatives are completely absent in *L. henryi* leaves. Li et al. (2009) reported the presence of mono 4-acylated CGAs in the flowers of *Lonicera* species. These observations suggested that *L. henryi* of *Lonicera* genus does not synthesize mono 4-acylated CGAs. A similar behavior was reported previously for gallium species (Rubeaceae family); e.g., *G. boreale* and *G. glaucum* do not produce 4-acylated CGA while *G. odoratum*, *G. verum*, *G. harcynicum* and *G. mollugo* produce it (Jaiswal et al., 2014).

2.2. Characterization of 3-O-hydroxydihydrocaffeoylquinic acid (M_r 372)

One peak was detected at *m/z* 371 (*m/z* 371.0972, $C_{16}H_{19}O_{10}$, Table 1) in the extracted and total ion chromatograms and was assigned as hydroxydihydrocaffeoylquinic acid **11**. It produced the MS^2 base peak at *m/z* 353 ($[caffeoyquinic\ acid\text{-}H^+]^-$) via the loss of H_2O (18 Da) and secondary peaks at *m/z* 191 ($[quinic\ acid\text{-}H^+]^-$), *m/z* 179 ($[caffeic\ acid\text{-}H^+]^-$), *m/z* 173 ($[quinic\ acid\text{-}H_2O\text{-}H^+]^-$), *m/z* 135 ($[caffeic\ acid\text{-}CO_2\text{-}H^+]^-$) and *m/z* 233 ($[acetylquinic\ acid\text{-}H^+]^-$). It produced the MS^3 base peak at *m/z* 191 ($[quinic\ acid\text{-}H^+]^-$) and secondary peaks at *m/z* 179, 173 and 135 (Table 1). The MS^3 spectrum was identical to the MS^2 spectrum of 3-*O*-caffeoylquinic acid (Table 1). The presence of the MS^2 ion at *m/z* 233 suggested that the –OH group was connected to the *β*-position (caffeoyl moiety) of the ester bond (Matei et al., 2012). Recently, we reported water addition products of the CGAs in brewed coffee (Matei et al., 2012). This compound showed retention times and fragmentation identical to previously reported 3-*O*-hydroxydihydrocaffeoylquinic acid (Matei et al., 2012). Based on the above arguments, this compound was assigned as 3-*O*-hydroxydihydrocaffeoylquinic acid **11**.

2.3. Characterization of 5-O-caffeoylquinic acid glucosides (M_r 516)

Four peaks were detected at *m/z* 515 (*m/z* 515.1395 – *m/z* 515.1431, $C_{22}H_{27}O_{14}$, Table 1) in the extracted ion chromatogram and were tentatively assigned as caffeoylquinic acid glucosides. The first and the third eluting isomers (**20** and **22**) produced the MS^2 base peak at *m/z* 353 ($[caffeoylquinic\ acid\text{-}H^+]^-$) via the loss of a glucosyl residue (162 Da); the secondary peaks occurred as follows: the peak at *m/z* 341 ($[caffeoyl\ glucoside\text{-}H^+]^-$) due to the loss of a quinic acid moiety (174 Da), the peak at *m/z* 323 ($[caffeoyl\ glucoside\text{-}H_2O\text{-}H^+]^-$) due to the loss of a quinic acid moiety (174 Da) followed by H_2O (18 Da), the peak at *m/z* 191 ($[quinic\ acid\text{-}H^+]^-$) due to the loss of a caffeoyl and a glucosyl residues and the peak at *m/z* 179 ($[caffeic\ acid\text{-}H^+]^-$) due to the loss of a glucosyl and a quinic acid moieties (Fig. 1 and Table 1). These compounds produced the MS^3 base peak at *m/z* 191 ($[quinic\ acid\text{-}H^+]^-$) via the loss of a caffeoyl residue (162 Da) (Fig. 1 and Table 1). The MS^2 and MS^3 spectra were identical to the MS^1 and MS^2 spectra of 5-*O*-caffeoylquinic acid (Fig. 1 and Table 1). The MS^2 peaks at *m/z* 353, 341 and 323 suggested that glucose was connected with the caffeic acid moiety by an ether linkage and caffeic acid was connected with quinic acid at C-5 by an ester bond.

The second and the last eluting isomers (**21** and **23**) produced the MS^2 base peak at *m/z* 323 ($[caffeoyl\ glucoside\text{-}H_2O\text{-}H^+]^-$) and secondary peaks at *m/z* 341 ($[caffeoyl\ glucoside\text{-}H^+]^-$), *m/z* 353 ($[caffeoylquinic\ acid\text{-}H^+]^-$), *m/z* 191 ($[quinic\ acid\text{-}H^+]^-$) and *m/z* 161 ($[caffeic\ acid\text{-}H_2O\text{-}H^+]^-$) (Fig. 1 and Table 1). They produced the MS^3 base peak at *m/z* 161 ($[caffeic\ acid\text{-}H_2O\text{-}H^+]^-$) and a secondary peak at *m/z* 133 ($[caffeic\ acid\text{-}CO\text{-}H_2O\text{-}H^+]^-$) which are

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