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Analytical Methods

Identification and quantification of (dihydro) hydroxycinnamic acids and their conjugates in potato by UHPLC–DAD–ESI-MS^n

Carlos-Eduardo Narváez-Cuenca [a,b], Jean-Paul Vincken [a], Harry Gruppen [a,*]

[a] Laboratory of Food Chemistry, Wageningen University, P.O. Box 8129, 6700EV Wageningen, The Netherlands
[b] Departamento de Química, Facultad de Ciencias, Universidad Nacional de Colombia, Carrera 30 # 45-03, Bogotá, Colombia

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ABSTRACT

Hydroxycinnamic acid conjugates (HCAcs) and dihydrohydroxycinamic acid conjugates (DHCAcs) were identified and quantified in potato tuber extracts by UHPLC–DAD–ESI-MS^n. The HCAcs and DHCAcs identification took place by screening for product ions and neutral losses in combination with UV spectra. Thirty-nine HCAcs/DHCAcs were detected, including 17 previously reported in potato. HCAs were found unconjugated, linked to hydroxyl-containing compounds including hexose, quinic acid and malic acid, to amino-containing compounds, such as putrescine and octopamine, and to unknown compounds. DHCAs were present linked to spermine, spermidine and to still unidentified compounds. Chlorogenic acid was the most abundant compound (25.43 ± 0.49 mg/g DW) followed by *crypto*chlorogenic acid (7.31 ± 0.38 mg/g DW), a non-hydrolyzable sinapic acid conjugate (2.80 ± 0.06 mg/g DW) and *neo*chlorogenic acid (2.41 ± 0.10 mg/g DW), in total accounting for 83% (w/w) of the total concentration of HCAs/DHCAs-containing compounds. Quantifications of HCAs released after alkaline hydrolysis matched well with the quantification of the unhydrolyzed molecules. The UHPLC–DAD–ESI-MS^n method showed a larger diversity of HCAcs and DHCAcs in potato than described before.

1. Introduction

Plants accumulate a large variety of secondary metabolites, which includes phenolic compounds, such as hydroxycinnamic acids (HCAs), their conjugates (HCAcs) and dihydrohydroxycinnamoyl conjugates (DHCAcs). HCAs/HCAcs/DHCAcs are related to resistance of plants to stress (Abdel-Farid et al., 2009; Edreva, 2005). These compounds also have technological importance, e.g. they can participate in browning reactions, and they can affect the applicability of proteins by binding to them (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). Furthermore, HCAs/HCAcs seem to have promising health benefits in humans, including their anti-hypertensive activity and prevention of atherosclerosis, type 2 diabetes, Alzheimer's disease and cancer (Zhao & Moghadasian, 2010). In addition, there is evidence of the inhibitory effect of HCAcs against HIV (Tamura et al., 2006), and of their antimicrobial activity (Muthuswamy & Rupasinghe, 2007).

HCAs/HCAcs/DHCAcs are the most abundant secondary metabolites found in potato (*Solanum tuberosum*), with chlorogenic acid (5-*O*-caffeoylquinic acid) as the major representative. Isomers, like *crypto*chlorogenic acid (4-*O*-caffeoylquinic acid) and *neo*chlorogenic acid (3-*O*-caffeoylquinic acid), as well as caffeic acid are frequently reported in potato (Friedman, 1997; Shakya & Navarre, 2006). Although identification and quantification of these major HCAs/HCAcs in potato is well reported, the large number of minor HCAs/HCAcs/DHCAcs is less documented (Parr, Mellon, Colquhoun, & Davies, 2005; Shakya & Navarre, 2006). Because of the potential benefits of HCAs/HCAcs/DHCAcs, it is important to have a suitable method to reveal and quantify the complete array of these compounds present in potato. Recently, ultra high performance liquid chromatography (UHPLC) has been shown to offer unprecedented resolution in the separation of phytochemicals. Besides UV–Vis data analysis, MS^n detection provides new opportunities for rapid identification of different phytochemicals (Harbaum et al., 2007; Moco et al., 2006). Therefore, in this study, UHPLC–DAD–ESI-MS^n was employed to identify and quantify HCAs/HCAcs/DHCAcs in aqueous methanolic potato tuber extracts, with emphasis on the minor constituents present.

2. Materials and methods

2.1. Chemicals

p-Coumaric acid, caffeic acid, ferulic acid, sinapic acid, and chlorogenic acid were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). UHPLC/MS grade acetonitrile (ACN) was purchased from Biosolve BV (Valkenswaard, The Netherlands).

* Corresponding author. Tel.: +31 317 483211; fax: +31 317 484893.
E-mail address: harry.gruppen@wur.nl (H. Gruppen).

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Water was obtained by a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were from Merck (Darmstadt, Germany).

2.2. Plant material

Potato tubers (Victoria variety) were purchased from a local supermarket in Wageningen, The Netherlands. Samples were washed with tap water and the surface was allowed to dry at room temperature (3 h). Whole tubers were manually sliced (0.3 mm thickness) and freeze-dried. Freeze-dried material was milled into a fine powder with a DCFH 48 vegetable grinder, with a sieve size of 0.5 mm (Wilten Woltil, de Meern, The Netherlands) and stored in sealed plastic containers at −20 °C until analysis.

2.3. Extraction of HCAs/HCAcs/DHCAcs

Freeze-dried samples (2 g) were extracted using 10 mL of water/methanol (50/50, v/v) containing 0.5% (v/v) acetic acid. The mixture was stirred at 500 rpm for 15 min at 4 °C and then centrifuged (15,000*g*; 15 min; 4 °C). This procedure was repeated four times. As the compounds investigated have absorption maxima ranging from 310 to 330 nm, the effectiveness of repetitive extractions was tested by measuring the absorbance at 320 nm. The fourth extraction represented less than 1% of the compounds that were extracted. The four supernatants were combined, filtered through a 0.45 μm filter (Schleicher & Schuell, Dassel, Germany), and stored at −20 °C until further analysis. This sample is further referred to as crude extract. Extractions were done in triplicates.

2.4. Alkaline hydrolysis of phenolic compounds

The crude extract, as such, was subjected to alkaline hydrolysis. Several concentrations of NaOH were tested: 0.0, 0.010, 0.025, 0.050, 0.075, 0.10, 0.25, 0.50 and 1.0 M. Crude extract (4 mL) was stirred with 4 mL of 0.0–2.0 M NaOH overnight at 20 °C; in all cases ascorbic acid and EDTA were added as antioxidants to final concentrations of 0.052 and 0.010 M, respectively. Afterwards, the mixture was acidified to pH 1–2 by adding 37% (w/v) HCl, and the volume was adjusted to 10 mL by adding the extraction solvent. The hydrolysates were analysed by UHPLC–ESI-MS^n (Section 2.5). To verify the stability of the HCAs under alkaline conditions the recovery of *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid was tested with authentic compounds at concentrations similar to those expected in the extract.

2.5. UHPLC–DAD–ESI-MS^n analysis

Samples, with and without alkaline hydrolysis, undiluted and 10× diluted, were analysed in a Thermo Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with pump, autosampler and photo-diode array detector (DAD) using a Hypersyl gold RP column (Thermo Scientific; 150 mm × 2.1 mm i.d.; particle size 1.9 μm) at 30 °C. The eluents used were water/acetonitrile/acetic acid (99:1:0.1, v/v/v) (eluent A) and acetronitrile/acetic acid (100:0.1, v/v) (eluent B). The elution program (elution condition I) was 0–5 min, 0% B; 5–23 min, 0–60% B; 23–24 min, 60–100% B; 24–27 min, 100% B; 27–28 min, 100–0% B; 28–35 min, 0% B. The flow rate was 400 μL/min; injection volume was 5 μL. MS^n analysis was performed on a Thermo Scientific LTQ-XL using electrospray ionisation (ESI) and detection in the negative ion mode, with a source voltage of 3.5 kV, and an ion transfer tube temperature of 350 °C. The instrument was tuned to optimise the ionisation process and sensitivity using chlorogenic acid. A full-scan mass spectrum over a range of *m*/*z* values of 150–1500 was recorded. The control of the instrument and data processing were done using Xcalibur 2.07 (Thermo Scientific). Identification was done based on the molecular mass of the parent ion, MS^n and UV–Vis spectra data. In a second set of runs (elution condition II) the elution program and temperature of the column were optimised to resolve feruloyl malate from sinapoyl malate, and ferulic acid from sinapic acid, which co-eluted using elution condition I. The elution condition II was 0–5 min, 0% B; 5–23 min, 50% B; 23–24 min, 50–100% B; 24–27 min, 100% B; 27–28 min, 100–0% B; 28–35 min, 0% B at 40 °C. Calibration curves were run at 320 nm for the external standards 5-*O*-caffeoylquinic acid, *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid at concentrations ranging from 0.05 to 5 μg/mL. 5-*O*-Caffeoylquinic acid was adopted as an external standard for the quantification of the chlorogenic acid isomers. Concentrations of compounds containing caffeic acid, different to chlorogenic acid isomers, were calculated by using caffeic acid. The concentrations of *p*-coumaric, ferulic and sinapic acid containing compounds were calculated by using *p*-coumaric, ferulic and sinapic acid, respectively. Furthermore, assuming that the response of the HCAcs is mainly determined by the HCA moiety, molecular weight (MW) correction factors were used, $MW_{HCAd}/MW_{external\ standard}$. Dihydrocaffeic acid-containing compounds were calculated by using calibration curves with caffeic acid, including the MW correction factor. Limits of detection (LOD) and quantification (LOQ) were determined as ten and three times the standard deviation of the noise, respectively.

2.6. Strategy used for the identification of HCAcs/DHCAcs

Fig. 1 shows the fragmentation pattern in negative mode of HCAcs and DHCAcs. When compounds containing HCAs are fragmented in negative ion mode, the ions $[HCA–H]^-$, $[HCA–H_2O–H]^-$ and/or $[HCA–CO_2–H]^-$ are diagnostic. When OCH_3 groups are present, as in ferulic acid and sinapic acid, the ions $[HCA–CH_3–H]^-$ and $[HCA–CO_2–CH_3–H]^-$ are expected (Clifford, Marks, Knight, & Kuhnert, 2006; Fan, Terrier, Hay, Martson, & Hostettmann, 2010; Harbaum et al., 2007; Lin & Harnly, 2010; Moco et al., 2006; Mullen, Borges, Lean, Roberts, & Crozier, 2010; Olsen, Aaby, & Borge, 2009; Shakya & Navarre, 2006; Simirgiotis, Caligari, & Schmeda-Hirschmann, 2009). For some HCA-containing compounds, none of the five ions described above are produced, which is the case for e.g. 3-*O*- and 4-*O*-feruloyl quinic acid (Clifford, Johnston, Knight, & Kuhnert, 2003), and glycosylated flavonoids containing HCAs (Ferreres et al., 2008; Harbaum et al., 2007). In that case, neutral losses corresponding to HCA or HCA-hexose are screened for (Fig. 1). Besides HCAs, DHCAs are also present, whose negative ions are rarely reported (Mullen et al., 2010). Compounds containing dihydrocaffeic acid are mainly identified by the neutral losses of 164 ($–C_9H_8O_3$) and 122 ($–C_7H_6O_2$), and those containing dihydroferulic acid by the neutral loss of 178 ($–C_9H_7O_3CH_3$) (Mullen et al., 2010; Shakya & Navarre, 2006).

The negative ions and neutral losses listed in Fig. 1 were screened in MS^2 and MS^3. Subsequently, the MW was calculated from the parent ion $[M–H]^-$, and the chemical identification was done. Furthermore, UV spectra were used to verify the annotations of compounds as HCAcs/DHCAcs (Inbaraj, Lu, Kao, & Chen, 2010; Parr et al., 2005). Compounds such as quercetin-3-*O*-glucosyl-rutinoside and rutin (quercetin-3-*O*-rutinoside), which have been reported in potato (Shakya & Navarre, 2006), give the negative ion *m*/*z* 179 and neutral losses of 162 and 308 that can be misinterpreted as corresponding to HCA-containing compounds. Therefore, when the screening reveals the presence of the mentioned negative ions and/or neutral losses, the UV absorption was checked. If a maximum UV absorption at 343–350 nm is observed, then the compounds are annotated as flavonol glycosides (Lin & Harnly, 2009). When HCAs are also present in these glycosylated flavonols,