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Investigation of phenolic acids in yacon (*Smallanthus sonchifolius*) leaves and tubers

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Abstract

Thin-layer chromatographic (TLC) screening of crude extracts of dried leaves and tubers of yacon (*Smallanthus sonchifolius*, Asteraceae) and products of acid hydrolysis of tubers on the silica gel HPTLC plates using the developing solvents ethyl acetate–formic acid–water (85:10:15, v/v/v) and *n*-hexane–ethyl acetate–formic acid (20:19:1, v/v/v) proved the presence of chlorogenic, caffeic and ferulic acid. These phenolic acids were isolated from the crude extract of yacon leaves by preparative TLC, and identified after elution by HPLC/MS, as well as by direct injection of the crude extract into the HPLC/MS system. Acid hydrolysis of tubers released the increased amount of phenolic acids (e.g. caffeic acid and ferulic acid), flavonoid quercetin and an unidentified flavonoid, which was detected by TLC analysis.

Ferulic acid, isomers of dicaffeoylquinic acid and still an unidentified derivative of chlorogenic acid ($M_r = 562$) as constituents of yacon leaves and ferulic acid as constituent of yacon tubers are reported here for the first time. These acids gave significant contribution to the radical scavenging activity detected directly on the TLC plate sprayed with 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Keywords: Plant materials; *Smallanthus sonchifolius*; Phenolic acids; Caffeic acid; Chlorogenic acid; Ferulic acid

1. Introduction

Yacon (*Smallanthus sonchifolius*, Asteraceae) is a tuber crop, which constitutes part of the diet of Indian tribes living in the high mountain areas of the Andes. It is food as well as acknowledged medicinal plant for the local population. Tubers are used as a source for the production of natural sweeteners and syrups suitable for persons suffering from digestive problems [1]. Anti-diabetic properties have been attributed to yacon leaves, which are dried and used in the preparation of tea. Hypoglycemic activity of the water extract was investigated in rats, however, the mechanism for reducing and maintaining a lower glucose blood level is not known [2]. Antifungal activities of the leaves extracts have recently been reported [3].

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Until the end of the 1980s, with the exception of Peru and Japan, the scientific community paid only vague attention to this plant. The tubers contain fructose, glucose, saccharose and β-(2-1)-fructo-oligosaccharides (inulin type oligofructans) [4–6]. The proportion of monosaccharides and oligofructans fluctuates during the growth cycle and following harvest. In a recent study, the antioxidative activity of tubers was studied, and tryptophan and chlorogenic acid were identified as major antioxidants [7]. Five caffeic acid derivatives have also been isolated from the tubers of yacon [8]. Recently, we have described antioxidant activity of two ethyl acetate extracts from yacon leaves (affected by the phenolics content) and their cytoprotective effect against *tert*-butylhydroperoxide induced oxidative damage of rat hepatocytes [9].

In Europe, the crop has been introduced for cultivation in Czech Republic. The aim of this work was to contribute to clarification of the chemical composition of yacon leaves and tubers, especially concerning the compounds with antioxidant activity.

2. Experimental

2.1. Chemicals

All the chemicals including solvents were of analytical grade. Milli-Q purified water was used. Standards quercetin, rutin, caffeic acid, ferulic acid, *p*-coumaric acid, protocatechuic acid, chlorogenic acid, and free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) were from Sigma (Deisenhofen, Germany); luteolin, apigenin, luteolin-7-glucoside, luteolin-3′,7-diglucoside were from Roth (Karlsruhe, Germany), diphenylboric acid 2-aminoethyl ester, L(+)ascorbic acid were from Merck (Darmstadt, Germany), butylated hydroxyanisole (BHA) was from Fluka (Buchs, Switzerland), Folin-Ciocalteau reagent from Lycom (Prague, Czech Republic) and sodium hydrogen carbonate from Lachema (Neratovice, Czech Republic). Separate solutions of standards (1 or 0.1 mg/ml in methanol) were prepared and were kept in a refrigerator.

2.2. Plant material

Smallanthus sonchifolius (yacon) plants, originally purchased from Ecuador, were grown in the Potato Research Institute in Havlickuv Brod, Czech Republic. Voucher specimens are deposited in the collection at the Institute of Medical Chemistry and Biochemistry in Olomouc, Czech Republic. The leaves and tubers were collected at harvest time. The leaves were dried separately from the tubers at room temperature; the tubers were first peeled, cut into 8 mm × 8 mm cubes, pre-dried for 12 min at 115 °C and then dried (31 min at 107 °C, 96 min at 100 °C and 100 min at 75 °C) according to the standardized commercial procedure as for carrots at Severofrukt Travcice, Czech Republic.

2.3. Extraction

2.3.1. Yacon leaves

Dried yacon leaves (20 g) were extracted as follows:

(1) *Ethyl acetate extract (SOX):* Using Soxhlet extractor with methanol (500 ml, 72 h). After evaporation of methanol, the extract was dispersed in water (300 ml) and chlorophyll was removed by extraction with petroleum ether (3 × 150 ml). The aqueous layer was then acidified and extracted by ethyl acetate (5 × 150 ml).
(2) *Decoction (DEC):* Material was kept in 200 ml of boiling distilled water under reflux for 20 min and then left to cool at room temperature.
(3) *Tea infusion extract (INF):* 1000 ml of boiling distilled water was poured onto the leaves and then allowed to extract for 20 min while cooling.

All the extracts were filtered and freeze dried. The extraction procedure was repeated three times for each sample and the reproducibility of the procedure was controlled by the measurement of total phenolic content [10]. Twenty-five microlitres of the tested extracts in distilled water or DMSO were mixed with 500 μl of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and maintained at room temperature for 5 min; 500 μl of sodium hydrogen carbonate (75 g/l) was added to the mixture. After 90 min at 30 °C, absorbance was measured at 725 nm. Results are expressed as gallic acid equivalents (GAE as percentage of leaf or tuber dry weight ± standard deviation from three determinations).

For the chromatographic investigation solutions with concentration 1 mg/ml in methanol with 0.1% BHA were prepared.

2.3.2. Yacon tubers

Extraction I: Pieces of dried yacon tubers were ground; 0.5 g of the ground material was extracted with the mixture consisted of 8.5 ml solution of 0.1% BHA in methanol and 1.5 ml of 10% acetic acid in water.

Extraction II: 0.5 g of ground yacon tubers was extracted with 10 ml of methanol containing 0.1% BHA.

Both extraction mixtures were ultrasonicated for 30 min and centrifuged. The supernatants were used as the test solution.

2.4. Acid hydrolysis of yacon tubers

Hydrolysis reagent: 10 ml concentrated HCl was mixed with 40 ml of water, 50 ml of methanol and 40 mg of ascorbic acid [11].

Hydrolysis procedure: 50 mg of ground yacon tubers was put into a flask together with 5 ml of hydrolysis reagent and heated at 80 °C under reflux for 2, 4 or 17 h. The content of the flask was cooled and transferred to a 10 ml volumetric flask and filled up with methanol.

Tuber extract I was also hydrolysed for 2 h under the same conditions. Different volumes were applied on TLC plate.

2.5. Thin-layer chromatography

Thin-layer chromatography was performed on 10 cm × 20 cm or 10 cm × 10 cm silica gel 60 HPTLC plates (Merck, Germany). Plates were developed in chloroform–methanol (1:1, v/v) to the top and dried at 110 °C for 30 min before use. All standards and samples were applied by means of Linomat IV (Camag, Muttenz, Switzerland). Plates were developed in horizontal or saturated twin trough developing chamber using *n*-hexane–ethyl acetate–formic acid (20:19:1, v/v/v) or ethyl acetate–water–formic acid (85:15:10, v/v/v) [12].

2.5.1. Detection and documentation

(1) Natural fluorescence at 366 nm.
(2) Spraying with diphenylboric acid 2-aminoethyl ester (NST reagent), 1% solution in methanol, enhancement and fixation of fluorescence by dipping into paraffin–*n*-hexane (1:2, v/v).
(3) Spraying with DPPH 0.04% solution in methanol.

In all the cases documentation of TLC plates was performed by Camag Video Documentation System, coupled to a Reprostar 3 transilluminator and a frame grabber system equipped with a 3 × 1/2 in. charge-coupled device (CCD) camera (model HV-C20, Hitachi Denshi, Japan) (1 in. = 2.54 cm). The Video Documentation System was operated with VideoStore 2 V2.30 software.

2.6. Isolation of phenolic acids by preparative TLC

About 6 mg of dry yacon SOX leaves extract (dissolved in 200 μl of methanol) was applied as 140 mm long band besides standards on the silica gel 60, 20 cm × 20 cm TLC plates (Merck, Germany), layer thickness 0.25 mm. Plate was developed in unsaturated twin trough developing chamber, using *n*-hexane–ethyl acetate–formic acid (20:20:1, v/v/v) for isolation of caffeic and ferulic acid and ethyl acetate–water–formic acid (85:15:10, v/v/v) for isolation of chlorogenic acid. Thereafter, the chromatographic bands containing the expected compounds were scrapped off and extracted with methanol. The methanol extract was again chromatographed for checking the extraction efficiency.

2.7. HPLC/MS conditions

The HPLC system consisted of a pump LDC ConstaMetric 4100 (TSP, Thermo Separation Product, Riviera Beach, CA, USA), an autosampler TSP (AS1000) with fixed 10 μl loop, Xcalibur® software and LCQ MS system (Finnigan, MAT, San Jose, CA, USA).

A stainless steel column (150 mm × 4.6 mm i.d.) packed with Luna C_{18} (2) stationary phase, particle size 3 μm was purchased from Phenomenex, USA. Binary mobile phase consisted from phase A: 10% (v/v) CH_3CN in water, 0.05% (v/v) of acetic acid and from phase B: 90% (v/v) CH_3CN in water, 0.05% (v/v) of acetic acid. Elution from the column was achieved with the following linear gradient: 0.0 min 100% A, flow 0.5 ml/min, 39.0 min 100% B, flow 0.5 ml/min, 40.0 min 100% B, flow rate 1.0 ml/min, 41.0 min 100% A, flow rate 1.0 ml/min, 46.0 min 100% A, flow rate 1.0 ml/min, 47.0 min 100% A, flow rate 0.5 ml/min. During the scanning of MS spectra the flow rate was 0.5 ml/min. The injection volume was 10 μl.

Mass spectra of phenolic acids were scanned with ion trap MS after electrospray ionization (ESI) in negative mode. ESI conditions for ionization were as follows: capillary temperature 200 °C, ion time: 5 ms, sheath gas flow at 0.8 MPa, auxillary gas flow at 0.2 MPa. Screening at negative ionization mode: source voltage 4.50 kV, source current 100.00 μA, capillary voltage −10.00 V, tube lens offset −50.00 V, *m*/*z* width: 1.0 mass unit.

3. Results and discussion

The choice of extraction conditions is very important for research of natural phenolic compounds [13,14]. Soxhlet extraction proved to be much more efficient for the extraction of phenolic compounds from yacon leaves than both versions of water extractions (Table 1). However, some qualitative differences were observed in TLC screening of SOX, DEC and INF extracts. Some more polar compounds appeared only in DEC and INF extracts (Fig. 1).

TLC screening (Figs. 1 and 2) of crude extracts of yacon leaves showed presence of phenolic acids. Co-chromatography with standards suggested chlorogenic, ferulic and caffeic or protocatechuic acid (Fig. 3). A flavonoid, giving brown–yellow fluorescence with the NST reagent, could only be seen in the extract obtained by Soxhlet extraction, but its R_f value differed from the R_f values of the applied flavonoid standards.

Table 1
Efficiency of extraction of phenolic compounds from yacon leaves

Type of extraction	Yield (%)	Total phenolic content[a]
SOX	1.3	0.32 ± 0.01
DEC	16.5	1.76 ± 0.06
INF	30.3	3.58 ± 0.11

[a] GAE as percentage of leaf dry weight ± standard deviation from three determinations.

After preparative TLC of the crude extract (SOX), seven fractions were obtained by extraction of silica gel from seven R_f regions on the plate. These fractions were analyzed by HPLC/MS, which confirmed the presence of the expected compounds (chlorogenic, ferulic and caffeic acid) separated in three fractions, but in other four fractions we could not find any known compound. Thereafter, it was also possible to detect and identify these compounds directly in the crude extract test solution by the same technique (Fig. 4). Selective ion chromatogram for chlorogenic acid at *m*/*z* 353 showed chromatographic peaks at five retention times, more than expected for isomers of chlorogenic acid. Chlorogenic acid at t_R 17.3 min gave the

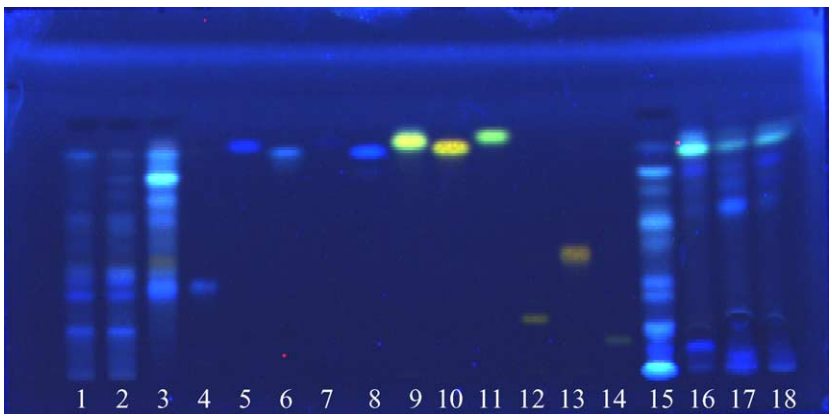


Fig. 1. TLC screening of phenolic acids and flavonoids from yacon leaves and tubers, developing solvent: ethyl acetate–water–formic acid (85:15:10, v/v/v), detection reagent NST. Applications: 1: extract DEC, 20 μl; 2: extract INF, 20 μl; 3: extract SOX, 10 μl; 4: chlorogenic acid, 0.2 μg; 5: ferulic acid, 0.2 μg; 6: caffeic acid, 0.1 μg; 7: *p*-coumaric acid, 0.5 μg; 8: protocatechuic acid, 0.2 μg; 9: quercetin, 0.5 μg; 10: luteolin, 0.5 μg; 11: apigenin, 0.5 μg; 12: rutin, 1 μg; 13: luteolin-7-glucoside, 0.5 μg; 14: luteolin-3′,7-diglucoside, 0.5 μg; 15: tuber extraction I, 20 μl; 16: acid hydrolysis of tuber 17 h, 5 μl; 17: acid hydrolysis of tuber extract I, 2 h, 20 μl; 18: acid hydrolysis of tuber, 2 h, 10 μl.

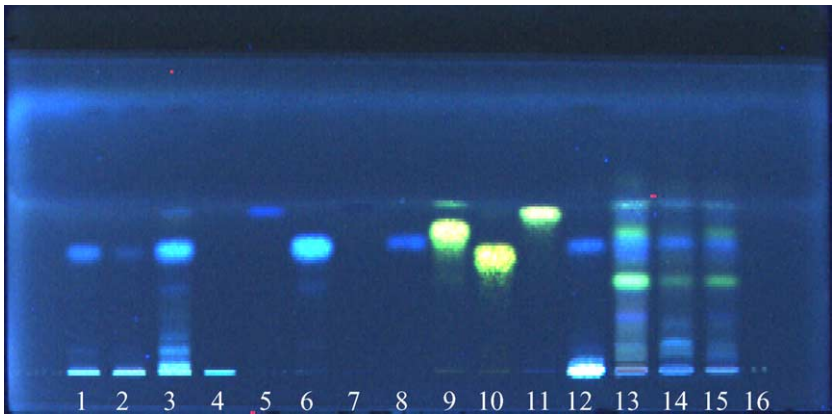
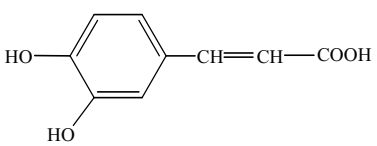
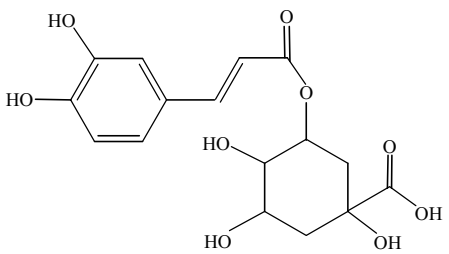


Fig. 2. TLC screening of phenolic acids and flavonoid aglycons from yacon leaves and tubers. Developing solvent: *n*-hexane–ethyl acetate–formic acid (20:19:1, v/v/v), detection reagent NST. Applications: 1: extract DEC, 20 μl; 2: extract INF, 20 μl; 3: extract SOX, 10 μl; 4: chlorogenic acid, 0.2 μg; 5: ferulic acid, 0.2 μg; 6: caffeic acid, 0.1 μg; 7: *p*-coumaric acid, 0.5 μg; 8: protocatechuic acid, 0.2 μg; 9: quercetin, 0.5 μg; 10: luteolin, 0.5 μg; 11: apigenin, 0.5 μg; 12: tuber extraction I, 20 μl; 13: acid hydrolysis of tuber 17 h, 5 μl; 14: acid hydrolysis of tuber extract I, 2 h, 20 μl; 15: acid hydrolysis of tuber, 2 h, 10 μl; 16: extraction solvent I, 10 μl.

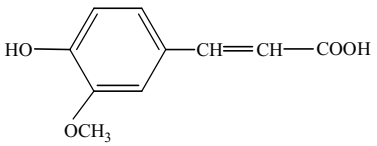
$[M-H]^-$ ion at *m/z* 353 and additional characteristic adduct ions $[M+M-H]^-$ of *m/z* 706.9 and $[M+M+M-H]^-$ *m/z* 1060.4 in standard and extract solution. The last two ions did not appear in the chromatographic peaks at other four retention times. Instead of them two intensive ions at *m/z* 515 and 1031 appeared at t_R 25.13, 26.07 and 32.09 min besides *m/z* 353 (Fig. 5). It was concluded that these peaks originated from three isomeric dicaffeoylquinic acids (nominal molecular mass 516). Peak at *m/z* 353 does not represent chlorogenic acid itself, but results from thermal fragmentation of deprotonated molecular ion of dicaffeoylquinic acid. Ion at *m/z* 1031 was an aduct ion $[M+M-H]^-$ that was formed by coupling of two molecules of dicaffeoylquinic acids. The fifth peak (Fig. 5) at retention time 29.39 or 29.23 min had



caffeic acid



chlorogenic acid



ferulic acid

Fig. 3. Structures of identified phenolic acids.

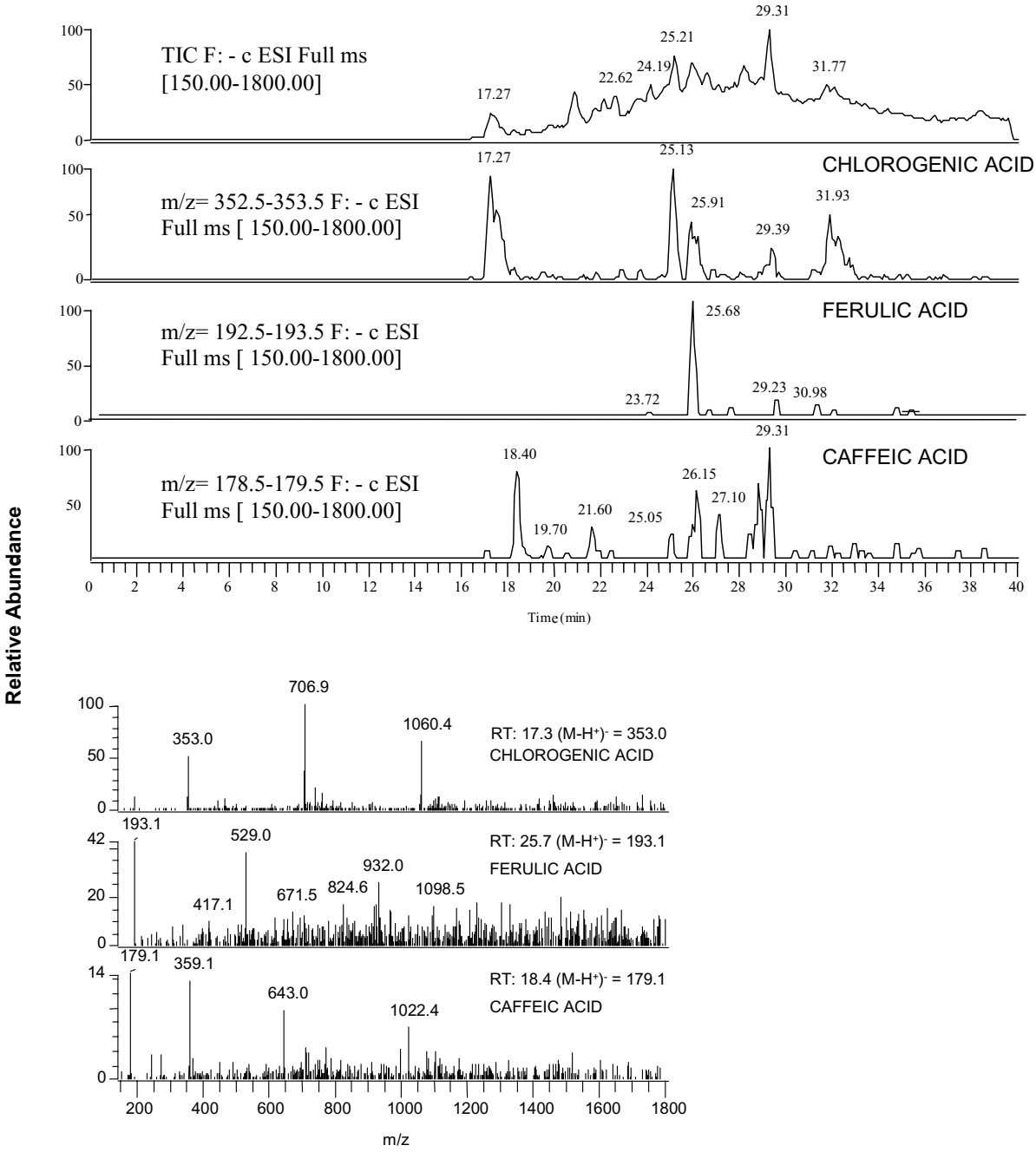


Fig. 4. HPLC/MS identification of phenolic acids in the extract of yacon leaves (30 mg/ml). Identified phenolic acids are as follows: at *m/z* 353 chlorogenic acid ($t_R = 17.3$ min), at *m/z* 193 ferulic acid ($t_R = 25.7$ min), at *m/z* 179 caffeic acid ($t_R = 18.4$ min).

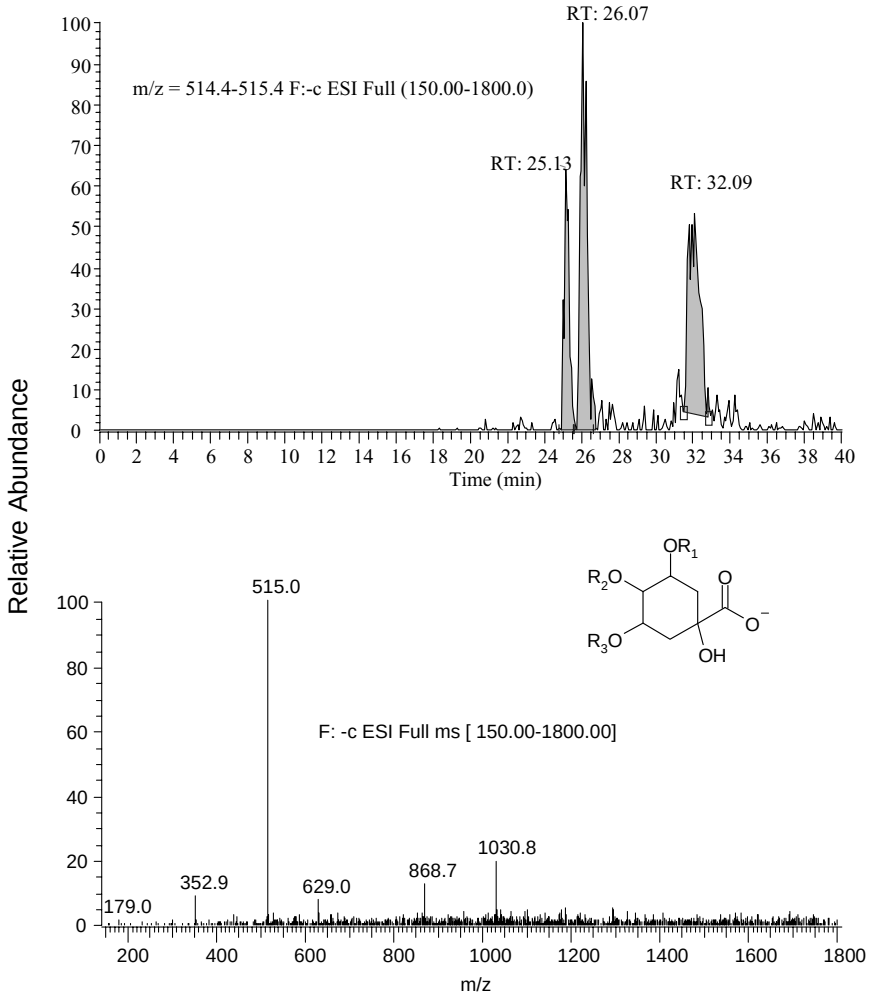


Fig. 5. HPLC/ESI/MS selected mass chromatogram of yacon leaves extract in negative ionization mode. Identified isomers of dicaffeoylquinic acid at *m*/*z* 515 appeared at t_R 25.13, 26.07 and 32.09 min. Peak at *m*/*z* 353 is a thermally fragmented ion of chlorogenic acid and adduct ion $[M + M - H]^-$ at *m*/*z* 1031 confirmed presence of deprotonated molecular ion $[M - H]^-$ at *m*/*z* 515. R_1, R_2 and R_3 pointed all three possible combinations ($R_{1,2}$, $R_{2,3}$ and $R_{1,3}$), where esterification with two molecules of caffeic acid can take place.

$[M - H]^-$ ion at *m*/*z* 561 and adduct ion at *m*/*z* 1123 (Fig. 6). Additional peaks at *m*/*z* 353 and 399 resulting from thermal fragmentation facilitated the identification of the compound as a derivative of chlorogenic acid, however, further investigation is needed for structural elucidation. To the best of our knowledge, this is the first report about the presence of three dicaffeoylquinic acids and additional still unknown derivative of chlorogenic acid ($M = 562$) in yacon leaves. Recently 3,5-dicaffeoylquinic acid was reported in yacon tubers [8]. These compounds, which were also found in some other plants from the family Asteraceae [15,16], have been recently established as a leading class of human immunodeficiency virus (HIV)-integrase inhibitors [17–20]. Nevertheless, further efforts are needed for the isolation, purification and structural elucidation of those isomers from yacon leaves. The composition of the other four fractions from the preparative TLC remained unknown.

TLC screening of antioxidants by DPPH in SOX extract of yacon leaves (lane 5, Fig. 7) revealed bands

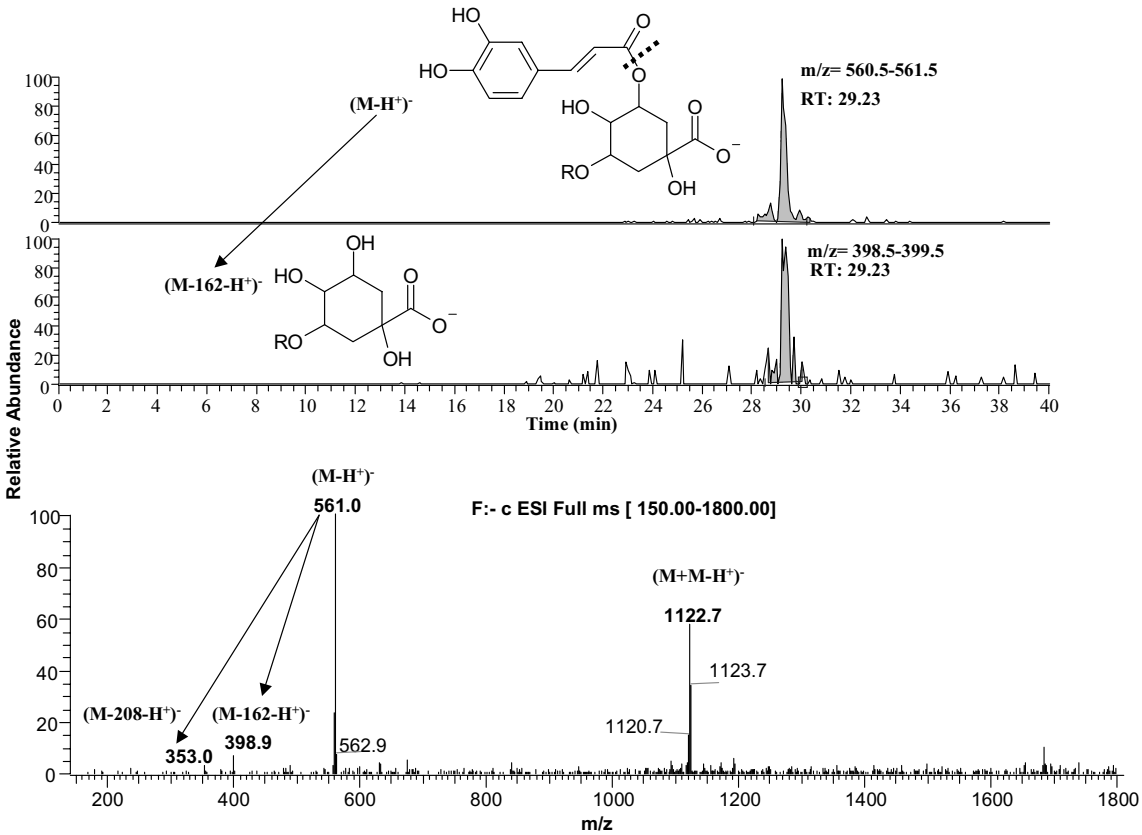


Fig. 6. HPLC/ESI/MS selected mass chromatogram of yacon leaves extract in negative ionization mode. Peak at *m*/*z* 353 is a thermally fragmented ion of chlorogenic acid and adduct ion $[M + M - H]^-$ at *m*/*z* 1123 confirmed presence of deprotonated molecular ion $[M - H]^-$ at *m*/*z* 561. The compound was identified as derivative of chlorogenic acid.

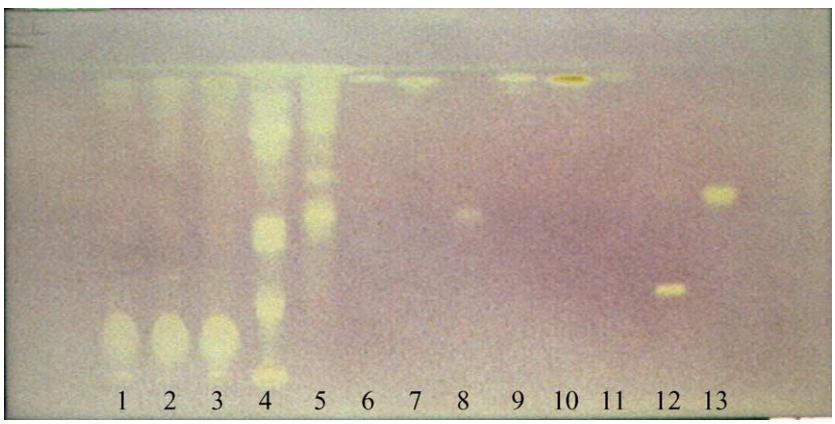


Fig. 7. TLC screening of antioxidants from yacon leaves and tubers. Developing solvent ethyl acetate–water–formic acid (85:15:10, v/v/v), detection reagent DPPH. Applications: 1: acid hydrolysis of tubers, 2 h, 5 μl; 2: acid hydrolysis of tubers, 4 h, 5 μl; 3: acid hydrolysis of tubers, 17 h, 5 μl; 4: tubers extraction I (without hydrolysis), 20 μl; 5: leaves extract SOX, 5 μl; 6: ferulic acid, 0,2 μg; 7: caffeic acid, 0,2 μg; 8: chlorogenic acid, 0,2 μg; 9: quercetin, 0.2 μg; 10: luteolin, 0.5 μg; 11: apigenin, 0.5 μg; 12: rutin, 1 μg; 13: luteolin-7-glucoside, 0.5 μg.

of already discussed phenolic acids, as well as bands of some unknown compounds. The band at the front rise from the antioxidant BHA added to the extract test solution.

The amount of phenolics obtained from dried tubers by extraction I (methanol–10% acetic acid) and extraction II (methanol) using Folin–Ciocalteu reagent, was determined to be 0.57 ± 0.05% and 0.35±0.03% of GAE calculated as percentage of tuber dry weight ± standard deviation from three determinations, respectively. In accordance with this finding, TLC screening of phenolic acids in tubers showed higher yield by extraction I than extraction II. TLC screening on phenolic acids, flavonoids and antioxidants showed numerous bands. Although qualitative differences between leaves and tuber extract could be observed in the chromatograms, it was confirmed that chlorogenic, ferulic and caffeic acid were present in both parts (leaves and tuber) of yacon (Figs. 1 and 2).

The standard 2-h acid hydrolysis [8] of tubers released a lot of phenolic acids, where caffeic acid prevailed (Figs. 1 and 2). The content of ferulic acid was increased after 17 h of hydrolysis compared to 2 h of hydrolysis of tubers. No flavonoid could be found in tubers before acid hydrolysis. The 2-h acid hydrolysis [11] of tubers released a flavonoid, which could be quercetin as stated by co-chromatography with standards (yellowish fluorescence with NST reagent at R_f 0.53, Fig. 2). The content of quercetin remained unchanged in all three hydrolysates (visual estimation from TLC chromatograms). An unknown flavonoid (yellow fluorescence at R_f 0.34, Fig. 2) was slowly releasing at acid hydrolysis of the tuber. Obviously, its content in the hydrolysate was still increasing after 17 h of acid hydrolysis compared to 2 and 4 h of acid hydrolysis. Minor content of a third flavonoid component was observed in the hydrolysates of tubers at R_f 0.19 (Fig. 2). Hydrolysis of tubers gave higher amounts of phenolic acids than hydrolysis of the tuber extract, because the cell walls release phenolic acids during the hydrolysis (bound phenolic acids [21]).

Acid hydrolysis of tubers and also extracts of tubers released big amount of free simple phenolic acids, where caffeic acid prevailed (Figs. 1, 2 and 7). Besides expected antioxidant activity originating from phenolic acids and flavonoids additional band with a strong antioxidant activity of an unknown polar compound appeared in the chromatogram of tuber hydrolysates near the start position (Fig. 7). DPPH test performed directly on the TLC plates was much more informative than previously reported spectrophotometric DPPH measurements [7], because it shows contributions to the antioxidant activity of different compounds separately.

To the best of our knowledge, ferulic acid, isomers of dicaffeoylquinic acid and a derivative of chlorogenic acid ($M_r = 562$) as constituents of yacon leaves and ferulic acid as constituents of yacon tubers are reported here for the first time. However, the analysis of the extracts of yacon leaves and tubers showed that both parts of this plant represent a rich source of phenolic acids and other radical scavenging compounds, therefore further work is needed for their identification.

Acknowledgements

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