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Isolation of ursolic acid from apple peels by high speed counter-current chromatography

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Abstract

Cuticular waxes of four varieties of *Malus domestica* were investigated regarding their content of ursolic acid. Peels from Fuji, Gala, Smith and Granny Smith apples were extracted with chloroform, ethyl acetate and/or ethanol. The crude extracts were purified by high speed counter-current chromatography (HSCCC), by using mobile and stationary phases derived from the two-phase solvent system composed by *n*-hexane:ethyl acetate:methanol:water in the proportion of 10:5:2.5:1. The phase proportions and the relative distribution of ursolic acid between the two-phases were optimized by TLC and optical densitometry, by comparison with an authentic sample of ursolic acid. The amount of ursolic acid present in the extracts as well as the characterization of the isolated compound were made by high resolution gas chromatography coupled to mass spectrometry (GC–MS), ^{13}C nuclear magnetic resonance (^{13}C NMR), Infrared; and by comparing thin layer chromatography and flame ionization detection gas chromatography (GC–FID) patterns with the commercial sample. The average content of ursolic acid of 0.8 mg/cm^2 in the peel (around 50 mg per medium sized fruit with a surface area of 50–70 cm^2) was found in the Fuji and Smith varieties, whereas 0.5 mg/cm^2 and 0.2 mg/cm^2 were the amounts calculated for Granny Smith and Gala, respectively. The HSCCC technique was shown to be a good method to purify free ursolic acid from apple peels and could represent a new technological tool to be developed to exploit industrially this source of product.
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Keywords: High speed counter-current chromatography (HSCCC); *Malus domestica*; Apple peel; Ursolic acid

1. Introduction

Most plants provide their more sensitive aerial organs with a micrometric polymeric lipophilic layer to protect themselves from water and nutrients losses caused by biotic and abiotic stress factors (Bringe, Schumacher, Schimitz-Eiberger, Steiner, & Oerke, 2006; Eigenbrode & Espelie, 1995). This cuticular layer is generally made up of aliphatic wax constituents mostly with up to around thirty carbon-atom chains, together with other compounds with emulsifying properties such as fatty acids, free alcohols and triterpenes, which promote functional co-existence with hydrophilic polymers also present (cutin, carbohydrate polymers, pectin, protein, etc.) (Ju & Bramlage, 1999). In the case of apples, the composition of the cuticular wax has been studied because of its relevance in avoiding fruit damage caused by insects and other pathogenic agents (Belding, Blankenship, Young, & Leidy, 1998; Eigenbrode & Espelie, 1995). The wax protection has also been correlated with antioxidant properties which prevent fruit scald induced by the spontaneous oxidation of α-farnesene, which also occurs in the peel (Whitaker, 1998). Variations

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of apple peel wax constituents according to climatic conditions, genetic factors and plant ontogenesis have been reported (Bringe et al., 2006). Usually, increasing wax content on the fruit surface is normally correlated to growth and maturation, although the distribution wax content/area remains at constant (Kolattukudy, 1984). This is an important post-harvesting parameter to be considered in order to guarantee shelf life and quality for commercialization.

Ursolic acid ((3β)-3-hydroxyurs-12-en-28-oic acid) is the main compound present in apple cuticular wax (Belding et al., 1998) as well as in the leaves. Its content may reach up to 32% of the total wax, depending on the ontogenetic period (Bringe et al., 2006). Beside betulinic and oleanolic acids, ursolic acid is a ubiquitous triterpenoid in the plant kingdom and its presence has been demonstrated in the wax-like coating of diverse other fruits as well as in several well-known medicinal plants (Chen, Xia, & Tan, 2003; Liu, 1995; Qi, Ding, Tian, Chen, & Hu, 2006). Adaxial young leaves of apple tree contained up to 26% of ursolic acid in the epicuticular wax; dropping to 20% during ontogenesis. Oleanolic acid also appears with around 7% of the total wax composition; (Bringe et al., 2006; Jetter & Schäffer, 2001).

The present study examines the application of high speed counter-current chromatography (HSCCC) to ursolic acid-rich substrates, represented by the pre-purified peel extracts of four varieties of apples: Fuji, Gala, Smith and Granny Smith. The choice of the developing system is fundamental to the HSCCC method (Hostettmann & Marston, 2001). Good separation requires (i) sample solubility in both solvent phases (ii) even distribution of ursolic acid in each phase (as close as possible to 1:1), and (iii) rapid separation of the phases after intense stirring. The optimum developing solvent system (stationary and mobile phases) for HSCCC separation was determined by dissolving the fraction to be chromatographed in the solvent mixture. The experimental conditions, previously developed for the purification of triterpene acids from leaves of *Eugenia brasiliensis* (Frighetto, Welendorf, Silva, Nakamura, & Siani, 2005a), have been successfully applied here to the chlorophyll-free extracts of apple peels, and the amounts of ursolic acid were calculated in function of the fruit area.

Derivatives of ursolic acid (and other triterpene acids) have been extensively studied either as pharmacological active molecules or as emulsifying agents in pharmaceuticals, foods and cosmetics (Liu, 1995; Merck Research Laboratories, 1989; Rona, Vailati, & Berardesca, 2004). Ursolic acid also plays the role of chemical marker in some medicinal plants, nutraceuticals (Chen et al., 2003; Cui et al., 2006; Takeoka et al., 2000) and phytopharmaceuticals (Dufour et al., 2007). A potential commercial source of ursolic acid would be represented by the "industrial apple", which fruits does not conform to the 'in natura' standards for the consumer and is transformed in juice, cider and vinegar (Paganini, Nogueira, Silva, & Wosiacki, 2005). These categories of products consume between 20% and 30% of the total production estimated in around 1 million ton/year in bulk in a planted area nowadays at approximately 33,000 hectares in Brazil. Gala and Fuji apples together make up around 95% of the total production that is exclusively concentrated in Southern regions (Nogueira, Biscaia, Wiecheteck, Denardi, & Wosiacki, 2006; Wosiack, Pholman, & Nogueira, 2004).

2. Materials and methods

2.1. Fruit material and semi-purified extracts

Commercially ripe Fuji, Gala, Smith and Granny Smith varieties of *Malus domestica* were bought in the municipal market in the city of Campinas, São Paulo, between September and November 2003. Twenty fruits of each variety were carefully handled and measured vertically and horizontally with a pachymeter in order to calculate the surface area ($4\pi r^2$), from the average radius (r) of the five closest measurements from eight different fruits. Following an adapted procedure (Belding et al., 1998), each whole fruit was immersed in *n*-hexane during 2 min in order to eliminate the wax compounds from the epithelium. The solvent was monitored by TLC (*n*-hexane:AcOEt 1:1) to ensure the absence of significant amounts of triterpene acids. After being removed from the hexane and air-dried, the fruit was then placed in a 500 mL beaker that was filled with 96% ethanol (the solvents ethyl acetate, $CHCl_3$ and CH_2Cl_2 were also examined) and maintained covered during 15 h. Removing of the fruit followed by solvent filtration and evaporation led to the crude extracts (Table 1). The ethanolic extract of a single fruit was suspended in $CHCl_3$:MeOH 9:1, absorbed onto a dry column

Table 1
Extract yields from peels of four apple varieties with different solvents

Solvent (time of immersion)	Total extract per apple variety (mg)[a]			
	Fuji	Gala	Smith	Granny Smith
n-Hexane (2 min)	20.70	39.90	60.30	63.70
EtOH (15 h)	2160 ± 71	1460 ± 23	2260 ± 93	2150 ± 204
$CHCl_3$ (15 h)	278.6 ± 26.4	212.8 ± 22.6	67.30 ± 7.30	74.70 ± 8.80
CH_2Cl_2 (15 h)	705.9 ± 37.4	446.8 ± 34.0	174.7 ± 19.6	273.6 ± 24.3
AcOEt (15 h)	401.0 ± 44.0	1270 ± 145	813.0 ± 65.0	84.10 ± 10.1

[a] One-fruit-average amount calculated from extracting six apples individually.

Table 2
Ursolic acid (UA) contents in crude extracts, pre- and post-HSCCC purified fraction from four apple varieties

Parameter	Apple variety			
	Fuji	Gala	Smith	Granny Smith
Crude extract (g)[a]	2.267	1.414	2.331	2.196
UA-enriched extract[b] (mg)	388	484	155	195
HSCCC purified UA (mg)	65	12	54	40
Average apple area[c] (cm^2)	84.50	55.94	65.90	81.07
Ursolic acid content (mg/cm^2)	0.77	0.21	0.82	0.49

[a] Initial amount of extract submitted to the dry-column.
[b] Amount of pre-purified extract submitted to HSCCC.
[c] For sampling and calculation see experimental.

(polyethylene, 40 cm × 2.0 cm) filled with silica gel G 60 (Merck) and eluted with $CHCl_3$ until the solvent front reached the lower end of the column. The column was then cut into six equivalent parts, and each one extracted with $CHCl_3$ and monitored by TLC for the presence of ursolic acid (silica gel plates Merck F_{254}; eluent *n*-hexane:AcOEt 5:1). Fractions containing ursolic acid were grouped together and the solvent was removed under vacuum. This procedure was applied to the four varieties of apple.

2.2. HSCCC conditions and TLC densitometric calibration

In order to optimize the purification of ursolic acid by HSCCC, several parameters such as the choice of stationary and mobile phases (MP), considering the upper and lower layers of the two-phase system selected, head-to-tail or tail-to-head solvent flow, clockwise or anti-clockwise coil rotation, relative volumes of stationary and mobile phases and sample concentration as assayed and described previously (Frighetto et al., 2005a). HSCCC experiments were carried out by using P.C. equipment (Potomac, USA) fitted with a multilayer coil separation column and a counter-weight, allowing injections of up to 100 mg (Conway, 1990). All experiments were carried out at 960 rpm with MP flows of 2.0–4.0 mL/min. The chosen mixture of solvents (*n*-hexane:AcOEt:MeOH:water 10:5:2.5:1) was homogenized, decanted and the resulting two-phases were separated out and degassed. The upper layer, consisting of the stationary phase, was introduced in a semi-preparative coil (80 mL). For each run, the ursolic acid-enriched fraction (see Table 2) was dissolved in a mixture of both phases (4 mL each) and injected 3.0 mL into the coil with the aid of a loop. The separation was then run using the lower layer as the mobile phase. Fractions (5 mL each) were collected and monitored by TLC and those fractions, which consisted of ursolic acid, were combined to afford this compound with a high degree of purity. At the end of each run, the stationary phase was completely removed to assure the recovery of all sample introduced into the coils. Relative amounts of ursolic acid in the extracts and in both mobile and stationary solvent phases were calculated by TLC using optical densitometry, with known concentrations of the samples and of commercial ursolic acid. This previously described assay procedure (Frighetto et al., 2005a) permitted determination of the optimum distribution of ursolic acid in the mobile:stationary phases, established as 1:1.16.

2.3. Characterization of ursolic acid and methyl ursolate

Evaporation of the combined fractions containing ursolic acid led to a white solid, which afforded an IR spectrum identical to the commercial sample. A sample of this solid (5–10 mg) was methylated with diazomethane (Diazald® Aldrich, USA) and the resulting methyl ursolate was analyzed in a Hewlett-Packard 6890 gas chromatograph with a capillary column HP-5 (30 m × 0.25 mm i.d. × 0.25 μm film thickness); using a flame ionization detector (FID) at 290 °C, coupled to a Hewlett-Packard 3396 integrator; oven temperature programmed from 150 °C to 290 °C at rate of 4 °C min^{-1} and then held for 30 min. GC–MS analyses were carried out in a Hewlett-Packard 5890 gas chromatograph coupled to mass spectrometer Model 5779, with a capillary column HP-5 MS (30 m × 0.32 mm i.d. × 0.25 μm film thickness), in the following conditions: helium as carrier gas at 1.0 mL min^{-1}; split ratio 1/20, injector at 290 °C; ion source at 250 °C and electron impact ionization at 70 eV; other chromatographic conditions were identical to the FID analysis. Injections were of 1 μL from a 5 mg/2.0 mL AcOEt solution. The infrared spectrum was obtained in a Nicolet 205 spectrophotometer and the ^{13}C RMN spectrum in a Bruker AC 200-A equipment.

The ursolic acid samples obtained from the HSCCC experiments from the semi-purified apple peel extracts of each different variety were compared with a commercial sample of the acid in TLC and GC and showed IR spectrum identical to the commercial sample (KBr, C=O η_{max} 1693 cm^{-1}) Silica gel GF 60 plates were run in $CHCl_3$:MeOH:Et_2O 90:5:5 (Rf 0.49) or benzene:acetone 75:25 (Rf 0.52) as eluents. The plates were revealed with iodine or *p*-anisaldehyde or vanillin in H_2SO_4. After methylation with CH_2N_2, GC analysis showed a unique signal in the chromatogram. ^{13}C NMR (50.3 MHz, $CDCl_3$, ppm) δ 79.7 (C-3; COH), 126.3 (C-12), 138.9 (C-13) e 178.8 (C-28; C=O) (Fig. 1). Mass spectra (GC–MS; 97% compatibility with Wiley electronic library pattern): *m/z* 470 (M^+), 455, 410, 262 (100%), 203, 189. Under the chromatographic conditions (GC–FID) described, methyl ursolate eluted at 33.60 min.

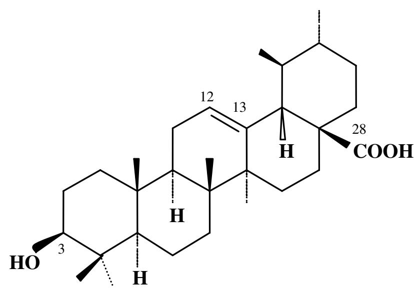

Fig. 1. Ursolic Acid.

3. Results and discussion

The potentiality of ursolic acid in therapeutics has drawn the scientific attention during last decade (Liu, 2005). This fact is evidenced by the exponentially increasing pharmacological research on the anti-inflammatory (Dufour et al., 2007), anti-cancer (Achiwa, Hasegawa, & Udagawa, 2005; Ma et al., 2005), and other activities of ursolic acid (Lee, Jin, Beak, Lee, & Kim, 2003). On the same hand, the potential of the apple as a functional food is presently based on the amount and type of its vitamin, phenolic and pectin content. Recent literature on studies with phenols, tannins or flavonoids supports the concept (Tsao, Yang, Xie, Sockovie, & Khanizadeh, 2005; Wolfe & Liu, 2003; Wolfe, Wu, & Liu, 2003). Ursolic acid itself is shown to have antioxidant properties (Adhikari, Schutzki, DeWitt, & Nair, 2006; Assimopoulou, Zlatanos, & Papageorgiou, 2005), but its eventual contribution to the apple's properties as a nutraceutical was not commented. Less polar extracts (acetone/butanol or butanone) of residues from plant sources have been reported as exhibiting higher *in vitro* antioxidant potential, when compared to those containing high percentages of phenolic compounds (ethanol/water or ethanol/butanol or butanone) (Peschel et al., 2006).

Extraction by immersing apples in chloroform has been used in comparative analysis of the compounds present in the epicuticular waxes of six apple cultivars (Belding et al., 1998; Lawrence, Iyengar, & Sun, 1985). However, since pentacyclic triterpenoids are located almost exclusively in the intracuticular compartment (Jetter & Schäffer, 2001) ethyl acetate and ethanol were also assessed. Ethyl acetate extraction has been reported as an effective process, when made by successive supersonication followed by defatting with petroleum ether (Ma et al., 2005). Apple immersion for 15 h in ethanol turned out to be better for extracting ursolic acid for the specific purpose of this study, since it provided quite complete removing from the peels (TLC monitory) and afforded results comparable to those obtained from macerating dry apple peels (data not shown). Although ethyl acetate afforded a more selective extraction, it was unable to exhaust the peels, based on TLC profiles, and led to smaller amounts of extract. Similar results were obtained by the use of chloroform or dichloromethane (Table 1). This first extraction step would be technologically relevant when obtaining of ursolic acid from this source.

The amount of crude extract obtained by EtOH extraction, the pre-purified fraction and the ursolic acid content calculated for each apple after the HSCCC separation are displayed in Table 2. The content of ursolic acid in the Granny apple was very close to the value of 50 mg per ripe fruit reported for Bramley apple (Silva-Fernandes, Baker, & Martin, 1964). The Fuji and Smith varieties were averaged the same (0.77 and 0.82 mg/cm^2, respectively), but the Gala apple presented a lower total value, as well as the lowest concentration per surface area (0.21 mg/cm^2). The isolated ursolic acid was well characterized by spectrometric methods as well as by comparing with an authentic sample in TLC and GC–FID, and showed purity degree $\geqslant$98% in all the HSCCC experiments, as indicated by the relative peak area in GC.

Separation of ursolic acid from apple peels by HSCCC was performed for the first time. This technique has been used before to purify many classes of analogous substances, but few specific procedures for purification of triterpene free acids have been reported to date (Frighetto et al., 2005a, Frighetto, Welendorf, Silva, Nakamura, & Siani, 2005b; Ito, Oka, Kitazume, Bhatnagar, & Lee, 1990). Besides corroborating the adequacy of the HSCCC technique for the purification of ursolic acid from apple peel extracts; the present study permitted assay of the amount of ursolic acid in each of four different apple varieties. Several ursolic acid derivatives have also been described as present in apple peel, and could be purified by conventional chromatographic methods (Ma et al., 2005). These compounds were not detected in the present work, indicating that the present protocol, optimized for ursolic acid, also serves to eliminate these probable artifacts from the final product. This work is the first to prospect ursolic acid in Brazilian apple cultivars.

4. Conclusions

The present study was focused on the purification of ursolic acid from apple peel and the estimation of its content in four varieties of apple growing in Brazil using analytical and semi-preparative HSCCC methodology, previously developed to purify triterpene acids from leaf extracts. The technique was successfully applied to semi-purified apple peel extracts. The highly pure ursolic acid, furnished by this technique; points to a new method for refining this valuable triterpenoid. The development of preparative and large-scale HSCCC (Hostettmann & Marston, 2001; Ito et al., 1990; Ma et al., 2006) applied to the pre-treated apple pomace extracts could be a useful route to ursolic acid for the food and pharmaceutical markets (Lu & Foo, 2000; Peschel et al., 2006).

Although methods (HPLC, GC, hyphenated techniques or others) for ursolic acid prospecting and quantification in plant or apple peel extracts there has been (Belding et al., 1998; Chen et al., 2003; Cui et al., 2006; Lawrence et al.,

1985; Liu et al., 2003; Qi et al., 2006; Takeoka et al., 2000), so far, no report has found about either the presence of residual ursolic acid in apple juice or cider or its eventual relevance in evaluating the nutritional value of apple derivatives. This remains a topic for further study in order to show what is the real contribution of ursolic acid to that old adage "An apple a day keeps the doctor away".

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