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Composition of phenolic acids content in apple (*Malus sp*) pomace

Composição do conteúdo de ácidos fenólicos no bagaço de maçã (*Malus sp*)

Márcia Christina Soares.¹; Érika Taciana Ribeiro¹; Eugênia Marta Kuskoski¹; Luciano Valdemiro Gonzaga¹; Alessandro Lima²; Jorge Mancini Filho^{2*}; Roseane Fett¹

Abstract

The present work aimed the study of phenolic acids composition in apple pomace of Gala and Fuji cultivars. Phenolic acids were fractionated in phenolic acids, esterified and insoluble and analyzed by gas chromatography–mass spectrometry (GC-MS). Sixteen phenolic acids were identified in apple pomace samples. Total phenolic acids in apple pomace from Gala and Fuji cultivars were, in dry weight, 93.94 mg/g and 68.38 mg/g, respectively. Content of free phenolic acids in apple pomace from Gala cultivar was 29.11 mg/g and the following acids were identified: salicylic, protocatequinic, quinic, p-coumaric, gallic, propylgallate and synapic. Content of free phenolic acids in apple pomace from Fuji cultivar was 16.03 mg/g and the following acids were identified: salicylic, protocatequinic, gallic, ferulic and sinapic. Salicylic was the predominant free phenolic acids found in both cultivars, consisting of 91.67% and 63.57% of the free phenolic acids in Gala and Fuji cultivars, respectively. Chlorogenic acid (1.147 mg/g) was found only in apple pomace from Fuji cultivar. Content of esterified phenolic acids in apple pomace from Gala and Fuji cultivars were 53.75 mg/g and 48.29 mg/g, respectively. It was verified that the predominant esterified phenolic acid in pomace from apple Gala is derived from salicylic acid (52.76 mg/g). Acids derived from gallic acid (0.175 mg/g), propylgallate acid (0.198 mg/g), ferulic acid (0.159 mg/g) and sinapic acid (0.140 mg/g) were also found in Gala cultivar. Regarding to pomace from cultivar Fuji, the main esterified phenolic acid found is also derived from salicylic acid (47.42 mg/g) followed by gallic acid (0.270 mg/g), benzoic acid (0.194 mg/g) and sinapic acid (0.115 mg/g). Content of insoluble phenolic acids in apple pomace from Gala and Fuji cultivars were, in dry weight, 11.08 mg/g and 4.05 mg/g, respectively. Insoluble phenolic acids derived from salicylic acid were found in higher concentrations in apple pomace from both cultivars.

Keywords: Phenolic acids, apple pomace

Resumo

O trabalho visou estudo da composição dos ácidos fenólicos de bagaço de maçã dos cultivares Gala e Fuji. Os ácidos fenólicos foram fracionados em ácidos fenólicos, esterificados e insolúveis e analisados por cromatografia a gás e espectrometria de massa (CG-MS). Foram identificados dezesseis ácidos fenólicos nas amostras de bagaço de maçã. Os teores de ácidos fenólicos totais nos bagaços de maçã dos cultivares Gala e Fuji foram respectivamente 93,94 e 68,38 mg/g (massa seca). O conteúdo de ácidos fenólicos livres no bagaço de maçã Gala foi 29,11 mg/g, sendo identificados os ácidos salicílico, protocatequínico, quínico, p-cumárico, gálico, propilgalato, sináptico. O teor de ácidos fenólicos livres no bagaço de maçã Fuji foi 16,03 mg/g, sendo identificados cinco (salicílico, protocatequínico, gálico, ferúlico, sináptico). O ácido salicílico foi o ácido fenólico livre predominante nas amostras de bagaço de maçã Gala e Fuji, constituindo 91,67% e 63,57% dos ácidos fenólicos livres, respectivamente. O ácido clorogênico (1,147 mg/g) foi encontrado somente no bagaço de maçã Fuji. Os teores de ácidos fenólicos

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esterificados do bagaço de maçã Gala e Fuji foram 53,75 e 48,29 mg/g, respectivamente. Verificou-se que o ácido fenólico esterificado predominante no bagaço de maçã Gala é derivado do ácido salicílico (52,76 mg/g) sendo também encontrados derivados de ácido gálico (0,175 mg/g), propilgalato (0,198 mg/g), ferúlico (0,159 mg/g) e sináptico (0,140 mg/g). No bagaço de maçã Fuji, o ácido fenólico esterificado encontrado em maior quantidade também é derivado do ácido salicílico (47,42 mg/g), seguido do ácido gálico (0,270 mg/g), ácido benzóico (0,194 mg/g) e ácido sináptico (0,115 mg/g). Os teores de ácidos fenólicos insolúveis nos bagaços de maçã Gala e Fuji foram respectivamente 11,08 e 4,05 mg/g (massa seca). Os ácidos fenólicos insolúveis derivados de ácido salicílico foram encontrados em maior quantidade nos bagaços de maçã de ambos cultivares.

Palavras-chave: Ácidos fenólicos, bagaço de maçã.

Introduction

Apple fruits are important dietary sources of potentially healthy phenolics. Epidemiological studies have shown an inverse relationship between the intake of fruits, vegetables and beverages rich in flavonoids and the incidence of coronary heart disease, but the relationship with cancer is not clear (BELL et al., 2000; HOLLMAN, 1997; RICE-EVANS; MILLER; PAGANGA, 1997). Apple fruits, especially skin, are rich in flavonoids (e.g. flavonols, catechins, phloridzin and anthocyanins) and contain considerable amounts of hydroxycinnamic acid derivatives, mainly represented by chlorogenic acid (AWAD; JAGER; VAN WESTING, 2000; LANCASTER, 1992; NICOLAS et al., 1994). Flavonoids and phenolic acids contribute to the quality aspects of apples. These compounds are involved in the quality characteristics of fresh fruits and its processed products, like texture, colour and taste, e.g. bitterness and astringency (LANCASTER, 1992; LEA; TIMBERLAKE, 1974; LIDSTE, et. al., 1986), and show a powerful antioxidant capacity both in *in vitro* and *in vivo* systems (COOK; SAMMAN, 1996; RICE-EVANS; MILLER; PAGANGA, 1997). Apple pomace, an heterogeneous mixture consisting of skin, core, seed, calyx, stem and soft tissue, has demonstrated to be a rich source of polyphenolics (LU; FOO, 1998; FOO; LU, 1999; EBERHARDT; LEE; LIU, 2000; LU; FOO, 1997; SCHIEBER; KELLER; CARLE, 2001). Apple polyphenolics are mainly found in the skin (DICK et al., 1987;

LOMMEN et al., 2000) and in the seeds (AWAD; JAGER; VAN WESTING, 2000; LU; FOO, 1998). Due to the low extraction yields during juice production (PRINCE et al., 1999), most of the phenolics remain in the pomace and contribute to its brown colour after oxidation. The aims of the present investigation were to fractionate the phenolic constituents of apple pomace into free, soluble, and insoluble forms and, after hydrolysis, to determine the relative proportions of the various phenolic acids.

Material and methods

Material

Samples of apple pomace from cultivars Gala and Fuji were supplied by Fischer, an industrial juice producer, Fraiburgo-SC, Brazil, were submitted to a thermal treatment of 80° for 10 min. in order to inactivate peroxidase and polyphenoloxidase enzymes (VALDERRAMA; MARANGONI; CLEMENTE, 2001). A portion of samples were, then, dehydrated at 45°C for 4 hours in an air circulation oven.

Isolation of phenolic compounds

Free phenolic, esterified and insoluble-bound phenolic compounds (Figure 1) were extracted and fractionated as previously described (KRYGIER; SOSULSKI; HOGGE, 1982) with some modifications (SOTERO, 2002).

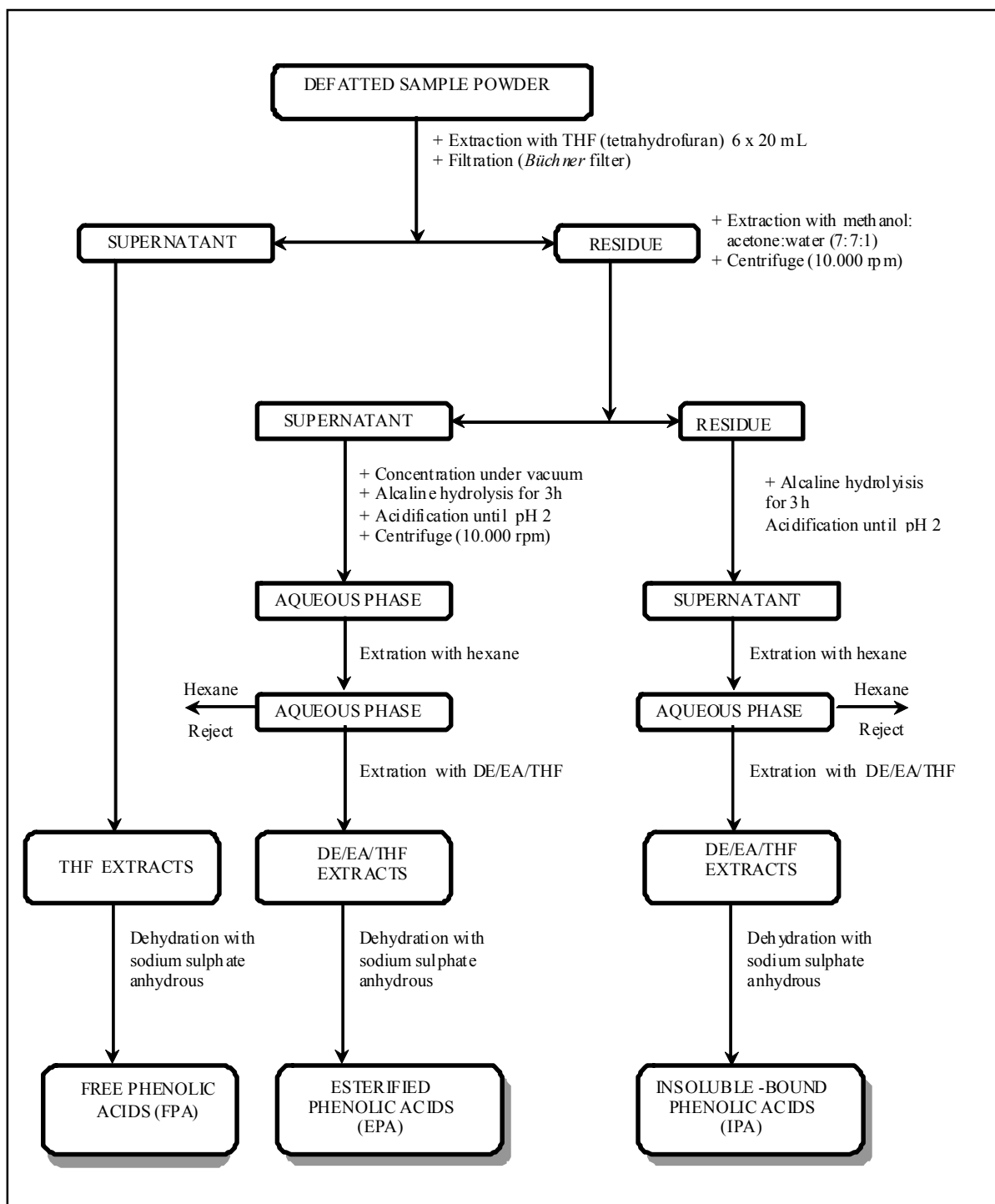


Figure 1. Procedure for the extraction and separation of free, esterified, and insoluble-bound phenolic compounds and their hydrolysis to phenolic acids (DE/EA/THF = diethyl ether-ethyl acetate-tetrahydrofuran) (SOTERO, 2002).

Free phenolic acids extraction

One gram of sample powder dry was submitted to delipidation with ethyl ether in Soxhlet; this sample was extracted six times with 20 mL tetrahydrofuran and mixed for 5 minutes in a vortex at room temperature ($\pm 23^{\circ}\text{C}$). The resulting supernatant of the extractions was filtered and dehydrated with anhydrous sodium sulphate. The fraction was concentrated in a rotary vacuum evaporator (Fisatom 802, São Paulo, Brasil), at 40°C and diluted in 5 mL tetrahydrofuran. The extract containing free phenolic acids fractions was stored under refrigeration and nitrogen atmosphere.

Esterified phenolic acids extraction

The residue from free phenolic acids extraction was used for new process of extraction. The soluble phenolics of the sample were extracted six times with 20 mL methanol:acetone:water solution (7:7:6). The sample was shaken for 5 minutes and centrifuged. At the end of the extraction, the supernatant was evaporated under vacuum, at 40°C , until aqueous phase. To release the soluble esters, which were esterified with proteins and polypeptides, an equal volume of 4N sodium hydroxide was added. After three hour hydrolysis at room temperature ($\pm 23^{\circ}\text{C}$) in the dark and nitrogen atmosphere, the pH was adjusted to 2 with 6N HCl, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was transferred to a separation funnel and extracted with hexane (1:1) to eliminate free fatty acids and other contaminants and also to extract the phenolics from the aqueous phase with a mixture of diethyl ether:ethyl acetate:tetrahydrofuran (1:1:1), for six times, under shaking. The extracted fraction was filtered and dehydrated with sodium sulphate anhydrous, rotaevaporated at 40°C , and made up to 5 mL with tetrahydrofuran. The storage followed the process described above.

Insoluble-bound phenolic acids extraction

The residue of the soluble phenolics extraction was hydrolyzed with 25 mL 4N NaOH at room temperature ($\pm 23^{\circ}\text{C}$) and in the dark for 3 hours. The insoluble-bound phenolic acid extract was acidified until pH 2 using 6N HCl, followed by centrifugation at 10,000 rpm for 10 minutes. From this step, the procedure was the same used in 2.2.2 item.

Identification of phenolic acids by gas chromatography

One-tenth mL of internal standard (methyl heptadecanoate-1.5mg/mL) was added to 0.5 mL of each fraction. The sample was derivatized by 0.2 mL N,O-bis (trimethylsilyl) acetamide (BSA) HEATED AT 60°C for 30 minutes, and after cooling to room temperature, was injected in a Shimadzu CG 17A, chromatograph, equipped with a flame ionization detector.

The identification of phenolics acids was based on matching retention time of standards. The chromatographic conditions followed the procedures described by Dabrowski and Sosulski (1984) with modifications made by Moreira and Mancini Filho (2003). The semipolar column DB5 (J & W®) (25 m x 0.25 mm) was used; operating conditions were as follows: initial temperature of the column 150°C , isothermic for 3 minutes; from 150 to 300°C at a rate of $5^{\circ}\text{C}/\text{min}$; isothermic 300°C for 3 minutes. The temperature of the injection camera was 250°C and of the detector was 300°C . A standard solution was prepared diluting the 18 phenolic acids (benzoic, p-hydroxybenzoic, salicylic, protocatechuic, chlorogenic, ellagic, *t*-cinnamic, vanillic, *o*-coumaric, gentisic, quinic, *p*-coumaric, cinnamic, gallic, propylgallate, ferulic, caffeic and synapic, were purchased from Sigma-Aldrich Co, St. Louis, MO, USA) in methanol, and an aliquot of this solution was added to an internal standard (methyl ester heptadecanoic acid) previously to their injection in

the chromatograph. The identifications were confirmed with the obtained mass spectra in the Phenolic library which was created with spectra of the phenolic standards (Sigma) in Hewlett Packard 5973 mass selective detector and registered in *Windows NT workstation 4.0*.

Statistical analysis

All analyses were performed in triplicate. One-way analysis of variance (ANOVA) and Tukey multiple comparisons were carried out to test any

significant differences among the means, by using Statistica® 6.0. Differences among means at 5% ($P < 0.05$) level were considered significant.

Results and discussion

Identification of phenolic acids

Figures 2 and 3 show free phenolic acids (A), esterified phenolic acids (B), and bound phenolic acids (C) extracted from apple pomace of Gala and Fuji cultivars.

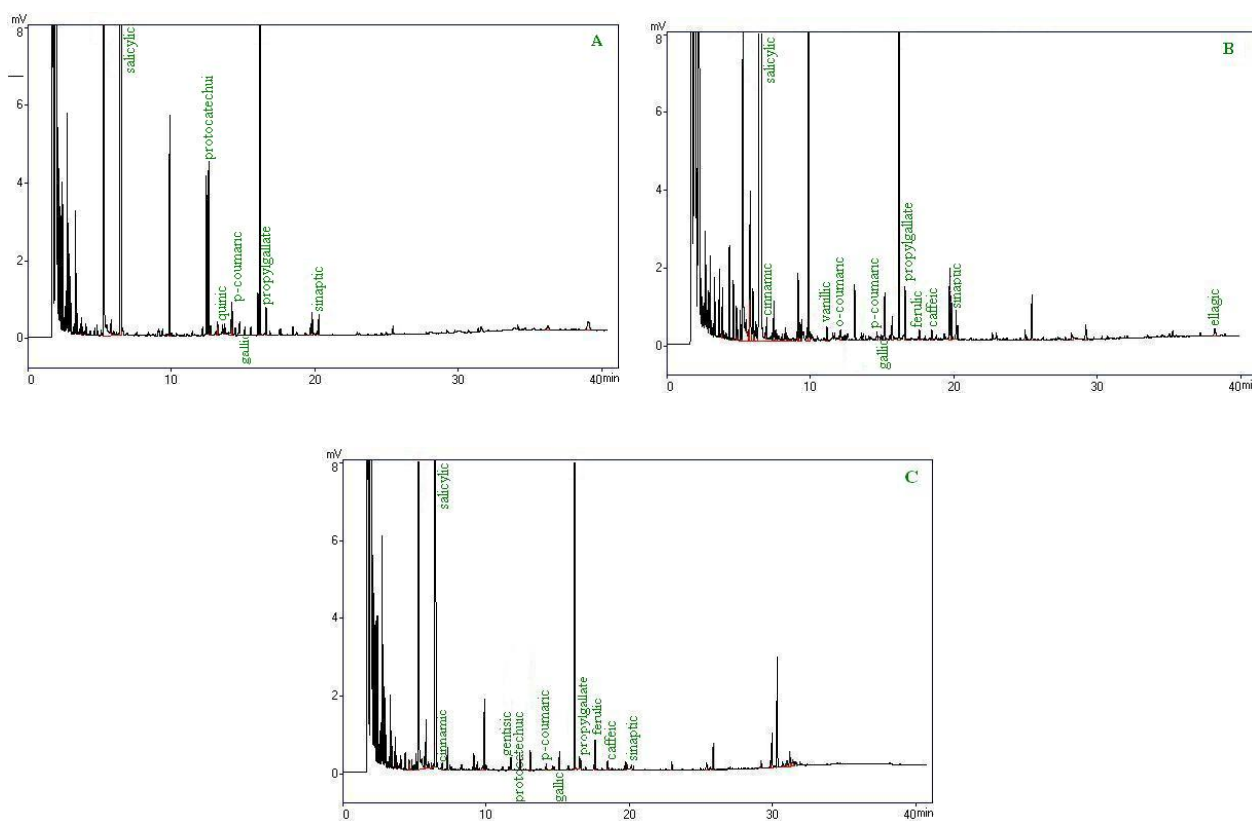


Figure 2. Gas chromatograms of free phenolic acids (A), esterified phenolic acids (B) and insoluble-bound phenolic acids (C) obtained for Gala cultivar.

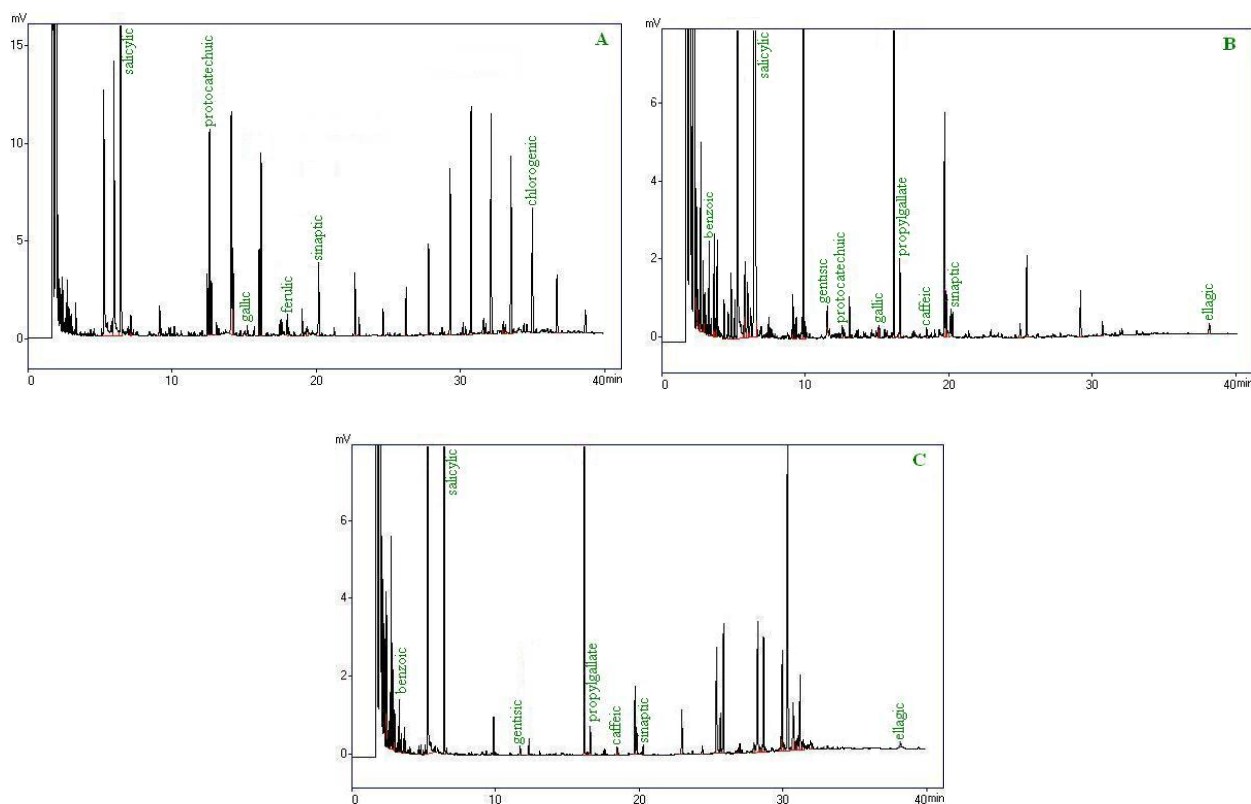


Figure 3. Gas chromatograms of free phenolic acids (A), esterified phenolic acids (B) and insoluble-bound phenolic acids (C) obtained for Fuji cultivar.

Phenolic acid contents

Tables 1 and 2 show free, esterified, and insoluble-bound phenolic acid contents in Gala and Fuji cultivars, respectively. Total phenolic acid contents in Gala pomace and Fuji pomace were very different, showing 93.94 mg/g and 68.38 mg/g, respectively. The results showed that esterified phenolic acids were the most abundant form, followed by free and insoluble-bound forms.

Regarding to Gala pomace, seven free phenolic acids were identified and its total content was 29.11 mg/g. Concerning to Fuji pomace, five free phenolic acids with a total content of 16.03 mg/g were identified. Salicylic acid was predominant in both varieties, presenting 85.71% and 60.89%, respectively.

Apple fruit also contains considerable amounts of hydroxycinnamic acid derivatives which are mainly represented by chlorogenic acid (LANCASTER, 1992; NICOLAS et al., 1994). In our study, chlorogenic acid was found at 1.147 mg/g in Fuji variety. Most of the phenolic acids was presented in esterified-form in both studied varieties. Total esterified phenolic acids contents in Gala and Fuji varieties were 53.75 and 48.30 mg/g, respectively.

In the present work, the main esterified phenolic acids present in Gala pomace were propylgallate acid, gallic acid, ferulic acid and sinapic acid, in Fuji pomace were propylgallate acid, benzoic acid and sinapic acid (Table 1 and 2). Esterified caffeic acid contents were 0.062 and 0.046 mg/g, respectively, i.e. considerably higher in Gala pomace. Salicylic acid was found in high levels and in esterified, free and insoluble forms, in both varieties.

Table 1. Phenolic acids composition (mg/g fresh weight) in apple pomace fractions from Gala^a cultivar.

Compound	Retention time (min)	free phenolic acids	esterified phenolic acids	Insoluble-bound phenolic acids
Salicylic acid	6.4	28.22 ± 2.10	52.760 ± 4.80	10.690 ± 0.80
Cinnamic acid	6.8	-	0.084 ± 0.03	0.032 ± 0.01
Vanillic acid	11.0	-	0.044 ± 0.02	-
Gentisic acid	11.6	-	-	0.050 ± 0.01
o-coumaric acid	12.0	-	0.028 ± 0.02	-
Protocatechuic acid	12.3	0.634 ± 0.09	-	0.055 ± 0.01
Quinic acid	13.6	0.032 ± 0.01	-	-
p-coumaric acid	14.6	0.070 ± 0.02	0.044 ± 0.0	-
Gallic acid	15.1	0.065 ± 0.01	0.175 ± 0.04	0.077 ± 0.01
Propylgallate	16.5	0.075 ± 0.02	0.198 ± 0.01	0.038 ± 0.00
Ferulic acid	17.5	-	0.159 ± 0.10	0.079 ± 0.02
Caffeic acid	18.4	-	0.062 ± 0.04	0.033 ± 0.00
Sinaptic acid	20.1	0.009 ± 0.08	0.140 ± 0.10	0.021 ± 0.01
Ellagic acid	38.1	-	0.055 ± 0.01	-
total phenolics		29.11	53.75	11.08

^aValues are the means ± standard deviation of triplicate determinations (n:3)

Total content of insoluble phenolic acids in Gala and Fuji pomace was 11.08 and 4.05 mg/g, respectively. Salicylic acid was also the main insoluble phenolic acid found out in both varieties. Nine insoluble phenolic acids (salicylic, cinnamic, gentisic, protocatechuic, gallic, propylgallate, ferulic, caffeic

and sinaptic) were detected in Gala pomace. Regarding to Fuji pomace, benzoic acid was detected in esterified fraction and cinnamic acid was not detected in any of the fractions. Total phenolic acids in Gala and Fuji pomace extracts were 93.94 and 68.38 mg/g, respectively.

Table 2. Phenolic acids composition (mg/g fresh weight) in apple pomace fractions from Fuji^a cultivar.

Compound	Retention time (min)	free phenolic acids	esterified phenolic acids	Insoluble-bound phenolic acids
Benzoic acid	3.2	-	0.194 ± 0.01	0.114 ± 0.02
Salicylic acid	6.4	12.450 ± 0.8	47.416 ± 1.00	3.704 ± 0.10
Gentisic acid	11.6	-	0.073 ± 0.01	0.026 ± 0.02
Protocatechuic acid	12.5	1.723 ± 0.01	0.057 ± 0.09	0.051 ± 0.00
Gallic acid	15.1	0.0804 ± 0.08	0.061 ± 0.10	-
Propylgallate	16.5	-	0.270 ± 0.02	0.088 ± 0.01
Ferulic acid	17.9	0.655 ± 0.90	-	-
Caffeic acid	18.4	-	0.046 ± 0.00	0.025 ± 0.00
Sinaptic acid	20.1	0.633 ± 0.01	0.115 ± 0.03	0.026 ± 0.00
Chlorogenic acid	34.9	1.147 ± 0.40	-	-
Ellagic acid	38.1	-	0.059 ± 0.01	0.022 ± 0.01
total phenolics		16.03	48.29	4.06

Conclusion

Phenolic compounds (benzoic acid, salicylic acid, *t*-cinnamic acid, vanillic acid, *o*-coumaric acid, gentisic acid, protocatechuic acid, quinic acid, *p*-coumaric acid, gallic acid, propylgallate ferulic acid, caffeic acid ellagic acid, chlorogenic acid and synapic acid) and its derivatives were identified in apple pomace.

In the three fractions obtained from apple Gala and Fuji cvs pomace were identified sixteen different phenolic acids as free, esterified and insoluble-bound forms. The total phenolic acids in Gala and Fuji pomace extracts were 93.94 and 68.38 mg/g and the principal component of both cultivars was salicylic acid.

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References

- AWAD, M. A.; DE JAGER, A.; VAN WESTING, L. M. Flavonoid and chlorogenic acid levels in apple fruit: characterisation of variation. *Scientia Horticulturae*, Amsterdam, v. **83**, n. 3, p. 249-263, 2000.
- BELL, J. C. R.; DONOVAN, J. L.; WONG, R.; WATERHOUSE, A. L.; GERMAN J. B.; WALZEM, J. R. (+)- Catechin in human plasma after ingestion of a single serving of reconstituted red wine. *American Journal of Clinical Nutrition*, Bethesda, v. **71**, n. 1, p.103-108, 2000.
- COOK, N. C.; SAMMAN, S. Flavonoids: chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of nutritional biochemistry*, Stoneham, v. **7**, n. 2, p. 66-76, Set. 1996.
- DABROWSKI, K. J.; SOSULSKI, F. Quantification of free and hydrolyzable phenolic acids in seeds by capillary gas-liquid chromatography. *Journal of agricultural and food chemistry*, Easton, v. **32**, n. 1, p. 123-127, 1984.
- DICK, A. J.; REDDEN, P. R.; DEMARCO, A. C.; LIDSTER, P. D.; GRINDLEY, T. B. Flavonoid glycosides of Spartan apple peel. *Journal of agricultural and food chemistry*, Easton, v. **35**, n. 4, p. 529-531, 1987.

- EBERHARDT, M. V.; LEE, C. Y.; LIU, R. H. Antioxidant activity of fresh apples. *Nature*, London, v. 405, n. 6789, p. 903-904, 2000.
- FOO, L. Y.; LU, Y. Isolation and identification of procyanidins in apple pomace. *Food Chemistry*, London, v. 64, n. 4, p. 511-518, 1999.
- HOLLMAN, P.C.H. *Determinants of the absorption of the dietary flavonoids quercetin in man*. 1997. Thesis (Ph.D) - Wageningen Agriculture University, Wageningen, The Netherlands.
- KRYGIER, K.; SOSULSKI, F.; HOGGE, L. Free, esterified and insoluble-bound phenolic acids. *Journal of agricultural and food chemistry*, Easton, v. 30, n. 2, p. 334-336, 1982.
- LANCASTER, J. E. Regulation of skin color in apples. *Critical review in plant science*, Boca Raton, v. 10, p. 487-502, 1992.
- LEA, A. G. H.; TIMBERLAKE, C. F. The phenolics of ciders. 1. Procyanidins. *Journal of the science of food and agriculture*, London, v. 25, n. 12, p. 1537-1545, 1974.
- LIDSTER, P.D.; DICK, A.J.; DEMARCO, A.; MCRAE, K.B. Application of flavonoid glycosides and phenolic acids to suppress firmness loss in apples. *Journal of the American Society for Horticultural Science*, Alexandria, v. 111, p. 892-896, 1986.
- LOMMEN, A.; GODEJOHANN, M.; VENEMA, D. P.; HOLLMAN, P. C. H.; SPRAUL, M. Application of directly coupled HPLC-NMR-MS to the identification and confirmation of quercetin glycosides and phloretin glycosides in apple peel. *Analytical Chemistry*, Washington, v. 72, n. 8, p. 1793-1797, 2000.
- LU, Y.; FOO, L. Y. Constitution of some chemical components of apple seed. *Food Chemistry*, London, v. 61, n. 1-2, Constitution of some chemical components of apple seed p. 29-33, 1998.
- _____. Identification and quantification of major polyphenols in apple pomace. *Food Chemistry*, London, v. 59, n. 2, p. 187-194, 1997.
- MOREIRA, A. V. B.; MANCINI FILHO, J. Atividade antioxidante das especiarias mostarda canela e erva-doce em sistema aquoso e lipídico. *Nutrire*, São Paulo, v. 25, n. 3, p. 31-46, 2003.
- NICOLAS, J. J.; RICHARD-FORGET, F. C.; GOUPY, P. M.; AMIOT, M. J.; AUBERT, S. Y. Enzymatic browning reaction in apple and apple products. *Critical reviews in food science and nutrition*, Boca Raton, v. 34, n. 2, p. 109-157, 1994.
- PRINCE, K. R.; PROSSER, T.; RICHETIN, A. M. F.; RHODES, M.J.C. A comparison of the flavonol content and composition in dessert, cooking and cider-making apples; distribution within the fruit and effect of juicing. *Food Chemistry*, London, v. 66, n. 4, p. 489-494, 1999.
- RICE-EVANS, A. C.; MILLER, N. J.; PAGANGA, G. Antioxidant properties of phenolic compounds. *Trends in Plant Science*, Oxford, v. 2, n. 4, p. 152-159, 1997.
- SOTERO, D. E. G. *Caracterização química e avaliação da atividade antioxidante de frutos da Amazônia: chope (*Gustavia augusta* L.), sacha mangua (*Grias neuberthii* Macbr.) e macambo (*Theobroma bicolor*)*. São Paulo: USP, 2002.
- SCHIEBER, A.; KELLER, P.; CARLE, R. Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *Journal of Chromatography A*, Amsterdam, v. 910, p. 265-273, 2001.
- VALDERRAMA, Patrícia; MARANGONI, Fabiane; CLEMENTE, Edmar. Efeito do tratamento térmico sobre a atividade de peroxidase (pod) e polifenoloxidase (ppo) em maçã (*Mallus comunis*). *Ciência e Tecnologia de Alimentos*, Campinas, v. 21, n.3, p. 321-325, 2001.