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Effect of mash maceration and ripening stage of apples on phenolic compounds and antioxidant power of cloudy juices: A study using chemometrics

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ABSTRACT

The effects of different enzymatic preparations on total phenolic content, phenolic profile (HPLC), and ferric reducing antioxidant power (FRAP) of cloudy juices from *Lis Gala* and *Fuji Suprema* apples varieties, at three ripening stages (unripe, ripe and senescent) were investigated using Principal Component Analysis and Hierarchical Cluster Analysis. The commercial preparations enzymatic (Ultrazym® AFPL; Pectinex® Ultra Clear; Pectinex® SMASH XXL; Panzym® YieldMASH) increased the total phenolic compounds and ferric reducing capacity of the cloudy juice from unripe and ripe *Lis Gala* (respectively by 67 and 49% for unripe apples, and 28 and 33% for ripe apples) and unripe *Fuji Suprema* apples (23 and 55%), while for the ripe *Fuji Suprema* apples only Pectinex® Ultra Clear and Panzym® YieldMASH had this effect. No significant ($p > 0.05$) was observed on senescent stage, whatever the enzymatic preparation. Enzymatic preparations could increase phenolic compounds concentration and antioxidant capacity of cloudy apple juice, but this effect depended on the maturity of the apples.

1. Introduction

The phenolic compounds in apple products, such as juice and cider, are considered to be important because they influence some important quality parameters, such as color, acidity and astringency, aromas and clarification (Mangas, Rodriguez, Suarez, Picinelli, & Dapena, 1999). There has been a growing interest in phenolic compounds present in many food preparations due to their antioxidant capacity, which contributes to protect human health from the deleterious effects of oxidative stress events (Oszmianski, Wojdylo, & Kolniak, 2009; Ribeiro, Henrique, Oliveira, Macedo, & Fleuri, 2010). The major classes of phenolic compounds found in apples are phenolic acids and flavonoids. The main representatives of phenolic acids are 5-caffeoylquinic acid (chlorogenic acid) and *p*-coumaroylquinic acid. The main flavonoids are flavan-3-ols (epicatechin, catechin and procyanidins), dihydrochalcones (phloretin as phloridzin, its glucoside, and xyloglucoside), flavonols (quercetin glycosides) and anthocyanins (cyanidin galactoside), the last two being as a general rule exclusively present in the peel (Awad, De Jager, & Van Westing, 2000; Tsao, Yang, Xie, Sockovie, & Khanizadeh, 2005; Vanzani et al., 2005).

The apple cultivar has a major effect on the juice phenolic composition since cultivars differ greatly in their phenolic compounds content (Will, Roth, Olk, Ludwig, & Dietrich, 2008). The application of pectinolytic enzymes in the production of apple juices results in a higher extraction of phenolic compounds, and a higher antioxidant capacity of the juice (Oszmianski, Wojdylo, & Kolniak, 2011; Oszmianski et al., 2009; Will, Ludwig, Dietrich, Schulz, & Otto, 2002). The ripening stage of the fruit to be processed also has some influence (Zhang, Li, & Cheng, 2010).

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Apples are harvested at different degrees of ripeness; fruits that are at an unripe stage (pre-maturation) are destined for cold-storage and ripe fruits are readily marketed (Harker & Hallett, 1992). Apples destined for the industrial sector are those that do not have acceptable physical characteristics (appearance, size and shape). In some countries, such as Brazil, apples that are rejected for marketing are processed during warm periods (25–35 °C) and fruits usually remain at the reception point for hours or even days, which speeds up the ripening process (Nogueira & Wosiacki, 2012). In this sense, there are three different ripening stages in apple processing: unripe (pre-maturation), ripe or full ripe, and senescent phase.

In apple juice processing, many different quality properties are usually studied, and this fact makes necessary the use of accurate and innovative classical mathematical and statistical approaches instead of only univariate comparisons among samples. Herein, the application of chemometric tools for the characterization, determination of origin, and quality control of food products has been increasingly used in food research. There are many applications of multivariate statistical techniques in order to explore and classify the antioxidant capacity and major phenolic compounds present in many foods, including apple-based products (Çam, Hisil, & Durmaz, 2009; Hossain, Patras, Barry-Ryan, Martin-Diana, & Brunton, 2011; Braga et al., 2013). Consequently, this study aimed to assess the effects of mash maceration with different commercial pectinases and ripening stage (unripe, ripe and senescent) of apples (*Lis Gala* and *Fuji Suprema*) on phenolic composition and ferric reducing antioxidant power of cloudy juices using multivariate statistical techniques.

2. Material and methods

2.1. Apple samples

Apples from *Lis Gala* and *Fuji Suprema* varieties, harvested in 2011–2012, were collected in Caçador, Santa Catarina, Brazil at the Experimental Station of the Agricultural and Rural Extension Company, Santa Catarina (EPAGRI) at three different ripening stages (unripe, ripe and senescent), with about 20 kg of samples for each ripening stage and each variety. Apples from the same cultivar were collected at different cardinal points from six different trees, and the top and the bottom of those trees, to homogenize the samples. The maturation index was determined by using the Starch-iodine test (Reid, Padfield, Watkins, & Harman, 1982). The fruits at different ripening stages were weighed (60 units for variety) and measured with a digital pachymeter (Insize, Waregem, Belgium) in relation to height and diameter (Table 1).

2.2. Chemicals and enzymes

Folin–Ciocalteau reagent; Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), TPTZ (2,4,6-Tri (2-pyridyl)-s-triazine), chlorogenic acid, phloridzin, (+)-catechin, procyanidin B1, procyanidin B2, quercetin-3-D-galactoside, quercetin-3-β-D-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-rutinoside were purchased from Sigma–Aldrich (Steinheim, Germany) and acetonitrile from Merck (Darmstadt, Germany). Commercial pectinolytic preparations: Ultrazym® AFPL; Pectinex® Ultra Clear; Pectinex® SMASH XXL were provided by LNF Bento Gonçalves – Novozymes Latin America and Panzym® YieldMASH was donated by Begerow (Germany).

Table 1
Physical characteristics of *Lis Gala* and *Fuji Suprema* at different ripening stages.

Variety	Ripening stage	[a]Maturation index	Weight (g)	Height (mm)	Diameter (mm)
Lis Gala	Unripe	1.0	110 ± 22	60 ± 4	56 ± 6
	Ripe	3.5	146 ± 31	68 ± 5	63 ± 6
	Senescent	4.5	165 ± 21	71 ± 55	66 ± 6
Fuji Suprema	Unripe	1.0	172 ± 42	75 ± 7	63 ± 7
	Ripe	3.5	183 ± 58	77 ± 9	64 ± 8
	Senescent	4.5	175 ± 44	76 ± 7	64 ± 7

Note: The physical measurements were performed on 60 fruit pieces and data are presented as mean ± standard deviation.

[a] The iodine regression test is an indicator of residual starch in fruits, lower values indicate less ripe. No standard deviation given as this was a visual comparison to the standard table of the maturity index for apples.

2.3. Enzymatic maceration in cloudy juice processing

The apples were selected, washed, sanitized (100 mg/L of sodium hypochlorite, 25 °C/15 min) and fragmented (ground) in an industrial blender LAR2 (Metvisa, Brusque, SC, Brazil). The apple pulp (100 g) was placed in each Erlenmeyer and conditioned at 35 °C in a shaker MA832 (Marconi, Piracicaba, SP, Brazil) under agitation (150 rpm/5 min). After temperature stabilization, enzymatic preparations were added to the apple pulp according to the manufacturer's recommendation and reaction was conducted for 1 h. Then, the Erlenmeyers were placed in a bath of boiling water (for 1 min) for the denaturation of the enzymes added. The supernatants, denominated laboratory juice in this study, were obtained by centrifugation (8000×*g*, 20 min) with a laboratory centrifuge HIMAC CR-GII (Hitachi, Ibaraki, Japan). Three replications were completed for each treatment. A triplicate control was performed under the same conditions but without enzyme addition. Juices were stored at −20 °C for further analysis.

2.4. HPLC analysis of phenolic compounds

The HPLC analysis of phenolic compounds (monomers and dimers) was based on the methodology described by Alberti et al. (2014). Apple juices (4 mL) were freeze-dried (model LD, Terroni, São Paulo, SP, Brazil) and reconstituted with a solution (2 mL) of 25 mL/L acetic acid and methanol. Then, samples were filtered through a syringe filter 0.2 μm (Nylon) prior to analysis. The HPLC apparatus was a 2695 Alliance (Waters, Milford, MA, USA), with photodiode array detector PDA 2998 (Waters, Milford, MA, USA), quaternary pump and auto-sampler. Separation was performed on a Symmetry C18 (4.6 × 150 mm, 3.5 μm) column (Waters, Milford, MA, USA) at 20 °C. The mobile phase was composed of solvent A (25 mL/L of acetic acid) and solvent B (acetonitrile). The following gradient was applied: 3–9% B (0–5 min), 9–16% B (5–15 min), 16–36.4% B (15–33 min), followed by washing and reconditioning of the column. The flow rate was 1.0 mL/min, and the runs were monitored at 280 nm (flavan-3-ols and dihydrochalcones), 320 nm (hydroxycinnamic acids), 350 nm (flavonols) and 520 nm (anthocyanins). Quantification was performed using calibration curves of standards (at least 5 concentrations were used to build the curves) described in chemicals (see Section 2.2). All determinations were performed in triplicate samples.

2.5. Total phenolic content

The total phenolic content was determined by colorimetric analysis using Folin–Ciocalteau reagent as described by Singleton and Rossi (1965). In a test tube, 8.4 mL of distilled water, 100 μL of sample or (+)-catechin (standard, 10–400 mg/L), and 500 μL of Folin–Ciocalteau reagent were added and the mixture was vortexed for 10 s. After 3 min, 1.0 mL of saturated sodium carbonate was added into each tube and the tube was agitated immediately in a vortex. After 1 h, the absorbance was measured using a spectrophotometer (model mini UV 1240, Shimadzu, Tokyo, Japan) at 720 nm. The total phenolic content was expressed as catechin

equivalents [mg catechin/100 mL of juice] of the sample. All determinations were performed in triplicate samples.

2.6. Ferric reducing/antioxidant power (FRAP) assay

To determine the antioxidant capacity, the FRAP spectrophotometric method was used, as described by Benzie and Strain (1996), with minor modifications. The absorbance was read at 593 nm at 37 °C. After the first reading (L1), which was performed within 5 s, the absorbance was monitored for 6 min (L6) every 15 s. To calculate the FRAP absorbance values, sample concentrations and the standard solution used as reference 6-hydroxy-2, 5,7,8-tetramethyl-chroman-2-carboxylic acid at a concentration of 1 mmol/L were correlated. A calibration curve was plotted for different concentrations of Trolox (0.1–1.0 mmol/L). All determinations were performed in triplicate samples.

2.7. Data analysis

Data were presented as mean ± standard deviation (SD) or mean ± pooled standard deviation (PSD). PSD for each response variable was calculated using Eq. (1)

$$\mathrm{PSD} = \frac{(n_1-1)s_1^2 + (n_2-1)s_2^2 + \cdots + (n_k-1)s_k^2}{n_1 + n_2 + \cdots + n_k - k} \quad (1)$$

where n_i sample size of the ith sample, s_i^2 is the variance of the ith sample, and k is the number of samples being combined.

Pearson products (r) were used to evaluate the strength of correlation among the response variables. Principal component analysis (PCA) and hierarchical cluster analysis (HCA), were the multivariate statistical methods used to analyze the results. For this purpose, a matrix composed of samples ($n = 30$) and responses ($n = 10$) was built, totaling 300 data points. The results obtained for each parameter were adopted as variables (columns) and the juice samples as individual (rows). Then, pre-treatment of data was carried to transform the mean values of each variable (average zero and standard deviation 1) into z-scores, equalizing thus the statistical importance of the response variables.

PCA was applied to separate the cloudy apple juice samples ($n = 30$) according to their values of phenolic compounds determined by HPLC, total phenols, and antioxidant capacity (FRAP). Analysis was based on linear correlations and variances were computed as sum of squares/($n - 1$). Eigenvalues higher than 1.0 were adopted to explain the projection of the samples on the factor plane, in which a two-dimensional graph was built to project both responses and juice samples (Zielinski et al., 2014).

HCA was performed to assess similarities among juice samples according to phenolic composition and antioxidant capacity. In this sense, sample similarities were calculated on the basis of the Euclidean metric, and the Ward's method was used to form and suggest groups of similar samples. The dendrogram imposes a hierarchy on this similarity so that it is possible to have a two-dimensional vision of the entire set of samples used in the study according to the selected responses (Granato, Katayama, & Castro, 2010). Subsequently, HCA was also applied to the variables: phenolic compounds determined by HPLC, total phenols and antioxidant capacity (FRAP). In order to compare the results within the four suggested clusters, Hartley's test was carried out to check for homogeneity of variances. One-way ANOVA and Fisher's LSD *post hoc* test were applied to highlight differences among the clusters. p-values below 0.05 were used to reject the null hypothesis. All statistical analyses were performed using the Statistica v.7 software (StatSoft, Tulsa, USA).

3. Results and discussion

3.1. Phenolic content at different ripening stages

The content of individual phenolic compounds and total phenols analyzed (Tables 2 and 3) were lower than those data found in another studies (Markowski, Mieszczakowska, & Plocharski, 2009; Nogueira et al., 2008; Oszmianski et al., 2011). Fruit variety, the cultivation conditions, climatic interactions, the ripening stage and juice extraction method can explain this variation in the concentration of these compounds (Spanos & Wrolstad, 1992; Will et al., 2008; Zhang et al., 2010). The total phenolic (monomeric and oligomeric forms) levels of Brazilian apple juices are between 100 and 900 mg/L (Wosiacki, Nogueira, Silva, Denardi, & Vieira, 2008), while European juices have levels from <100 up to 3000 mg/L (Gökmen, Artık, Acar, Kahraman, & Poyrazoglu, 2001; Markowski, Kolodziejczyk, Król, Plocharski, & Rutkowski, 2007; Nogueira et al., 2008).

The levels of phenolic compounds and antioxidant capacity (FRAP) in the cloudy juices increased with maturity for both cultivars (Tables 2 and 3). Juices from the unripe apples had lower phenolic content, probably due to oxidation of these compounds during processing, given that unripe fruits are more susceptible to enzymatic browning due to a low content of ascorbic acid and a high activity of polyphenoloxidase (PPO) (Murata, Tsurutani, Tomita, Homma, & Kaneko, 1995).

Chlorogenic acid and phloridzin were the monomeric phenolics found in the highest concentration in both cultivars. Other authors have reported high levels of these phenolic compounds in apples (Awad, De Jager, Van Der Plas, & Van Der Krol, 2001; Kondo, Tsuda, Muto, & Ueda, 2002; Veberic et al., 2005).

Zhang et al. (2010) found that the levels of procyanidin B1 increased during the ripening of apples. In the present study, flavonols (quercetin-3-D-galactoside, quercetin-3-β-D-glucoside, quercetin-3-*O*-rhamnoside and quercetin-3-rutinoside) were found in the highest levels in samples of unripe apples of the *Fuji Suprema* variety (Table 2) and senescent apples of the *Lis Gala* variety (Table 3). Awad et al. (2001) reported that quercetin glycoside levels vary according to the cultivar and the position of the fruit on the tree (sun exposure). Fruits of *Fuji Suprema* show a specific characteristic as compared to other varieties; after full blooming, the fruit epidermis is evenly covered with a dark red color, even in the shaded parts of the plant. During ripening they lose this coloration and turn a lighter shade of red. However, anthocyanins were not found in the samples analyzed (absence of peaks in chromatogram at 520 nm) probably due to enzymatic oxidation and loss during the process (data not shown).

3.2. Enzymatic maceration in phenolic extraction

In the processing of apple juice by the traditional process of pressing, most of the phenolic compounds present in the fruits are retained in the pomace due to non-covalent bonds within the cell wall (Le Bourvellec, Le Quéré, & Renard, 2007). In the present study and others studies with enzymatic treatments (Markowski et al., 2009; Oszmianski et al., 2011), there was a significant increase ($p < 0.05$) in the phenolic content of the juices (Tables 2 and 3).

The increase in total phenols in the enzymatically treatment juices of unripe and ripe apples was, on average, 23% (230–308 mg/L) and 9% (239–256 mg/L) respectively for *Fuji Suprema* whereas for the *Lis Gala* variety it was 67% (366–415 mg/L) and 28% (365–395 mg/L), respectively. This indicates that the enzymatic treatment acted most effectively on the *Lis Gala* variety.

The highest levels of total phenolic compounds were obtained for the juices of ripe and unripe apples which had been treated with

Table 2
Effect of enzymatic treatment on the phenolic compounds (mg/L), total phenols (mg/L) and antioxidant capacity (FRAP) (μmol/L) in apple juice processing with fruit at three different ripening stages of the *Fuji Suprema* variety.

Samples		CQA	PLZ	PB1	PB2	QGA	QGL	QRH	QRU	TPC	FRAP
Unripe	Control	11	21	8	nd	8	2	5	11	231	2407
	Ultrazym® AFPL	12	23	8	4	7	2	4	10	230	3864
	Pectinex® Ultra Clear	18	26	8	nd	4	2	3	10	288	3990
	Pectinex® SMASH XXL	12	26	8	nd	8	2	5	10	283	3539
	Panzym® YieldMASH	16	28	10	3	10	2	6	11	308	3577
	PSD	3	3	1	2	2	0	1	0	36	627
Ripe	Control	10	19	6	nd	5	2	3	10	228	2799
	Ultrazym® AFPL	11	22	7	4	5	2	3	10	243	2979
	Pectinex® Ultra Clear	10	21	5	4	6	2	4	10	256	3319
	Pectinex® SMASH XXL	9	24	5	nd	4	2	3	10	239	2774
	Panzym® YieldMASH	11	22	9	7	8	2	5	10	254	3430
	PSD	1	2	2	3	2	0	1	0	12	300
Senescent	Control	18	25	11	nd	6	2	3	10	304	3882
	Ultrazym® AFPL	19	29	6	nd	5	2	3	10	335	4158
	Pectinex® Ultra Clear	12	23	6	6	5	2	3	10	288	4112
	Pectinex® SMASH XXL	14	31	2	nd	5	2	3	11	316	3706
	Panzym® YieldMASH	11	22	5	nd	6	2	4	10	290	3645
	PSD	3	4	3	2	1	0	0	0	20	231

CQA, chlorogenic acid; PLZ, phloridzin; PB1, procyanidin B1; PB2, procyanidin B2; QGA, quercetin-3-D-galactoside; QGL, quercetin-3-β-D-glucoside; QRH, quercetin-3-*O*-rhamnoside; QRU, quercetin-3-rutinoside; TPC: total phenolic compounds; FRAP: ferric reducing antioxidant power; PSD: pooled standard deviation; nd: not detected or values below the limit of detection. All determinations were performed in triplicate.

Pectinex® Ultra Clear and Panzym® YieldMASH. These two enzymatic preparations are declared by the manufacturer to be rich in PG activity. The enzymatic preparations which are declared to be rich in pectinlyase activity (Ultrazym® AFPL and Pectinex® SMASH XXL) were less efficient for increasing polyphenol extraction to cloudy juices. In the samples from senescent fruits, this was not observed. Goulao, Santos, de Sousa, and Oliveira (2007) observed an increase of the enzymes activity like exo-polygalacturonase, β-galactosidase, pectin methylesterase and α-L-arabinofuranosidase during apple ripening. These enzymes are related to pectin solubilization and then, the addition of pectinase in senescent fruit does not result in an increase in the extraction of phenolic compounds compared to the control. In some treatments (Tables 2 and 3) the amount of total phenols decreased, probably due to a secondary enzymatic capacity of the preparations employed, as corroborates by Mihalev, Schieber, Mollov, and Carle (2004).

3.3. The effect of enzymatic maceration on antioxidant capacity

The effect of enzyme treatment on antioxidant capacity was measured as the ability of reduction of iron using the FRAP assay. The treatments with pectinase resulted in increased ($p < 0.05$) antioxidant capacity of juices from the unripe and ripe fruits, compared to controls. On average, an increase of 55% (3538–3989 μmol/L) and 12% (2774–3430 μmol/L) was found for fruit at the unripe and ripe stages, respectively, for the *Fuji Suprema* variety (Table 2), and the *Lis Gala* variety (Table 3), which represented an average increase of 49% (3094–4141 μmol/L) and 33% (3376–5430 μmol/L), for the same ripening stages, respectively. In the juices made with senescent apples with enzyme treatment there was not a significant variation in the antioxidant activities, probably due to the content of endogenous enzymes (Pectolytic), as previously mentioned.

Table 3
Effect of enzymatic treatment on the phenolic compounds (mg/L), total phenols (mg/L) and antioxidant capacity (FRAP) (μmol/L) in apple juice processing with fruit at three different ripening stages of the *Lis Gala* variety.

Samples		CQA	PLZ	PB1	PB2	QGA	QGL	QRH	QRU	TPC	FRAP
Unripe	Control	16	14	6	nd	3	2	2	9	234	2659
	Ultrazym® AFPL	21	28	9	nd	9	2	5	11	374	3882
	Pectinex® Ultra Clear	25	28	13	11	13	3	7	11	408	4043
	Pectinex® SMASH XXL	29	27	9	9	9	2	6	10	416	4141
	Panzym® YieldMASH	21	26	6	7	10	2	6	10	366	3094
	PSD	5	9	3	5	3	0	2	1	73	653
Ripe	Control	16	15	7	nd	9	2	5	11	301	2782
	Ultrazym® AFPL	21	19	6	6	10	2	5	11	365	3726
	Pectinex® Ultra Clear	20	22	10	nd	12	3	6	11	391	5430
	Pectinex® SMASH XXL	23	17	11	4	11	3	6	11	395	3691
	Panzym® YieldMASH	18	19	9	nd	12	3	6	11	380	3376
	PSD	3	3	2	3	1	0	1	0	38	986
Senescent	Control	27	16	7	nd	9	2	5	11	438	5004
	Ultrazym® AFPL	38	20	12	nd	8	2	5	10	482	5143
	Pectinex® Ultra Clear	24	20	9	8	7	2	5	10	377	4844
	Pectinex® SMASH XXL	31	19	8	nd	9	2	5	10	399	3680
	Panzym® YieldMASH	29	18	11	nd	8	2	5	10	466	5317
	PSD	5	2	2	4	1	0	0	0.25	44	649

CQA, chlorogenic acid; PLZ, phloridzin; PB1, procyanidin B1; PB2, procyanidin B2; QGA, quercetin-3-D-galactoside; QGL, quercetin-3-β-D-glucoside; QRH, quercetin-3-*O*-rhamnoside; QRU, quercetin-3-rutinoside; TPC: total phenolic compounds; FRAP: ferric reducing antioxidant power; I (Control); II (Ultrazym® AFPL); III (Pectinex® Ultra Clear); IV (Pectinex® SMASH XXL); V (Panzym® YieldMASH); PSD: pooled standard deviation; nd: not detected or values below the limit of detection. All determinations were performed in triplicate.

3.4. Multivariate analysis of data

Principal component analysis (PCA) was applied in order to evaluate the data of principal individual phenolic compounds determined by HPLC, total phenols and antioxidant capacity. PC1 explained up to 54.90% of total variance and PC2 explained 14.39%, totaling 69.29%. Samples were separated along the first principal component (PC1) by differences observed in chlorogenic acid, procyanidin B1, quercetin-3-D-galactoside, quercetin-3-β-D-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-rutinoside, total phenolic compounds and FRAP. The second PC classified the samples related to their chlorogenic acid, TPC, and FRAP (all strongly correlated) opposed to flavonols contents.

The scatter plot (Fig. 1) shows suggested reasons for the locations of the juices processed with or without enzymatic preparation on the basis of their phenolic composition and antioxidant capacity. PC1 showed related to the cultivar effect, with *Fuji Suprema* and *Lis Gala* each on one side of PC2 (*Fuji* juices had less polyphenols than *Gala* juices, and this is the main effect, not the enzymes nor the ripeness). Changes in position along PC1 can be linked to global effects on polyphenols concentration, while along PC2 indicated a modification of the equilibrium among chlorogenic acid and flavonols.

The results of Pearson's correlation analysis showed a significant ($p < 0.01$) association between antioxidant capacity and chlorogenic acid ($r = 0.66$) and total phenolic compounds ($r = 0.92$). The similarity of sample was evaluated using hierarchical cluster analysis (HCA) applied to the samples and four clusters were suggested (Fig. 2).

The juices in Cluster 1 (Table 4) contained samples with the highest levels of chlorogenic acid, procyanidin B1, quercetin-3-β-D-glucoside, total phenolic compounds and antioxidant capacity. This cluster was characterized by the samples from the *Lis Gala* variety at the ripe and senescent stages, with the application of enzymes and senescent control juice. Samples included in Cluster 2 presents the highest content of quercetin-3-D-galactoside, quercetin-3-β-D-glucoside, quercetin-3-*O*-rhamnoside and quercetin-3-rutinoside (Table 4). Cluster 2 was also characterized by samples from the *Lis Gala* variety at all the ripening stages (samples received enzymatic treatment with the four tested enzymes). Cluster 3 was composed mostly by *Fuji Suprema* variety and showed the highest levels of phloridzin, and intermediate values of other individual phenols, total phenolic compounds and antioxidant capacity. Cluster 4 included the samples with the lowest phenolic content and antioxidant power, that is, control sample and juices made with unripe and ripe apples from both varieties (Table 4). According to Alonso-Salces et al. (2005) and Zhang et al. (2010), during the ripening of apples the increase of phenolic compounds occurs and this fact depends on the variety. Several authors have reported that the application of pectinolytic enzymes in juice processing also results in increased phenolic compounds (Cheynier, 2006; Markowski et al., 2009; Oszmianski et al., 2009), however this was not observed for senescent fruits.

According to the HCA, the four enzymatic preparations were effective in increasing the total phenolic concentration and antioxidant activity of unripe and ripe *Lis Gala* cloudy apple juices. For

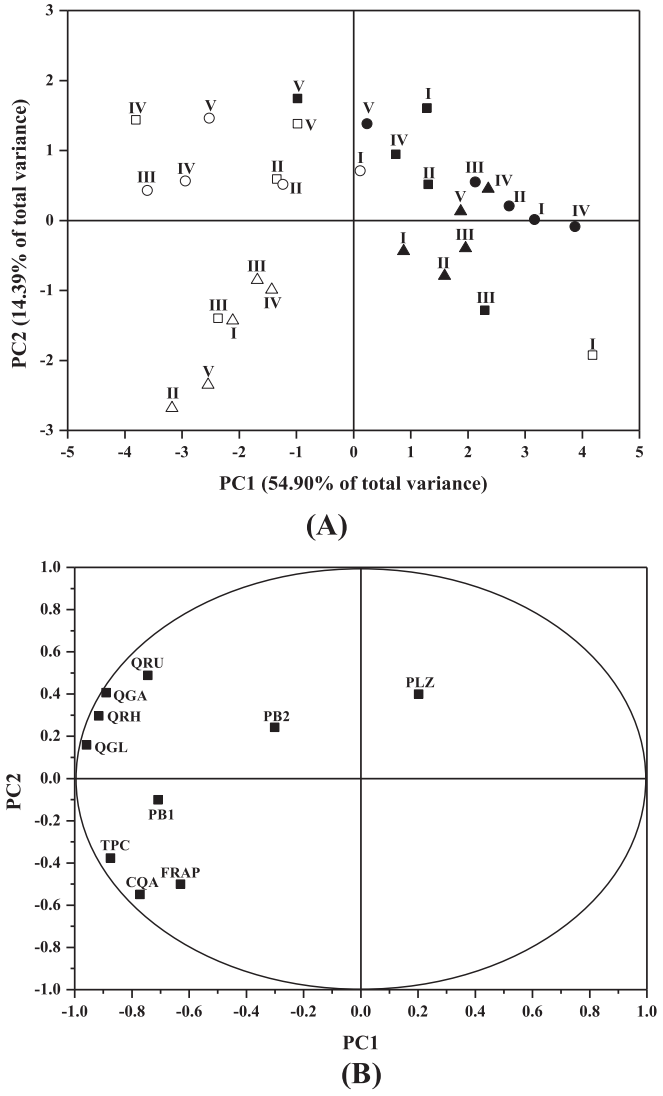

Fig. 1. A scatter plot PC1 vs. PC2 on the main sources of variability between the apple juice samples. (A) *Scores* and (B) *Loadings* plots. Note: I (Control); II (Ultrazym® AFPL); III (Pectinex® Ultra Clear); IV (Pectinex® SMASH XXL); V (Panzym® YieldMASH); black forms correspond to *Fuji Suprema* variety and unfilled forms correspond to *Lis Gala* variety, (■) unripe; (●) ripe; (▲) senescent; (□) unripe; (○) ripe; (△) senescent; CQA, chlorogenic acid; PLZ, phloridzin; PB1, procyanidin B1; PB2, procyanidin B2; QGA, quercetin-3-D-galactoside; QGL, quercetin-3-β-D-glucoside; QRH, quercetin-3-*O*-rhamnoside; QRU, quercetin-3-rutinoside; TPC: total phenolic compounds; FRAP: ferric reducing antioxidant power.

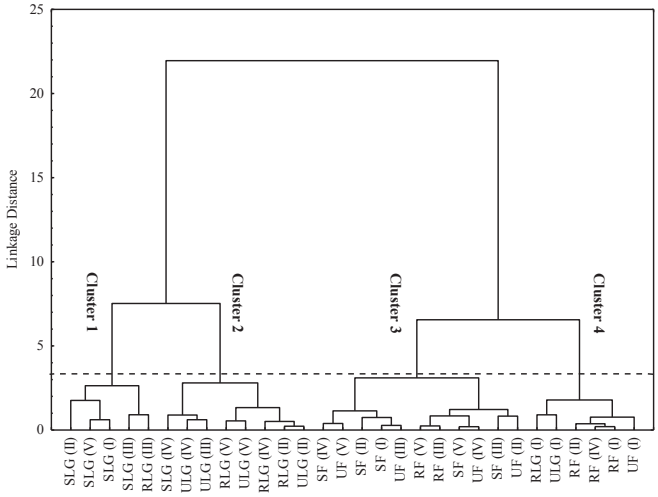

Fig. 2. Dendrogram for juice samples obtained from hierarchical cluster analysis. Note: UF: unripe *Fuji Suprema*; RF: ripe *Fuji Suprema*; SF: senescent *Fuji Suprema*; ULG: unripe *Lis Gala*; RLG: ripe *Lis Gala*; SLG: senescent *Lis Gala*; I (Control); II (Ultrazym® AFPL); III (Pectinex® Ultra Clear); IV (Pectinex® SMASH XXL); V (Panzym® YieldMASH).

Table 4
Principal phenolic compounds (mg/L), total phenols (mg/L) and antioxidant capacity (μmol/L) of apple juices from apple juices classified using hierarchical cluster analysis.

Variables	Cluster 1 ($n = 5$)	Cluster 2 ($n = 8$)	Cluster 3 ($n = 11$)	Cluster 4 ($n = 6$)	PSD	p-value*	p-value**
Chlorogenic acid	27.39[a]	23.61[a]	13.92[b]	12.15[b]	7.25	0.26	<0.001
Phloridzin	19.18[b]	20.20[b]	25.06[a]	19.11[b]	5.11	0.07	0.03
Procyanidin B1	10.03[a]	8.88[b]	7.19[b]	6.59[b]	2.45	0.29	0.04
Procyanidin B2	1.69	4.72	2.17	0.68	3.41	0.17	0.13
Quercetin-3-D-galactoside	8.80[a]	9.92[a]	6.40[b]	5.62[b]	2.39	0.48	<0.001
Quercetin-3-β-D-glucoside	2.36[a]	2.36[a]	1.81[b]	1.71[b]	0.35	0.18	<0.001
Quercetin-3-*O*-rhamnoside	5.42[a]	5.80[a]	3.70[b]	3.35[b]	1.34	0.56	<0.001
Quercetin-3-rutinoside	10.48[a]	10.62[a]	10.24[ab]	9.86[b]	0.47	0.29	0.01
Total phenolic compounds	430.91[a]	387.69[b]	286.58[c]	245.93[d]	75.35	0.28	<0.001
Ferric reducing antioxidant power	5147.79[a]	3704.11[b]	3747.29[b]	2733.54[c]	786.91	0.59	<0.001

FRAP: ferric reducing antioxidant power; PSD: pooled standard deviation; *Probability values obtained by Hartley test (*F*-max) for homogeneity of variances; **Probability values obtained by one-way ANOVA. Different letters in the same line represent statistically different results ($p < 0.05$).

the *Fuji Suprema* variety, all enzymatic preparations were effective at the unripe stage, while for the ripe stage only Pectinex® Ultra Clear and Panzym® YieldMASH were able to increase the levels of phenolic compounds and antioxidant capacity of apple juices. No alterations were observed at the senescent stage with the application of enzymes due to the fact that the presence of endogenous enzymes may have minimized the action of the added preparation (Ben-Arie, Kislev, & Frenkel, 1979; Goulao et al., 2007).

The antioxidant capacity of polyphenols is related to factors such as their reactivity as electron or hydrogen-donating agents (Rice-Evans, Miller, & Paganga, 1996). The reducing ability is due to two main reasons: to the number and acidity of their phenolic hydroxyl groups, and to the resonance between the free electron pair on the phenolic oxygen and the benzene ring. These chemical features increase the electron delocalization and provides a partial negative charge upon the substitution position adjacent to the hydroxyl group, increasing thus the antioxidant power of the sample (Cheynier, 2006; Pietta & Simonetti, 1999).

The most abundant phenolic acid in apples is chlorogenic acid (Veberic et al., 2005). In our study, this compound showed significant difference among the clusters ($p < 0.01$), and a positive and significant correlation ($r = 0.66$, $p < 0.001$) with antioxidant capacity. The antioxidant capacity of hydroxycinnamic acids is related to the presence of catechol moiety which generates a semiquinone stabilized by hydrogen bonds when a hydrogen atom is abstracted from the chemical structure (Amorati, Pedulli, Cabrini, Zambonin, & Landi, 2006).

Procyanidin B1 ($r = 0.47$, $p < 0.01$), quercetin-3-β-D-glucoside ($r = 0.50$, $p < 0.01$) and quercetin-3-*O*-rhamnoside ($r = 0.37$, $p < 0.05$) were correlated with the antioxidant capacity measured by FRAP, with significant difference among clusters. According to Tsao et al. (2005), procyanidins showed higher antioxidant capacity measured by FRAP, while quercetin glycosides were a group with moderate antioxidant capacity. The antioxidant capacity of procyanidins is due to the presence of the catechol unit on the aromatic B-ring (Rice-Evans et al., 1996), which stabilizes free radicals and their ability to chelate metals and proteins due to several *o*-dihydroxy phenolic groups in such a high molecular weight structure (Santos-Buelga & Scalbert, 2000), while for flavonols (quercetin glycosides) the combination of the catechol moiety with double bond at C2–C3 and 3-OH provides an extremely active free-radical scavenger (Van Acker et al., 1996).

4. Conclusion

Mash maceration with different enzymes had a positive effect on the production of cloudy apple juice. It improved not only the concentration of phenolic compounds but also the antioxidant capacity when applied to unripe and ripe, but not senescent, apples. The chemometric approach allowed observing the influence of apple ripening stage on the efficiency of enzymatic preparations. Multivariate statistical techniques appear to be suitable tools to monitor technological and agronomical properties of apples and juices. Both the ripening stage of each apple variety and the enzymatic preparation used in the apple juice processing are essential technological factors to be controlled in order to obtain a high efficiency in the process.

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