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Efficiency of extraction of antioxidant compounds in by-product from red guava processing

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ABSTRACT

Studies that investigate efficient methods for extraction of antioxidant compounds in by-products of fruits can contribute to their appreciation in food products. This study aimed comparing two methods for phenolic extraction: single extraction (single solvent) and sequential (different solvents) in by-product from Paluma guava processing, evaluating the influence of the extracts on the stabilization of radicals by DPPH and ORAC assays. Sequential extraction showed higher efficiency in obtaining phenolic (2.60>0.56 mg GAE g^{-1}), allowing the extraction of free phenolic, conjugated and fat-soluble, as well as determining the highest antioxidant capacity in these extracts. Acetone was characterized to be the responsible solvent for higher extraction of phenolic in the sequential extraction, being necessary 0.90 mg mL^{-1} of extract to reduce 50% the action of DPPH• radicals. Thus, sequential extraction of phenolic is a potential method for better use of antioxidant compounds in the guava processing by-product.

Keywords: Paluma; By-products; Phenolic compounds; Radicals.

INTRODUCTION

The processing of guava generates high volume of waste, known as a by-product, which is comprised primarily by seeds, bagasse and barks (MANTOVANI *et al.*, 2004).

Studies show that most of the nutrients are concentrated in the barks and seeds of the fruits (SOUSA *et al.*, 2011), which could be applied to the development of new food products such as cookies, cakes and cereal bars (SOUSA; VIEIRA; LIMA, 2011). However, the components present in fruits and their properties should be better studied to better use of by-product.

The by-product from guava is considered a waste with great antioxidant capacity due to the presence of phenolic compounds (SOUSA; VIEIRA; LIMA, 2011). According to Naczk and Shahidi (2004) the efficiency of extraction of phenolic compounds in plants depends on a few factors such as chemical nature of phytochemicals and solvents, the extraction method employed and solvent, the size of the particle sample, the time and storage conditions, and the presence of interfering substances. Furthermore, the phenolic may be complexed with carbohydrates, with proteins and other nutrients present in the plant or be in its insoluble form in the matrix.

Thus, the objective of this study was to compare the efficiency of extraction of phenolic compounds in by-product from the Paluma guava processing by two extraction methods - single and sequential, evaluating the efficiency of extraction of antioxidant compounds as well as the influence of extraction in the capacity to stabilize free radical of by-product extracts by DPPH and ORAC assays.

MATERIAL AND METHODS

MATERIAL

The by-product from the Paluma guava processing, characterized by seeds, barks, stems and part of the discarded pulp during the pulping of fruit, was donated by a producer industry of juices and fruit nectars (De Marchi), located in Jundiaí – SP, being conducted under refrigeration until the Bioaromas Laboratory and Bioactive Compounds of the Department of Food Science, Food Engineering - FEA / UNICAMP and frozen until the phenolic compounds extraction.

OBTAINING EXTRACTS

The extracts of the samples were obtained in triplicate for two methods of extraction with solvent only (90% hydroethanol) and sequential (solvents different).

SINGLE EXTRACTION

The single extraction (SiE) was performed according to Roesler *et al.* (2007), with modifications. Fresh samples (0.5 g) were shaken in a shaker New Brunswick Scientific (Model C76, Edison, NJ, USA) at 180 rpm for 30 minutes in dark and cold room with 3 mL of 90% ethanol solvent (10:90, v v^{-1} distilled water:ethanol) and after were centrifuged in a Hettich Zentrifugen (model 460R Rotanta, Germany) centrifuge for 15 minutes at 4750 rpm and at 4°C. The residue was re-extracted under the same conditions, totaling 60 minutes of extraction. The resulting supernatants were combined and the volume adjusted to 10 mL.

SEQUENTIAL EXTRACTION

Sequential extraction (SE) was performed according to Sun *et al.* (2002) and Dewanto, Wu, Liu (2002), with modifications, allowing us to obtain three fractions. The free phenolic compounds (FPE) were extracted using 50 g fresh weight of the homogenized sample with 100 mL of 80% hydroacetonic solution (v v^{-1}) in blender Skymsen Industrial (LI-N-1.5 model, Brazil) for 5 minutes and further 3 minutes at ultra-turrax Tron Poly (model PT - MR 2100, Switzerland), and subsequently centrifuged at 5°C for 15 minutes at 5000 rpm. The supernatant was evaporated in a Büchi rotary evaporator (Model R-210) coupled chiller Büchi (Model F108) at 45°C until evaporation to about 90% of the supernatant and resuspended in deionized water (Milipori Bedford, Q3UV model CT, USA) to a final volume of 50 mL. In the residue obtained from the centrifugation of sample we added 20 mL of 2N NaOH solution and homogenized in a shaker at room temperature for 1 hour (200 rpm) to hydrolysis of the binding of the conjugated compounds. The medium was acidified with concentrated HCl to pH 2 and washed with 25 mL hexane to fat removal, centrifuged and the supernatant collected for analysis (extract of fat-soluble phenolic – FSPE). The residue obtained in the second centrifugation was washed six times with ethyl acetate, centrifuged, evaporated on rotary evaporator at 45°C until complete solvent evaporation and resuspended in deionized water to a final volume of 10 mL, obtaining conjugated phenolics (CPE).

PHENOLIC COMPOSITION

The concentration of phenolic compounds in the extracts was determined in NOVOSTAR fluorometer (model S / N 700-0120, Switzerland) at 760 nm using Folin-Ciocalteau solution, according to the methodology described by Roesler *et al.* (2007) with modifications. For colorimetric reaction, an aliquot of 30 µL of methanol extract solution was homogenized with 120 µL Folin Ciocalteau solution and 130 µL of 5% sodium carbonate solution, and incubated at 50°C for 5 minutes in a water bath New Brunswick Scientific (Model C76, Edison, NJ, USA) for color development. After cooled in ice bath we performed reading, being the white composed by 50% hydromethanol solution (v v^{-1}). We determined the quantitation of phenolic compounds through a calibration curve constructed using as standard gallic acid (Sigma Aldrich, USA) 100 µg mL^{-1} in the concentrations from 10 to 70 µg mL^{-1} in methanol and the results were expressed in mg of gallic acid equivalents (GAE) per g of by-product.

IN VITRO ANTIOXIDANTS ASSAYS

DPPH (2,2-DIPHENYL-1-PICRYLHYDRAZYL)

The DPPH radical scavenging activity was performed according to the methodology described by Roesler *et al.* (2007) with modifications. The stock solution of 0.004% DPPH prepared in methanol had absorbance measured on fluorometer between 0.8 and 1.2 at 517 nm. The standard curve was prepared with the water soluble analogue of vitamin E, Trolox (Sigma Aldrich, USA) in 1500 µmol in methanol in the concentrations from 10 to 250 µmol. Fifty microliters of homogenate extract in methanol, which were submitted for 30 minutes to an ultrasound Ultrasonic Cleaner (model M/UNIQUE, Brazil), was diluted in serial sequence and reacted with 250 µL of 0.004% DPPH for 30 minutes. Then, we performed the reading of absorbance at 517 nm. The results were express in IC_{50} values, by constructing linear curve between the antioxidant capacity of the extract and its corresponding concentration for each extract concentration.

ORAC (OXYGEN RADICAL ABSORBANCE CAPACITY)

We performed the assay based on the methodology presented by Dávalos, Gómez-Cordovéz and Bartolomé (2004), with modifications. Readings were performed on fluorometer and we analyzed the hydrophilic and lipophilic phenolic compounds. The standard curve was prepared with 1500 µmol Trolox, dissolved in 75 mM potassium phosphate buffer, pH 7.4 (hydrophilic) and randomly methylated β-cyclodextrin solution 7% (RMCD) (Sigma Aldrich, USA) (lipophilic) at concentrations of 50 to 800 µmol. The reaction consisted of 20 µL extract, 120 µL of fluorescein solution, 60 µL of AAPH (2.2'-azobis(2-amidino-propane) dihydrochloride) diluted in potassium phosphate buffer, resulting in a final total volume of 200 µL. For white the extract was replaced by phosphate buffer. The reading was performed in conditions of fluorescein at 485 nmEX/520 nmEM, every 60 seconds in a cycle equal to 80 minutes at 37°C. The determination of the decay curve from fluorescence of each reaction was calculated by the formula of area under the fluorescence curve (Equation 1), where f_0 represents the fluorescence obtained at time 0 and f_i the fluorescence in the intermediate times between 0 and 80 minutes. The results were expressed through decay curve of fluorescence intensity of fluorescein (AUC) versus time in minutes.

$$AUC = 1 + f_i/f_0 \ldots + f_i/f_0 \ldots + f_{80}/f_0 \quad \text{(Equation 1)}$$

STATISTICAL ANALYSIS

Statistical analysis was performed with three replicates by analysis, being the results expressed in mean ± standard deviation. Statistically significant differences between the extracts were made by the STATISTICA software, version 7.0

(STATSOFT, 2004), and analysis of variance (one-way ANOVA) followed Tukey test, at 5% (n = 3, p <0.05).

RESULTS AND DISCUSSION

PHENOLIC COMPOSITION

Most of PC total presented high affinity with solvent 80% acetone (FPE) used in the sequential extraction method (SE) (Figure 1). Acetone is considered a good solvent for dissolving lipophilic compounds with relatively polar range (WU *et al*., 2004) - miscible in water with relative polarity equal to 0.355 (MUROV, 2010), being responsible for extracting the free aglycones and glycosylated forms from sample, characterizing the free phenolic compounds (SUN *et al.*, 2002; DEWANTO; WU; LIU, 2002).

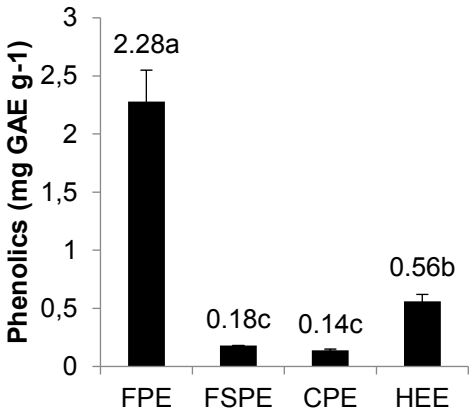

Figure 1 - Contents of phenolic compounds in by-product extracts from Paluma guava processing obtained through sequential and single extraction, expressed in fresh base. NOTE: * FPE - extract of free phenolic; FSPE - extract of fat-soluble phenolic; CPE - extract of conjugated phenolic; HEE - hydroethanolic extract.

The application of hydroethanol solution in PC extraction is one of the most used procedures in fruit and other plant foods (ALOTHMAN; BHAT; KARIM, 2009; SOUSA; VIEIRA; LIMA, 2011; CHIARI *et al*., 2012; MARTÍNEZ *et al.*, 2012; ARAÚJO *et al*., 2014) due to its ease of handling, low toxicity and efficiency in extraction. However, in this study it had less extraction efficiency than the acetone used in the SE method.

When determining the best solvent for the PC extraction in *Limonium delicatulum* plant, Medini *et al.* (2014) also observed greater efficiency in 80% acetone, then 80% methanol, 90% ethanol, water and finally hexane to PC. Soares *et al.* (2008) analyzed the effect of methanol, ethanol and acetone solvents in different concentrations on apple bagasse and noted that extraction with acetone at concentrations of 75% and 100% (v v^{-1}) presented higher content of phenolics (4.67 mg GAE g^{-1} and 5.23 ± 0.04 mg GAE g^{-1}, expressed on a dry basis) than other solvents, which was in accordance with the results obtained in this study.

The reduction of content of total PC in extracts obtained in SE (FPE, FSPE and CPE) can be explained by the solubility of the compounds according to affinity with the solvent applied, being the polarity in descending order of solvent applied in the SE - acetone (0.355) > ethyl acetate (0.228) > hexane (0.009) (MUROV, 2010).

PC can be present in food in free or bound form, as determined by SE. There is no much search on bonded phenolic compounds or conjugated of the determinations of phenolic compounds contents, in general, the determination of total phenolic compounds is most commonly used.

Conjugated phenolics are compounds of low molecular weight, soluble in water, present in the cytosol or fat-soluble forms, associated to waxes of plant surface, and can be found as esters and amides, rarely occurring as glycosides (MIRA *et al.*, 2008).

The conjugated PC can be liberated from the compounds which are linked as lignin, pectin and structural proteins, for example (ACOSTA-ESTRADA; GUTIÉRREZ-URIBE; SERNA-SALDÍVAR, 2014), by alkaline processes, acidic or enzymatic treatments from samples (LIYANA-PATHIRANA; SHAHIDI, 2006). In the SE of the guava by-product we used NaOH solution in order to promote hydrolysis of the phenolic-binding compounds link and, thus, releasing the compounds conjugated to measure the antioxidant capacity as suggested by Sun *et al.* (2002) and Dewanto, Wu and Liu (2002).

In humans the PC conjugated, for resisting the actions of the human stomach acidity and digestion in the small intestine, reach the colon intact, and its bioactivity is best used to health (SUN *et al.*, 2002). Some studies showed that conjugated PC of insoluble character has shown antioxidant capacity significantly higher when compared with free and conjugated (ACOSTA-ESTRADA; GUTIÉRREZ-URIBE; SERNA-SALDÍVAR, 2014).

Thus, the determination of both free PC as conjugated allows a more complete estimation of phenolic composition which, by chromatographic methods, could be characterized and conducted a study of the importance of each.

Among the free PC there are some phenolic acids that can be extracted as aqueous-organic solutions such as methanol, ethanol or acetone (MIRA *et al.*, 2008), and they may be conjugated in the sample as indicated by Soares *et al.* (2008), in which, in apple gala bagasse, the presence of salicylic acid, gallic, synaptic and p-coumaric could be verified, both in the free phenolic acids fraction as in the conjugated fractions; ferulic acid, cinnamic, vanillic, caffeic and ellagic are considered in this last fraction.

The by-product of the industrial processing from guava presented higher representativeness of PC of free character (FPE) than conjugated (CPE) (Figure 1). In fruits the free soluble form has greater representativeness, result obtained by a survey conducted by Sun *et al.* (2002) that determined the free and conjugated PC content in apple, banana, red grape, orange, lime, strawberry, peach, and pear. However, Dewanto, Wu and Liu (2002) have noted a higher content of conjugated compounds in sweet corn, which demonstrates that each raw material is characterized by different proportions of phenolic compounds.

IN VITRO ANTIOXIDANTS ASSAYS

DPPH ASSAY

The results of the DPPH assay can be expressed in three ways - IC_{50}, µmol Trolox per gram of sample and percentage of inhibition; IC_{50} is much expressed in scientific research. IC_{50} represents the minimum concentration necessary of a sample/extract with bioactive compounds of interest to lead to a 50% reduction of $DPPH^{\bullet}$ radicals present in the matrix analyzed (LIM; LIM; TEE, 2007).

Analyzing Table 1, it appears that FPE extract showed the lowest value concentration required to reduce by 50% the action of $DPPH^{\bullet}$ (0.90 mg mL^{-1}), in other words, compared to other extracts it presented better potential in sequester the free radical DPPH, consistently with the levels of phenolic compounds shown in Figure 1.

Table 1. IC_{50} value (mg mL^{-1}) of the extracts from the by-product of industrial guava processing.

FPE	FSPE	CPE	HEE
0.90[1]	193.29	38.17	7.44

NOTE: [1] Average of three repetitions* FPE - extract of free phenolics; FSPE - extract of fat-soluble phenolics; CPE - extract of conjugated phenolics; HEE - hydroethanolic extract.

In by-product of the industrial guava processing some studies point IC_{50} values lower than those obtained in extracts – 0.142 mg mL^{-1} (fresh base) (SOUSA; VIEIRA; LIMA, 2011) and 0.169 mg mL^{-1} (dry base) (ARAÚJO *et al.*, 2014), however, the extracts showed good antioxidant potential.

IC_{50} referring to CPE and FSPE extracts were higher than the presented in the literature, because they are fractions obtained by SE. Due the exhaustive extraction process, the tendency to extract PC with affinity by the solvent is lowered, thus, it is expected higher levels of the extracts for 50% reduction of $DPPH^{\bullet}$ radicals. Due the array effect of the FSPE extract, in other words, the presence of possible interfering substances extracted with the hexane solvent, such as carotenoids and lipids or even the turbidity characteristic of the extract, this presented higher IC_{50} value.

The differences of levels are due to extrinsic factors as the type of solvent extractor, proportion solvent and sample, extraction time, extraction mechanism, number of extraction stages and intrinsic factors such as variety of the sample and type of crop and fruit maturation stage for obtaining the guava by-product, making it difficult to accurately compare the data obtained with those presented in scientific research.

Taking into consideration that the extracts which had higher antioxidant capacity were free phenolics extract (FPE) and hydroethanolic extract (HEE), we chose these extracts to compare the antioxidant activity obtained from extracts among the samples, and the results present in antioxidant activity – % inhibition.

The concentration we used for comparison between samples of the same extract was 3.33 mg mL^{-1} (Figure 2).

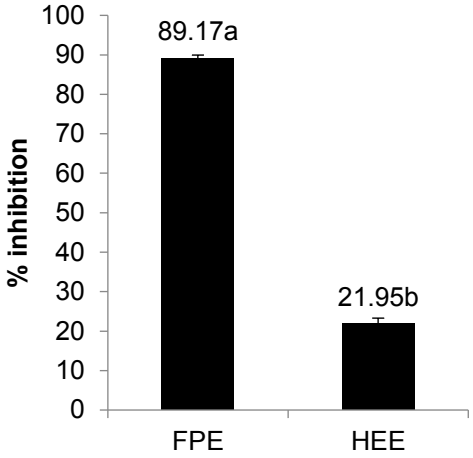

Figure 2 - Antioxidant activity (%) of the extracts from the by-product of industrial processing of Paluma guava in concentration 3.33 mg mL^{-1}, in fresh base, by the DPPH free radical sequestration method (mean ± standard deviation, minimum n = 3).
NOTE: * FPE - free phenolics extract obtained by method of sequential extraction; HEE - hydroethanolic extract; Means followed by different letters differ significantly ($p \leq 0.05$) by Tukey test.

Melo *et al.* (2008) classified the strength of activity of fruit extracts according to the percentage of inhibition. Activities above 70% were considered effective in sequestering DPPH$^{\bullet}$ free radical, between 50 and 70%, moderate action and below 50%, weak action. As in Figure 2, FPE extract presented effective antioxidant activity before the stabilization of DPPH$^{\bullet}$ radical, however the HEE extract had weak action. Ongphimai *et al.* (2013) analyzed the antioxidant capacity of guava extract by free radical DPPH$^{\bullet}$ and also found low percentages of inhibition being 22% to insoluble PC and 20% to soluble PC. The authors suggested that possibly the determined low activity is due to the slow rate of reaction between DPPH molecules and the phenolic of food matrix extract.

ORAC ASSAY

The ORAC assay compared to DPPH and ABTS methods presents the advantage of using the peroxyl radical, which is associated with free lipid oxidation, being, thus, closely involved in biological functions of chain scission (MAHATTANATAWE *et al.*, 2006).

The ORAC method allows the determination of antioxidant capacity of both hydrophilic and lipophilic compounds, so as ABTS assay for also being soluble in aqueous and organic solvents (APAK *et al.*, 2007), thus two decay curves (AUC) of loss fluorescence were obtained according to the reaction time between the phenolic compounds present in the extracts and peroxyl radical from reaction (Figure 3).

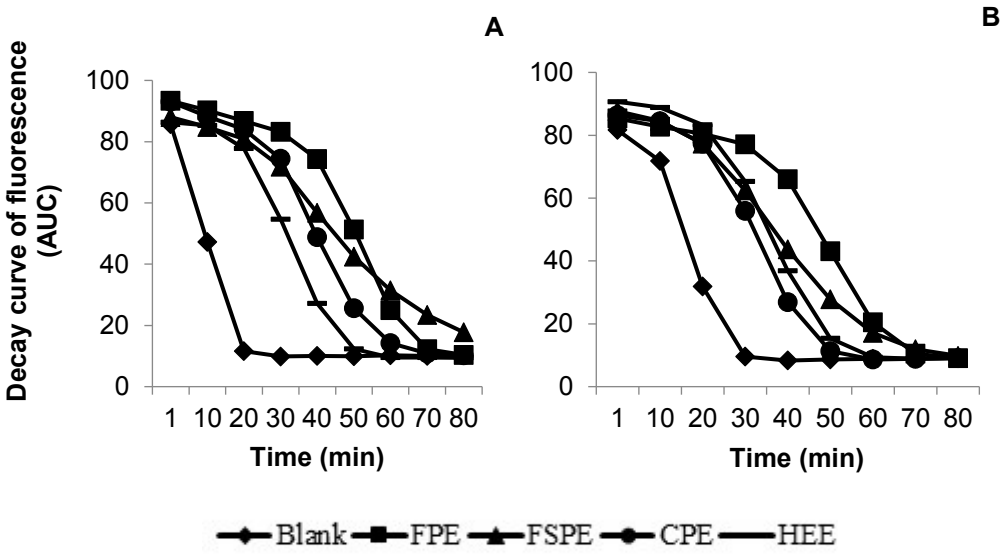

Figure 3 - Intensity decay area of extracts relative to the blank in the ORAC assay for hydrophilic and lipophilic phenolic compounds.
NOTE: * A - ORAC hydrophilic; B - ORAC lipophilic; FPE - free phenolic extract; FSPE - fat-soluble phenolic extract; CPE - conjugated phenolic extract; HEE - hydroethanolic extract.

As the loss of intensity of fluorescein is not linear with time but exponential, an area under the decrease curve (AUC) is used. The highest delay time of fluorescence intensity fall in the assay indicates higher efficiency of antioxidant compounds present in the extract in transferring hydrogen atoms to the peroxyl radical and thus inhibiting the speed of loss reaction of the fluorescein intensity. In Figure 3, we verified better antioxidant activity in FPE extract and, therefore, after 40 minutes greater decreases in fluorescence occurred. Both hydrophilic assay as lipophilic, unlike FSPE and CPE extracts (30 minutes) and HEE (20 minutes) in the hydrophilic assay and all extracts of 20 minutes in the lipophilic assay, showed better efficiency in the extracts from SE.

INFLUENCE OF THE TYPE OF EXTRACTION OBTAINING ANTIOXIDANT COMPOUNDS

The biological properties of PC as well as functional effects to health intensified research efforts of more effective methods of extraction, separation and identification from natural sources for applying these compounds in food; extraction is a stage of extreme importance in the study of compounds which can determine the results obtained in others stages (quantification, antioxidant activity) subsequent to this.

In the extraction process several factors can influence the content of obtained compounds such as temperature, liquid-solid ratio, flow rate of the particle size, and kind of the compound (IGNAT; VOLF; POPA, 2011).

Due to PC diversity and their different interactions with solvents there is no single method and appropriate universal standard for extracting PC from fruits and by-products.

The PC solubility in a solvent is a characteristic of the bioactive compound bound to its molecular weight (PELLEGRINI *et al*., 2007), the polarity of the phenolic, beyond of the polymerization degree, interaction with other constituents (APAK *et al.*, 2007) and the type of functional groups as –OH and –COOH, and is due to the diversity of these compounds in food with different properties that the methods to evaluate the antioxidant activity in food are strongly influenced by the solvents used during extraction (PELLEGRINI *et al*., 2007).

In order to extract some classes of phenolic compounds as phenolic acids, hydroxycinnamic acids, flavonoids and carotenoids, the application of order decreasing polarity of the solvent is required, and a combination of different polarities can be applied for specific purposes (APAK *et al*., 2007).

The most widely used solvents for PC extraction in food by descending polarity order are water, 80% methanol or 70% ethanol, 80% acetone and ethyl acetate (APAK *et al*., 2007). The concentration and its efficacy depend of factors as the polarity of the phytochemicals present in the sample, the degree of polymerization and the interaction with other constituents (CAETANO *et al.*, 2009).

For PC extraction two types can be performed - single extraction (SiE) and sequential extraction (SE). In SiE procedures, methanol is the most efficient solvent for PC extraction, however, ethanol may be safer than methanol to human health, so it is more preferable in the food industry (IGNAT; VOLF; POPA, 2011), and therefore, ethanol application was the single extraction we performed in this work.

However, taking into account that plants presented varied PC classes with chemical structures and different polarities, the SE method, by using the same sample for extraction of phenol with solvents of different degrees of polarity, allows a better and more efficient extraction of different compounds exhaustively (CAETANO *et al.*, 2009) - free and conjugated compounds (DEWANTO; WU; LIU, 2002; SUN *et al.,* 2002), in other words, a better estimation of PC levels present in the food matrix, especially some of the compounds that are linked in compounds in plants, being often their quantification excluded of single extraction analysis, resulting in underestimated phenolic content (BALASUNDRAM; SUNDRAM; SAMMAN, 2006).

Therefore, a proven fact in the results presented in this study, where the SE showed greater efficiency in the extraction of phenolic compounds (Figure 4) present in the by-product of the industrial guava processing, culminating in the highest antioxidant activity measured by ORAC assay, since it comprises wider range of PC, which shows the importance of extracting the maximum possible of bioactive compounds of different polarity for this type of extraction (MELO *et al.*, 2008).

The order of solvent efficiency in the PC extraction to SE in by-product from guava was 80% acetone > hexane > ethyl acetate, being the content obtained in the single extraction with 90% ethanol between hexane and 80% acetone.

Acetone efficiency, in the extraction of phenolic compounds was also found by Caetano *et al.* (2009) by evidencing that in agro-industrial by-product of Barbados cherry, 80% acetone extracted the greatest amount of these

compounds, when compared with 80% methanol, water and 80% ethanol. However, if the order of application of the solvent is changed, it is possible that the biggest efficiency of the acetone solution in the extraction may be different.

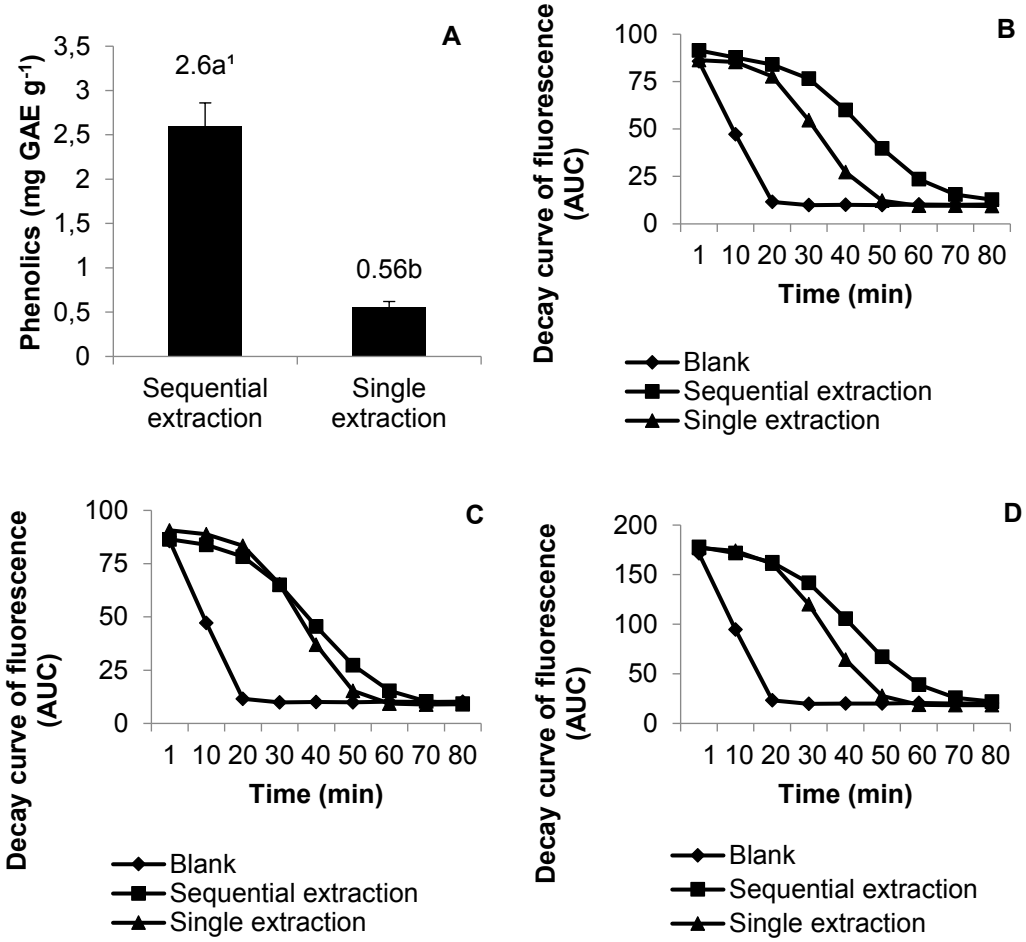

Figure 4 - (A) Content of phenolic compounds; fluorescence decay curve of the fluorescein determined by ORAC assay (B) hydrophilic, (C) lipophilic and (D) hydrophilic + lipophilic in by-product of industrial processing from Paluma guava in fresh base (mean ± standard deviation, minimum n = 3).
NOTE: * Sequential Extraction - sum of extract of free phenolics (FPE), fat-soluble phenolics (FSPE) and conjugated phenolics (CPE); Unique Extraction - Extraction with 90% ethanol ($v.v^{-1}$); ¹ Different letters between extractions for phenolic differ significantly ($p \leq 0.05$) by Tukey test.

Alothman, Bhat and Karim (2009), by quantifying PC content in guava pulp with different solvents (methanol, ethanol, acetone and water) at different concentrations (50, 70 and 90%) reported that 90% acetone could extract with increased efficiency the PC presents in the sample (1.91 mg GAE g^{-1}), followed by 90% ethanol (1.85 mg GAE g^{-1}), 70% methanol (1.55 mg GAE g^{-1}) and water (1.53 mg GAE g^{-1}), consistently with the results obtained in this study. The acetone solvent we used in the study had concentration 80% which may explain the difference of the PC content by comparing the result of the authors, in addition of

factors as extraction mechanisms, extraction time, sample relationship and solvent, feedstock cultivar, among others.

However, there are other families of bioactive compounds present in the extracts which influence the elimination of free radicals such as carotenoids, tocopherols (vitamin E), vitamin C, chlorophyll, and other (PELLEGRINI *et al*., 2007), which would have to be measured by influencing the antioxidant activity.

CONCLUSION

The sequential extraction of phenolic compounds present in the by-product obtained from processing Paluma guava were more efficient in the recovery of antioxidants compounds, since the application of solvents of different polarities in a sample allows different interaction of various phenolic present in the matrix, allowing even the quantification of conjugated compounds which, by single extraction, cannot be determined, thus allowing better utilization of antioxidant benefits of the by-product.

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