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PII: S0023-6438(20)31086-0

DOI: https://doi.org/10.1016/j.lwt.2020.110097

Reference: YFSTL 110097

To appear in: *LWT - Food Science and Technology*

Received Date: 25 May 2020

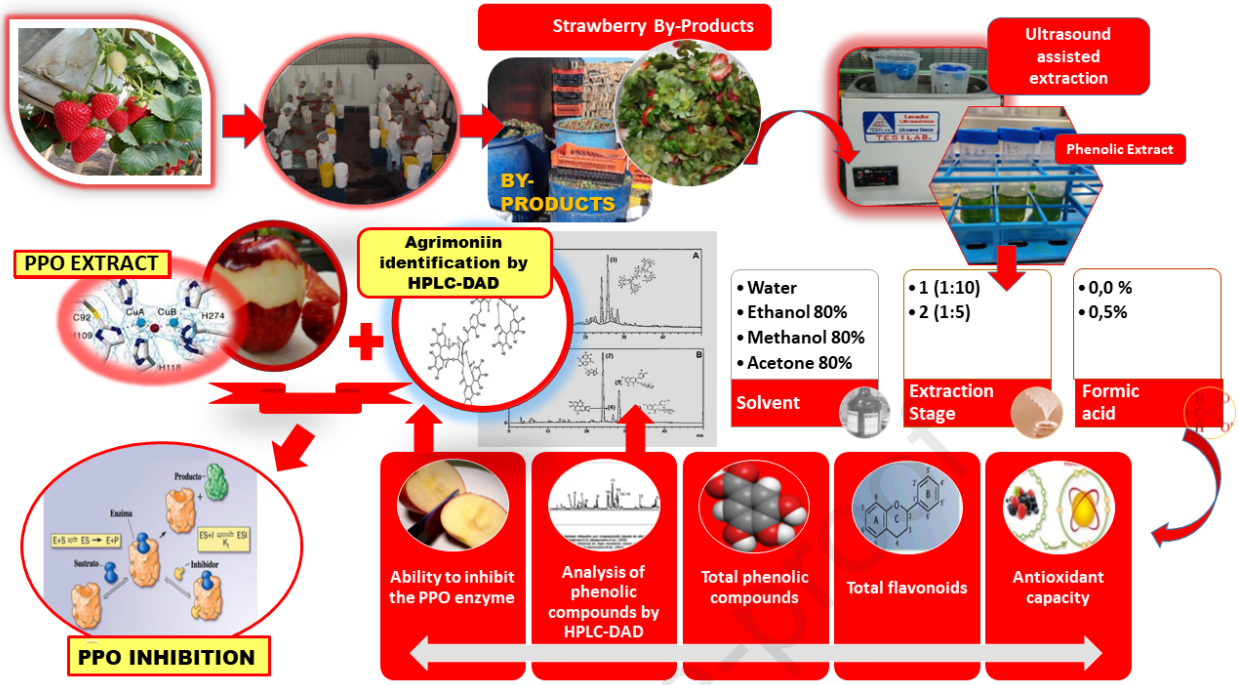
Revised Date: 24 July 2020

Accepted Date: 18 August 2020

Please cite this article as: Villamil-Galindo, E., Van de Velde, F., Piagentini, A.M., Extracts from strawberry by-products rich in phenolic compounds reduce the activity of apple polyphenol oxidase, *LWT - Food Science and Technology* (2020), doi: https://doi.org/10.1016/j.lwt.2020.110097.

CRediT authorship contribution statement

Esteban Villamil-Galindo: Conceptualization, Data curation, Investigation, Writing - original draft, Formal analysis. **Franco Van de Velde:** Methodology, Formal analysis, Supervision, Writing - Review & Editing. **Andrea M. Piagentini:** Methodology, Writing - Reviewing and Editing, Supervision, Project administration, Funding acquisition



Extracts from strawberry by-products rich in phenolic compounds reduce the activity of apple polyphenol oxidase

Esteban Villamil-Galindo[a,b], Franco Van de Velde[a,b], Andrea M. Piagentini[a]

[a]Instituto de Tecnología de Alimentos - Facultad de Ingeniería Química – Universidad Nacional del Litoral. Santiago del Estero 2829, 3000, Santa Fe, Argentina.

[b]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

*corresponding author: ampiagen@fiq.unl.edu.ar

Abstract

The ultrasound-assisted extraction of phenolic compounds from strawberry by-products was studied varying the solvent type (water, ethanol 80%, methanol 80%, and acetone 80%), the formic acid concentration (0 and 0.5%), and the number of extraction steps (1 and 2). Total phenolic and total flavonoid compounds were determined spectrophotometrically and analyzed by PAD-HPLC. The antioxidant capacity (DPPH and FRAP assays) and the ability of extracts to inhibit apple (*Malus domestica* cv. 'Red Delicious') polyphenol oxidase (PPO) were also investigated. Extracts with acidified methanol in two-steps yielded the highest phenolic compound concentration (15.01 g/kg), and the highest antioxidant capacity. Agrimoniin was the major polyphenol found, and the extraction with acetone in two-steps produced the highest yield (2.45 g/kg). This ellagitannin was the only polyphenol that correlated (R^2>0.80, p<0.05) with the antioxidant capacity. Water and ethanol showed the lowest phenolic compound yields.

Abbreviations

S, type of solvent; C_{FA}, formic acid concentration; ES, number of extraction steps; PPO, polyphenol oxidase enzyme; W, water; EtOH, Ethanol 80%; MeOH, methanol 80%; Ac, acetone 80%; TPC, total phenolic content; GAE, gallic acid equivalents; TF, total flavonoids; QE, quercetin equivalent; DPPH, antioxidant capacity by DPPH; FRAP, ferric reducing antioxidant power; I%, inhibition percentage; *vo*, PPO rate without the inhibitor (abs/min); *vo'*, PPO rate affected by the inhibitor (abs/min); Km, Michaelis-Menten constant for the PPO; Vmax, maximum rate of PPO; Km', Michaelis-Menten constant for the PPO with inhibitor; Vmax', maximum rate of PPO with inhibitor; Ki; inhibition constant; AA, ascorbic acid.

However, extraction with these green solvents (water or ethanol) in two-steps showed polyphenol contents similar to those obtained with methanol or acetone in one-step (≈9 g/kg). Additionally, extracted polyphenols (0.24 g/L) produced 30% apple PPO inhibition, in a reversible 'uncompetitive' inhibition. Results showed the high revalorization potential of strawberry by-products as a low-cost source of polyphenols, with antioxidant and anti-browning effects.

Keywords: Polyphenols; by-product revalorization; ultrasound-assisted extraction; antioxidant capacity; polyphenol oxidase inhibition.

1. Introduction

Strawberry (*Fragaria x ananassa* Duch.) is a widely grown hybrid species of the genus *Fragaria* that includes more than 20 species and many cultivars (Gunduz & Özdemir, 2014). This fruit has a great demand in the world, not only for its taste but also for the benefits it brings to health. Strawberry is a recognized source of nutrients and phytochemicals such as vitamin C and phenolic compounds, respectively, with antioxidant, anti-inflammatory, and other health-related properties (Giampieri et al., 2015). Argentina is the second largest strawberry producer in MERCOSUR (Southern Common Market), after Brazil. About 60% of strawberry from Argentina is consumed fresh, while the remaining production is intended for processing mainly as frozen fruit (Van de Velde, Vaccari, Piagentini, & Pirovani, 2016a). Consequently, by-products such as leaves, calyxes, stems, and rest of vegetable tissue are generated, representing a high percentage of the processed fruit.

Sánchez, Murillo, & Méndez (2010) reported that strawberry by-products are rich in phenolic compounds with high antioxidant activity. Hence, the use of these by-products would generate industrial opportunities, obtaining raw material at low cost with an interesting added value, which in turn, may reduce the cost of its final disposition. In this regard, the leaves of *Fragaria chiloensis* have been used as medicine by the

Mapuche aborigines in Chile and Argentina (Simirgiotis & Schmeda-Hirschmann, 2010). Additionally, Katalinic, Milos, Kulisic, & Jukic (2006) reported that the leaves of raspberries, blackberries, and strawberries had the highest antioxidant and the highest phenolic content among 70 medicinal plants. As a consequence, there would be a growing interest in determining the relationship between the composition of phenolic compounds of strawberry by-products (sepal, peduncle, stems, etc.) and their beneficial properties for health, considering these by-products as an alternative source of bioactive compounds (Ferlemi & Lamari, 2016).

Solid-liquid extraction is the most reported method for obtaining phenolic compounds from plant materials, and it is mainly affected by solvent, acidity, temperature, and time, among others (Ignat, Volf, & Popa, 2011).

The use of different organic solvents such as ethanol, methanol, acetone, and/or their water solutions, showed significant differences in the polyphenol extraction efficiency, depending on the solvent system employed. According to Mokrani & Madani (2016), a single dissolvent cannot extract all classes of phenolic compounds simultaneously and at a maximum concentration. Therefore, innovative extraction technologies such as high hydrostatic pressure, high-frequency ultrasound standing waves (400 – 600 kHz), supercritical fluid extraction, among others, have been widely explored for isolation of bioactive compounds from plant material (Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016; Pinela et al., 2018; Zhang, Poojary, Choudhary, Rai, & Tiwari, 2018). However, solid-liquid extraction using an appropriate combination of solvents becomes one of the most affordable options for a low-cost raw material like the strawberry by-products.

In this regard, the solvent polarities and the synergistic interaction between them govern the solid-liquid extraction process. Water, ethanol and methanol are polar protic solvents and mainly extract low molecular weight compounds, such as phenolic glucoside and non-glucoside compounds. Acetone is a polar aprotic solvent and is considered an intermediate solvent. It can solvate low and high molecular weight

compounds with protonatable functional groups, like tannins, proanthocyanidins, and flavonols (Martínez-Ramos et al., 2020). Therefore, the right selection of the solvent can greatly improve the efficient extraction of antioxidants from strawberry by-products becoming a promising opportunity to obtain bioactive compounds with a high added value. Moreover, depending on the toxicity of the solvent employed, this by-product revalorization may reduce, at the same time, the environmental impact (Joshi & Adhikari, 2019).

In addition, phenolic compound recovery from plant materials can be enhanced by ultrasound-assisted solvent extraction (≈ 40 kHz), a green efficient way to increase mass transfer (Safdar et al., 2017).

On the other hand, enzymatic browning is recognized as one of the main problems of fruit and vegetable processing and is mainly due to the activity of the enzyme polyphenol oxidase (PPO) which catalyzes the oxidation of phenolic compounds into dark pigments (Singh et al., 2018). To attend this problem, there has been an increasing demand for finding natural anti-browning agents. Several natural extracts and various families of compounds whose origins are natural products are potential effective anti-browning agents. It is known that phenolic compounds can interact with proteins, leading to inhibition of enzymes. Therefore, some phenolic compounds can prevent their oxidation by inhibition of the PPO enzyme (Bobo-García, Arroqui, Merino, & Vírseda, 2019). In that sense, extracts of rice brans and *yerba mate* (Ilex paraguariensis St. Hil. Aquifoliaceae), both rich in phenolic compounds, successfully delayed the enzymatic browning of apple, producing an inhibitory effect on PPO (Rodríguez-Arzuaga & Piagentini, 2018; Sukhonthara, Kaewka, & Theerakulkait, 2016). Therefore, phenolic compound extract from strawberry by-products could represent a promising alternative to inhibit PPO in vegetable products, but this effect has not been previously reported and deserves to be explored.

Consequently, the main objective of this work was to study the effect of different ultrasound-assisted extraction systems based on water and mixtures of organic solvents and water on the content and profile of phenolic compounds from strawberry by-products. Moreover, the antioxidant capacity and the ability of extracts to inhibit the PPO enzyme obtained from apple flesh were also investigated.

2. Materials and methods

2.1. Plant material

The by-products of strawberry (*Fragaria x ananassa* Duch.) cv. 'Festival' were obtained from one field at Coronda (31°58′00″S 60°55′00″W). Samples, with a moisture content of 89.2 ± 0.07 %, were packed in polyethylene bags, and frozen at -20 °C until the processing day. Before each extraction assay, 100 g of frozen strawberry by-product was ground in a mortar using CO_2 (particle size ≤1 mm).

2.2. Extraction procedure

2.2.1. Experimental design

Strawberry by-product extractions were carried out through solid-liquid extractions, following a factorial design of three variables: type of solvent (4 levels), formic acid concentration (2 levels), and the number of extraction steps (2 levels) maintaining the same final solid/solvent ratio (Table 1). All extractions (16 experimental runs) were performed in triplicate at 20 °C. The responses evaluated in each extract were the contents of total phenolic and total flavonoid compounds, individual phenolic compounds, and the antioxidant capacity.

2.2.2. Preparation of extracts

To carry out one-step extraction, the mixture of ground strawberry by-products plus the solvent (1:10 w/v) was sonicated (40 kHz and 160 W, Ultrasonic Cleaner, Testlab, Buenos Aires, Argentina) at 20 °C in continues mode for 15 min, and then centrifuged at 12000 *g* for 20 min at 20 °C (Neofuge 18R Heal Force centrifuge, Shanghai, China). The resulting supernatant was separated and reserved for analysis.

The two-step extraction consisted of sonicating for 15 min at 20 °C, a mixture of the ground sample plus the solvent (1:5 w/v) (first step), and then centrifuged at 12000 *g* for 20 min at 20 °C. The supernatant was collected in a volumetric flask, and the pellet resulting was re-extracted with another aliquot of solvent (1:5 w/v) (second step). Then, the mixture was sonicated, centrifuged, and the supernatant was separated. Both supernatants (first and second steps) were pooled and reserved for analysis (final solid/solvent ratio in two-step extraction: 1:10 w/v).

2.3. Total phenolic content (TPC)

Total phenolic content was determined by the Folin-Ciocalteu method according to Rodriguez-Arzuaga et al. (2016). TPC analysis was performed by triplicate in each sample and the results were expressed as g of gallic acid equivalents (GAE) per kilogram of strawberry by-product (g GAE/kg).

2.4. Total flavonoid content (TF)

The determination was made in triplicate according to Dowd (1959), using aluminum chloride solution as the specific reagent for flavonoid determination. Results were expressed as g of quercetin equivalent (QE) per kilogram of strawberry by-product (g QE/kg).

2.5. Determination of phenolic compound by HPLC

An LC-20AT Prominence Liquid Chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a photodiode array detector (PAD), and a reversed-phase Phenomenex Gemini column, 25 mm × 4.6 mm, with 5 µm particle size (Phenomenex, Torrance, CA, USA) was used for phenolic compound determination**.** The analysis was conducted as described by Simirgiotis & Schmeda-Hirschmann (2010). Retention times and UV-PAD spectra of pure standard compounds and previously identified peaks obtained through mass spectrometry (Van de Velde, Grace, Pirovani, & Lila, 2016b) were used to identify the phenolic compounds in the extracts. Identified phenolic compounds were quantified through the external standard method using calibration curves of ellagic acid

(0.03-0.50 mg/mL, R^2 = 0.9916), kaempferol-3-*O*-glucoside (0.06-0.50 mg/mL, R^2 = 0.9972) and quercetin-3-*O*-glucoside (0.06-1.00 mg/mL, R^2 = 0.9979). Results were expressed in g per kg of strawberry by-product (g/kg).

2.6. Antioxidant capacity

The antioxidant capacity was determined by analyzing the ability of samples to scavenge the free-radical DPPH according to Teow et al. (2007). The determinations were made in triplicate and results were expressed as mmol Trolox/Kg (DPPH).

Moreover, the antioxidant capacity was determined through the ferric reducing antioxidant power (FRAP) assay, according to Rodríguez-Arzuaga & Piagentini (2018). Results were expressed as mmol Fe^{2+}/kg (FRAP).

2.7. Apple PPO inhibition assay

2.7.1. Extraction and enzymatic activity of PPO

Extracts were obtained according to Rodríguez-Arzuaga, Ríos, & Piagentini (2019), with some modifications. Briefly, 50 g of apple flesh (*Malus domestica* cv. 'Red Delicious') were homogenized with 100 mL potassium phosphate buffer (0.1 mol/L, pH = 7.0) containing Triton X100 (0.45 g/L) and polyvinylpolypyrrolidone (6.67 g/L) at 4°C. The homogenates were centrifuged (12000 *g*, 20 min, 4°C) and the supernatants were separated and used for enzyme activity determination and inhibition assays.

The PPO (EC 1.10.3.1) activity was determined by measuring the increase in absorbance (Genesys 10s UV-Vis, Thermo Scientific™, Waltham, MA, USA) of reaction at 405 nm. The reaction mixture consisted of 0.05 mL enzyme extract, 1.5 mL sodium acetate buffer (pH = 5.5), catechol solution (5-40 mmol/L), and water. Results were expressed as Δabs/min per kg of apple flesh.

2.7.2. Effect of inhibitors

The extract rich in polyphenols from strawberry by-product was evaluated for its effectiveness as inhibitor of apple PPO activity using catechol 10 mmol/L as the substrate. To calculate the inhibition (Eq. 1), 0.25 mL of the extract was added to the

reaction mixture (*section 2.7.1*). Additionally, ascorbic acid was employed as a reference inhibitor of the PPO (Table 5).

$$I\ (\%) = \left(1 - \frac{vo'}{vo}\right) * 100 \quad (1)$$

Where (*I*) is the inhibition (%), *vo'* is the PPO activity affected by the inhibitor (abs/min), and *vo* is the PPO activity without the inhibitor.

2.7.3. Inhibition kinetics

The PPO inhibition kinetic was determined by measuring the increase in absorbance of reaction at 405 nm for 3 min in the presence of inhibitor. The reaction mixture consisted of 0.05 mL enzyme extract, 1.5 mL sodium acetate buffer (pH = 5.5), catechol (5-40 mmol/L), 0.25 mL of inhibitor (strawberry by-product polyphenol extract or ascorbic acid) and water. The Michaelis-Menten constants for the PPO with (Km', Vmax') or without inhibitor (Km, Vmax) were calculated through the Lineweaver–Burk linearization (1/activity versus 1/substrate concentration) (Lineweaver & Burk, 1934). Inhibition constants (Ki), and type of inhibition were determined according to Dixon (1953).

2.8. Statistical analysis

All data were analyzed statistically using analysis of variance (ANOVA). Significant differences among means were determined by Tukey´s test at 5% level of significance. Pearson correlation was performed to determine correlations between the studied variables**.** Analyzes were performed with STATGRAPHICS Centurion XV (StatPoint Technologies Inc., Warrenton. VA, USA).

3. Results and discussion

3.1. Total phenolics, total flavonoids, and antioxidant capacity

Different ultrasound-assisted extraction systems were studied to determine the better options for obtaining phenolic compounds from the plant tissue produced as a discard of the conditioning of strawberries cv. 'Festival' intended for industrial processing.

The type of solvent (S), formic acid concentration (C_{FA}), and the number of extraction steps (ES) affected ($p \leq 0.001$) the contents of total phenolic compounds (TPC), total flavonoids (TF) and the antioxidant capacity (DPPH and FRAP) (Table 2). As can be seen, the interactions of $S.C_{FA}$ and S.ES resulted also highly significant for all the evaluated responses. Additionally, the interaction of C_{FA}.ES was only significant ($p \leq 0.001$) for TF and for the antioxidant capacity (DPPH); and $S.C_{FA}$.ES interaction was only significant for the contents of TPC and TF ($p \leq 0.001$), and for the antioxidant capacity (DPPH) ($p \leq 0.05$). As most of the interaction terms were statistically significant, the effect of each factor over response variables could not be analyzed separately from the other factors. However, considering that mean squares for the individual factors were greater than that for the interaction terms (Table 2), it could be suggested that the individual factors were dominant (Ellison, Barwick, & Farrant, 2009).

In general, the increase in C_{FA} from 0 to 0.5 % produced an increase in the TPC content and the antioxidant capacity of extracts for all the solvents studied. However, the content of TF of the extracts was lower for all the solvents as the concentration of acid increased. Moreover, the content of TPC and the antioxidant capacity of extracts were always higher in two- than in one-step extractions, for all the acidic (C_{FA} = 0.5%) solvents. Oppositely, TF content was always higher in two- than in one-step extractions, for all the solvents without acid (C_{FA} = 0).

Table 3 shows that the extractions in one-step (C_{FA} = 0%) with MeOH (8.98 g GAE/kg) or Ac (9.15 g GAE/kg) presented the highest TPC extraction yields among solvents, with no differences between them ($p \geq 0.05$). MeOH and Ac have low viscosities and therefore, high diffusivity, facilitating the extraction of bioactive compounds (Wijekoon, Bhat, & Karim, 2011). The extraction of the strawberry by-products in one-step with C_{FA} = 0.5% increased the TPC content in all extracts between 2 to 28%, compared to the same extracts without acid. For these samples, MeOH extract had the highest TPC yield (11.69 g/kg). Polyphenols are more stable at low pH, as the acidic condition helps

phenolic compounds to stay neutral, thus readily extracted into organic solvents (Rajbhar, Dawda, & Mukundan, 2015). Therefore, a decrease on solvent pH, for instance for MeOH, from 6.78 (C_{FA} = 0%) to 3.77 (C_{FA} = 0.5%) produced an increase in the phenolic compound recovery of around 25% (Table 3).

On the other hand, the extraction of the strawberry by-product with MeOH without acid in two-steps (pooled extract) showed the highest TPC content (12.8 g GAE/kg) among extracts obtained with the other solvents in the same conditions (Table 3). This extract showed TPC content 1.4 times higher than that obtained with the same solvent but in one-step (Table 3). Therefore, the two-step extraction in strawberry by-products rendered higher yields of phenolic compounds than the extraction in one-step with the same volume of solvent. Obtaining a higher extraction efficiency with the same solid/ solvent ratio is likely due to a sequential release of polyphenols from the matrix to the solvent, and the concentration gradient as the driving force that dominates this extraction phenomenon (Azmir et al., 2013). However, this phenomenon depends on plant material, for instance, the optimal phenolic compound content was obtained in a single extraction for apple, broccoli, and leek, but the two-step extraction showed the best results for orange (Michiels, Kevers, Pincemail, Olivier, & Dommes, 2012).

In two-step extractions, formic acid (C_{FA} = 0.5%) increased TPC in all samples, 10.0-16.2% higher than those obtained for the same samples without acid (Table 3). The extraction of the strawberry by-products with acidified methanol in two-steps yielded the highest phenolic compounds concentration (15.01 g/kg), among all extraction systems (Table 3). Similarly, Simirgiotis & Schmeda-Hirschmann (2010) reported a TPC concentration of around 20 g/kg in strawberry leaves extracted with methanol-formic acid (99:1, v/v) for 1h three times.

According to Kapasakalidis, Rastall, & Gordon (2006), the extraction of plant materials with acidified methanol is the most efficient method to extract phenolic compounds. Methanol polarity and the good solubility of phenolic compounds of different plant

tissues could be the reason for the high extraction yields observed. In fact, it was reported that in polyphenolic extractions from grape pulp, the extraction with methanol was 20% more effective than with ethanol, and 73% more effective than with water extraction (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). It is well known that solvent polarity plays a key role in increasing the solubility of phenolic compounds, which in turn allows their migration from the matrix into the solvent system, increasing the extraction yield (Martínez-Ramos et al., 2020). Although water is the most polar solvent of all, it did not extract the higher content of total phenolic compounds. This phenomenon could be attributed to the higher viscosity of water than that of the other solvents, which is a matter of mass transfer (Şahin & Şamli, 2013). However, water seems to be important for phenolic compound extraction, acting as the plant swelling agent, allowing the solvent to more completely penetrate the plant material, as reported by Fernández-Agulló et al., (2013), who obtained higher TPC content using water as solvent in blueberry leaves. Moreover, water solutions of solvents such as methanol, ethanol, and acetone, as employed in this work (Table 1), increased the solvent polarities due to the high dielectric constant of water, likely yielding higher content of phenolic compound than that obtained with the pure solvents (Şahin and Şamli, 2013).

Considering that water and ethanol are recognized as Generally Recognized as Safe solvents (Fernández-Agulló et al., 2013), the extractions of strawberry by-product with W or EtOH in two-steps could enhance the polyphenol extraction efficiency, reaching phenolic compound contents in the order of those obtained with MeOH or Ac in one step (Table 3).

Regarding flavonoids, their extraction from strawberry by-products with Ac in one-step without acid yielded the highest content (1.12 g QE/kg) compared with the rest of the solvents in the same conditions (Table 3). According to Fan, Xu, Shen, & Zhang (2015), this can be attributed to the fact that acetone has strong hydrogen bond

acceptance (HBA) ability and can form hydrogen bonds easily with the hydroxyl groups of flavonoids such as quercetin and kaempferol identified in this work (Section 3.2). However, flavonoid solubility could be lower in methanol, ethanol and/or water because they can also form strong hydrogen bonds between solvent molecules. In these solvents not only the hydrogen bond donation (HBD) but also the hydrogen bond acceptance (HBA) ability are strong (Marcus, 1993).

Two-step extractions increased the TF in the extracts obtained with all solvents as expected, being Ac with C_{FA} = 0% the best extraction system (TF = 1.90 g QE/kg).

The addition of formic acid to the solvents in one- or - two-step extractions did not increase the yields of flavonoids, as obtained for total phenolic compounds (Table 3). Although polyphenols are supposed to be more stable at low pH, maintaining structures neutral which are easily extracted into organic solvents (Rajbhar et al., 2015), flavonoid molecules could be unstable in acidic solvent solutions. Quercetin-based flavonols from grape skin, as found in strawberry by-products (Section 3.2), were unstable in mildly acidic (formic, acetic, citric, and maleic acids) methanol and highly labile in 1% hydrochloric acid in methanol. The 50% mixture of methanol and water (v/v) was the most efficacious solvent, extracting from grape skin 30 to 50% more quercetin-3-*O*-glucoside than any of the other solvents (Downey, Mazza, & Krstic, 2007). Differently, Putnik, Bursać Kovačević, & Verica (2015) reported that the addition of hydrochloric acid (0.5-1%) improved the extraction of flavonoids from a grape by-product using ethanol 50% as solvent.

Regarding the antioxidant capacities of strawberry by-product extracts obtained with MeOH and Ac, with or without formic acid, in one- or two-steps, yielded the best results, measured through DPPH and FRAP methods. The antioxidant capacity contents of the extracts obtained with MeOH with acid in two-steps were 124.49 mmol Trolox/kg (DPPH) and 100.79 mmol Fe^{2+}/kg (FRAP). These results were 13 and 21 times higher than the antioxidant capacity of the extract of strawberry fruit (80 %

methanol: 20 % water, 0.5 % acetic acid) determined by DPPH (9.96 mmol Trolox/kg) and FRAP (4.8 mmol Fe^{2+}/kg) methods, respectively (Van de Velde et al., 2019). Latter results confirm the high potential of strawberry by-products as a source of antioxidant compounds.

On the other hand, intending to encourage the use of green solvents, it is important to highlight that the extraction of strawberry by-product with W in two-steps with C_{FA} = 0.5% showed values of antioxidant capacity (DPPH: 90.76 mmolTrolox/kg, FRAP: 61.26 mmol Fe^{2+}/kg) similar to those obtained with acidified MeOH and Ac in one-step (Table 4). Finally, there was a high correlation between antioxidant capacity and TPC (R^2>0.81) for all extractions systems (Tables material supplementary 2, 3, 4, and 5).

3.2. Phenolic compound identification

Six phenolic compounds were identified and quantified as the main hydrolysable tannins, ellagic acid derivatives, and flavonoids from by-products of 'Festival' strawberry. Extracts contained two major hydrolysable tannins: tetragalloyglucose isomer (peak 1) and a dimer of galloyl-bis-HHDP-glucose (agrimoniin isomer) (peak 3). Additionally, ellagic acid pentoside (peak 2), free ellagic acid (peak 4), and the flavonols quercetin-3-*O*-glucuronide (peak 5) and kaempferol-3-*O*-glucuronide (peak 6) were also identified in the samples (Figure 1).

The highest concentration of quercetin-3-*O*-glucuronide (5) and kaempferol-3-*O*-glucuronide (6) were obtained with Ac in two-steps, regardless of the presence of acid in the extracts (Table supplementary material 1). However, for some solvents both in one- and two-steps, the presence of formic acid in the extracts rendered, slightly lesser flavonol contents in comparison with extracts obtained without acid in the same conditions (Table supplementary material 1). This is in agreement with Downey et al. (2007), who reported that the extracts of quercetin-based flavonols from grape skin were unstable in acidic methanol solutions.

Agrimoniin (3) was the major phenolic compound found in the strawberry by-products, in agreement with that reported by Kårlund et al. (2014) for leaves of strawberry. This compound is one of the most representative polyphenols of the family *Rosaceae* and is considered a taxonomic marker (Grochowski et al., 2017). As shown in Table 3, the extraction in two-step with Ac and C_{FA} = 0% produced the highest yield of agrimoniin (2.45 g/kg). However, there were no differences in the agrimoniin isomer content extracted with W, MeOH, and Ac with C_{FA} = 0.5% (≈ 2 g/kg), pointing out the opportunity of extracting this compound with a green solvent such as acidified water. Furthermore, this ellagitannin was the only phenolic compound that correlated (R^2>0.80, p<0.05) with the antioxidant capacity (FRAP and DPPH) in samples extracted in one-step with acid (Table supplementary material 3); and with the antioxidant capacity (DPPH) in samples extracted in two-steps with or without acid (Tables supplementary material 4 and 5).

Remarkable, the content of agrimoniin extracted in this way resulted in about 117 times higher than the content extracted in the whole strawberry (0.017 g/kg) (Van de Velde et al., 2016b). Tannins have diverse effects on biological systems since they are potential metal ion chelators, protein precipitating agents, and biological antioxidants (Ignat et al., 2011). Therefore, according to these results, strawberry by-product may represent an excellent ellagitannin source with potential biological uses.

3.3. Apple PPO inhibition

The Michaelis-Menten constant (Km) and maximum velocity (Vmax) values of 'Red Delicious' apple PPO were 0.1136 Δabs/min and 9.48 mmol/L, respectively. Both values were within the range of those reported by Nicolas, Richard-Forget, Goupy, Amiot, & Aubert (1994).

Polyphenols extracted from strawberry by-products (0.24 g GAE/L) produced 30% inhibition of apple PPO activity (Table 5). This result is in agreement with Soysal (2009) who reported inhibition of 42% on 'Golden Delicious' apple PPO activity by an extract of

green tea (0.35 g GAE/L). Similarly, a 20 g/L *yerba mate* extract (1,09 g GAE/L) reduced by 50% the PPO activity of 'Princesa' apples (Rodríguez-Arzuaga & Piagentini, 2018). Likewise, rice bran extracts, rich in polyphenols, inhibited apple PPO by 18-47% (Sukhonthara et al., 2016). On the other hand, a solution of ascorbic acid (0.4 g/L), a reference antioxidant, showed a greater inhibition of apple PPO activity of around 45% (Table 5).

Regarding the type of inhibition, reversible inhibition of 'uncompetitive' type was obtained for the strawberry by-product extract, since Km' and Vmax' values decreased in the presence of the inhibitor. This is likely due to the coupling of phenolic compound hydroxyl groups to the enzyme-substrate complex, which facilitates electron transferences and the concomitant reduction of oxidized compounds, preventing the generation of pigment products (Rotbart, Reuveni, & Urbakh, 2018).

Ascorbic acid inhibited apple PPO in a 'competitive' form, the inhibition mechanism in which Vmax' remained constant but Km' increased (Cox & Nelson, 2000). Ascorbic acid bonds to the free enzyme and competes for the active site with the substrate, avoiding in this way the product formation. Besides, ascorbic acid lowers pH, making the enzyme catalysis more difficult (Piagentini, Güemes, & Pirovani, 2003).

Finally, the inhibition constant (Ki), which is equal to the inhibitor concentration necessary to inhibit 50% of PPO enzyme activity (I_{50}) was higher for the strawberry by-product extract than for ascorbic acid (Table 5).

4. Conclusions

Strawberry by-products extraction in two-steps rendered higher yields of phenolic compounds than the extraction with the same volume of solvent but in one single step. Acidified methanol extracted the highest contents of antioxidant phenolic compounds among all the solvent systems studied. Agrimoniin was the major polyphenol found in samples, and the extraction in two-steps with acetone produced the highest yield, being the obtained content more than 100-times higher than the content extracted from

the whole fruit. Encouraging extractions with green solvents, strawberry by-product extraction in two-steps with water or ethanol showed polyphenol concentrations similar to those obtained with methanol or acetone in one-step.

Strawberry by-product extracts (0.24 g GAE/L) produced 30% apple PPO inhibition, in a reversible inhibition of 'uncompetitive' type, 15% lower than the inhibition reached with a solution of higher concentration of ascorbic acid (0.40 g/L).

The results obtained showed the high revalorization potential of strawberry by-products as a low-cost source of phenolic compounds with antioxidant activity. Furthermore, the extracts represent a promising natural alternative to avoid the enzymatic browning of fruits and vegetables, one of the main problems of processing these products.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgments

The authors acknowledge María Sordo for providing strawberry by-product tissues, and the ANPCyT (Argentina) for financial support through PICT 2017-406. Esteban Villamil-Galindo was supported with a doctoral grant from CONICET (Argentina).

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601 **Figure Captions:**

602 **Figure 1.** Typical reversed phase HPLC-UV chromatograms of strawberry by-product
603 extracts obtained with acidic methanol in two-steps at 254 nm (**A**) and 360 nm (**B**).
604 Peak identification: (**1**) tetragalloyglucose isomer (**2**) ellagic acid pentoside, (**3**) dimer of
605 galloyl-bis-HHDP-glucose (agrimoniin isomer), (**4**) free ellagic acid, (**5**) quercetin-3-*O*-
606 glucuronide, (**6**) kaempferol-3-*O*-glucuronide.

607

Table 1. Factors and levels of the experimental design

Factor	Levels
Solvent (S)	Water (100%) (W) 80 % Ethanol: 20 % water (EtOH) 80 % Methanol: 20 % water (MeOH) 80 % Acetone: 20 % water (Ac)
Formic acid concentration (C_{FA}, %)	0.0 0.5
Extraction steps (ES)	1 2

Table 2. ANOVA for the contents of total phenolics (TPC), total flavonoids (TF), and the antioxidant capacity (DPPH and FRAP)

Variation source		Sum of squares			
	DG	TPC	TF	DPPH	FRAP
S	3	155.84***	3.56***	0.014***	0.007***
C_{FA}	1	29.34***	0.61***	0.001***	0.001***
ES	1	100.08***	0.62***	0.074***	0.006***
S.C_{FA}	3	4.48***	0.14***	0.009***	0.0004***
S.ES	3	4.39***	0.05***	0.002***	0.0002***
C_{FA}.ES	1	0.007^{ns}	0.47***	0.0003***	0.00002^{ns}
S. C_{FA}.ES	3	4.65***	0.29***	0.0004*	0.00008^{ns}
Residues	32	4.71	0.05	0.005	0.0004
Total	47	303.53	5.80	0.028	0.015

S: solvent, C_{FA}: formic acid concentration, ES: extraction steps, DG: degree of freedom.
*: $p \leq 0.05$; **: $p \leq 0.01$;***: $p \leq 0.001$. ns: p> 0.05.

Table 3. Total phenolic compounds (TPC), Total flavonoid content (TF) and Dimer of galloyl-bis-HHDP-glucose (agrimoniin isomer) of different extracts of the strawberry by-products

S	ES	TPC (g AGE/kg)		TF (g quercetin/kg)		(3), agrimoniin isomer (g/kg)	
		C_{FA} (%)		C_{FA} (%)		C_{FA} (%)	
		0	0.5	0	0.5	0	0.5
W	1	5.70 ± 0.20 fB	7.90 ± 0.21 eA	0.54 ± 0.03 fA	0.37 ± 0.02 fB	0.04 ± 0.02 bcB	1.73 ± 0.43 abcA
	2	8.60 ± 0.37 dB	9.49 ± 0.05 dB	0.84 ± 0.09 dB	0.48 ± 0.01 eA	0.03 ± 0.01 cB	2.04 ± 0.06 abA
EOH	1	6.96 ± 0.02 eB	7.67 ± 0.03 eA	0.72 ± 0.01 deB	0.76 ± 0.02 cA	0.23 ± 0.25 bcB	0.28 ± 0.18 cB
	2	9.40 ± 0.22 cB	10.01 ± 0.05 bB	1.00 ± 0.05 cA	0.82 ± 0.05 cB	0.49 ± 0.26 bcB	0.47 ± 0.35 bcB
MeOH	1	9.00 ± 0.08 cdB	11.69 ± 0.09 cA	0.69 ± 0.01 eA	0.66 ± 0.01 dB	0.94 ± 0.83 bcB	1.22 ± 0.14 abcB
	2	12.80 ± 0.15 aB	15.01 ± 0.03 aA	1.11 ± 0.03 bA	0.80 ± 0.20 cdB	1.09 ± 0.01 abcB	2.28 ± 0.96 aA
Ac	1	9.20 ± 0.16 cdB	9.30 ± 0.19 dB	1.12 ± 0.09 bB	1.20 ± 0.06 aB	1.42 ± 0.38 abB	2.08 ± 0.37 abB
	2	11.64 ± 0.08 bB	13.89 ± 0.04 bA	1.90 ± 0.08 aA	1.00 ± 0.04 bB	2.45 ± 0.01 aB	2.11 ± 0.20 abB

S: solvent, C_{FA}: formic acid concentration, ES: extraction steps. Mean (n=3).
Different capital letters and lowercase letters indicate significant differences ($p < 0.05$) by Tukey's test, between formic acid concentration, and among extraction systems, respectively.

Table 4. Antioxidant capacity of different extracts of the strawberry by-products by DPPH and FRAP methods

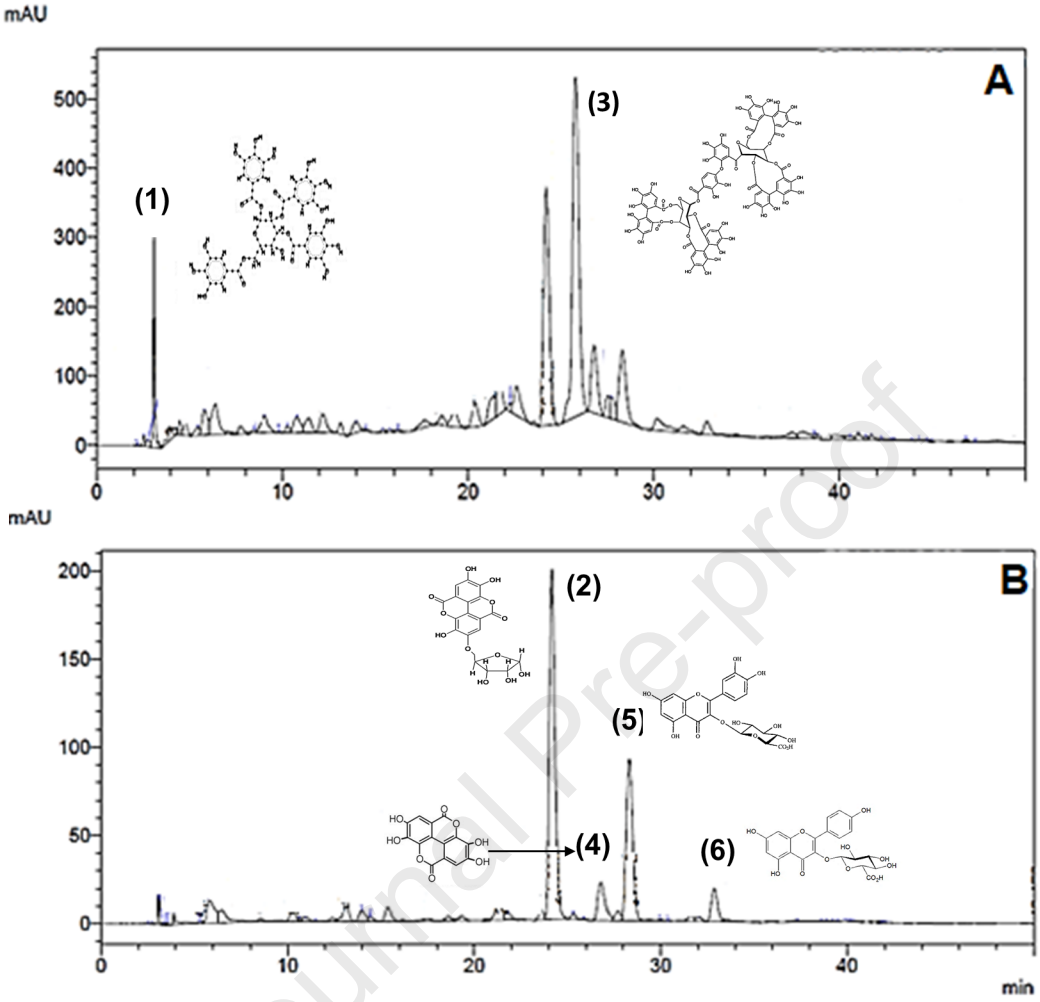
S	ES	DPPH (mmol Trolox/Kg)		FRAP (mmol Fe^{2+}/Kg)	
		C_{FA} (%)		C_{FA} (%)	
		0	0.5	0	0.5
W	1	43,52 ± 9,38 dB	64,63 ± 6,66 dA	34,97 ± 0,18 dB	44, 42 ± 2,08 dA
	2	88,09 ±13,0 bcB	90,76 ± 5,12 cA	60,34 ± 2,01 bB	61, 23 ± 1,14 cB
EOH	1	66,7 ± 6,81 bcB	67,48 ± 0,86 dB	38,99 ± 1,35 dB	41, 65 ± 2,46 dA
	2	79,55 ± 9,33 bcB	86,12 ± 9,74 dA	56,26 ± 1,70 cB	62 ,06 ± 3,36 cA
MeOH	1	70,33 ± 12,44 cB	101,22 ± 7,28 cA	53,70 ± 1,29 cB	67,05 ± 5,76 cA
	2	121,12 ± 3,94 aB	124,49 ± 9,79 bB	82,98 ± 0,80 aB	100,79 ± 2,31 aA
Ac	1	90,45 ± 7,01 bB	101,93 ± 7,31 cA	56,43 ± 3,86 cB	66 ,17 ± 7,14 cA
	2	118,55 ± 6,21 aB	120,81 ± 3,66 bB	77,62 ± 2,28 aB	90,23 ± 1,11 bA

S: solvent, C_{FA}: formic acid concentration, ES: extraction steps. Mean (n=3).
Different capital letters and lowercase letters indicate significant differences ($p < 0.05$) by Tukey's test, between formic acid concentration, and among extraction systems, respectively.

Table 5. Inhibition of the 'Red Delicious' apple PPO by polyphenols extracted from strawberry by-products (P) and ascorbic acid (AA)

Inhibitor	Concentration (g/L)	Inhibition (%)	Type of inhibition	Ki (mM)
P	0.24	30.26	Uncompetitive	17.16
AA	0.40	44.61	Competitive	1.44

Ki: inhibition constant.



Polyphenols from strawberry by-products were obtained by different extraction systems

Acidified methanol in two-steps yielded the highest phenolic compound concentration

Agrimoniin, the major polyphenol found, was better extracted in 1-step with acetone

Extracts reduced PPO activity, in a reversible inhibition of uncompetitive type

Strawberry by-products represent a promising low-cost source of polyphenols

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: