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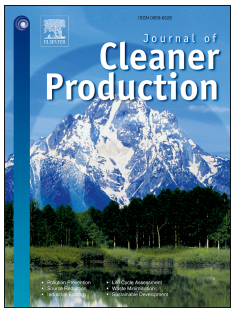

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Waste Mitigation: from an effluent of apple juice concentrate industry to a valuable ingredient for food and feed applications

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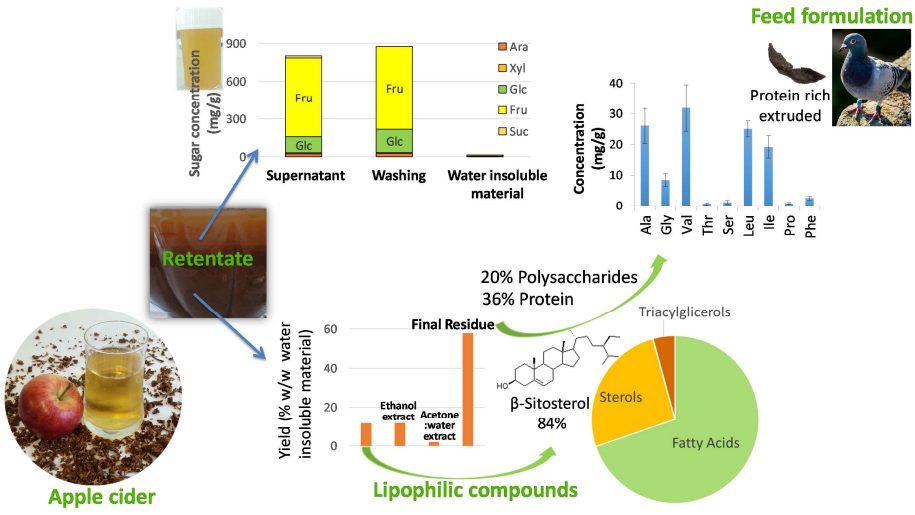
Abstract

Retentate is a by-product of the concentrate apple juice industry, resultant from clarification through an ultrafiltration process. It has a liquid to sludgy appearance, with 8% of total solids content, usually discarded as industrial waste, conveyed as wastewater directly to the sewage plant. However, its origin and expected composition may allow to define its use as a source of valuable compounds as well as its application in food and feed. The present work shows that retentate is very rich in carbohydrates, namely 40% fructose (dry weight), glucose (8%), oligosaccharides (5%), and polysaccharides (3%). It is also rich in protein (8%) and an available and inexpensive source of β-sitosterol (0.6%). This was the major sterol identified, accounting for 21% of total lipophilic compounds recovered by *n*-hexane. The retentate suspension was successfully used as nutritive medium for production of cider by a microbrewery and the protein-rich water insoluble material (28% of protein) was successfully used in feed formulations for racing pigeons.

Keywords: Retentate; Carbohydrates; Amino acids composition; Lipophilic compounds; β-Sistosterol; Apple cider.



1. Introduction

According to the Food and Agriculture Organization (FAO) of the United Nations, the global production of apples was 85 million tons in 2014 (FAOSTAT, 2017). The majority are consumed as fresh fruit, while 25 to 30% are converted into processed products, with apple juice concentrate as the main product (65%) (Bhushan et al., 2008).

Worldwide, the apple juice is the second most popular juice, after orange juice (European Fruit Juice Association, 2014; Huebner and Kienzle, 2001; Protzman, 2016; United States Department of Agriculture, 2016). After extraction of apple juice, large quantities of by-products are generated, namely apple pomace, which accounts approximately to 25% of fresh apple weight. In recent years, a broad range of applications have been described for apple pomace - namely animal feed, pectin recovery (O'Shea et al., 2012), extraction of phytochemicals (Bhushan et al., 2008; O'Shea et al., 2012), bioproduction of high value-added products, such as enzymes, organic acids, and biofuels (Dhillon et al., 2013) and recently multi-valorisation including scaffold for tissue engineering (Yates et al., 2017). As new environmental rules for industrial waste disposal have become stricter, the reusing of residues and by-products is an advantage for economically viable solutions. Recently, agro-industrial wastes and by-products reutilization has been adopted in a sustainable process, using the biorefinery concept (Mirabella et al., 2014). The valuation of food industry wastes, using a multi-valorisation approach with integral utilization of apple pomace (Yates et al., 2017) or an integrated approach for pineapple waste valorisation (Seguí Gil and Fito Maupoey, 2018), has been proposed.

Since the majority of apple juice (90%) is consumed as clarified juice (Huebner and Kienzle, 2001), a mode of consumption that still persists nowadays, ultrafiltration is an important step in large-scale industrial production, aiming to remove suspended solids and substances that cause haze and turbidity (Bruijn and Bórquez, 2006). The accumulation of carbohydrates, polyphenols and/or proteins on the ultrafiltration membrane, results in an organic filtration residue named retentate (Bruijn and Bórquez, 2006). The retentate has liquid to sludgy appearance with a total solids content

between 5 and 15% whereas the permeate occurs as a concentrate of 70% of total solids and is the main product of the concentrated apple juice industry. The profitability of the process is closely related to the higher recovery of sugars from the fruits to the juice.

Unlike apple pomace, few applications have been described for the by-product released after juice clarification. The retentate has been proposed for use in agriculture, mainly applied to fields or as animal fodder, or applied directly into the soil (Huebner and Kienzle, 2001). However, this by-product is usually disposed by landfill, incineration, or industrial wastewater treatment plants, which have a cost to the producer and create environmental problems. The management of apple juice wastewater has been improved, however, with low profitable solutions (Barrantes Leiva et al., 2014; Virmond et al., 2012). The juice processing industry is claiming attention due to the generation of large amounts of effluents with properties that turn them into potential pollution sources if improperly discarded. In a sustainable process, reutilization should be adopted, namely by mitigation of the amount of residue from juice clarification (retentate) that is conveyed directly to the sewage plant. This will allow driving the retentate within the industrial process, maintaining its food grade, into new applications in food and feed fields, with advantage of reducing the organic charge of wastewater. The studies already available concerning the retentate valuation as a by-product, described as apple pomace sludge, exploit its use for bioproduction of citric acid (Dhillon et al., 2011; Dhillon et al., 2013) and insect diets (Dhillon et al., 2013).

The retentate has a total solids content of 115-135 g L^{-1}, from which 56-66 g L^{-1} are carbohydrates, 29-34 g L^{-1} protein, and 5-6 g L^{-1} lipids; it also contains a large range of micronutrient elements (Dhillon et al., 2013). To enhance knowledge on the retentate composition, retentate samples obtained directly from an apple juice concentrate industry were analysed concerning the type of carbohydrates, amino acids, fat and sterols composition. This allowed to obtain fractions rich in compounds with possible application in food and feed industry, namely its use as nutritive medium for production of apple cider, as a β-sitosterol source for nutraceutical applications, and as

protein-rich material for production of feed formulations, driving towards a "zero waste" approach in the apple juice industry.

2. Materials and methods

2.1. Sequential extraction of the retentate

The retentate samples were supplied by Indumape, S.A., Portugal, throughout one year. The retentate resulted industrially from the ultrafiltration of the apple juice followed by the washing of the retained material with water, in a process named diafiltration. From these, for the complete analysis performed, two samples of retentate, collected in different seasons of the same year, were used. The retentate suspensions (150 g) were centrifuged at 15,000 rpm, at 4 ^{o}C during 20 min to separate soluble and insoluble material. Then, the insoluble material was sequentially washed with water (40 ^{o}C, 120 mL, 15 min). Due to the yields reproducibility of the retentates analysed, only one sample of water insoluble material was used for the extraction with: [1] *n*-hexane (soxhlet, 150 mL, 5 h); [2] ethanol with 1% (v/v) of acetic acid (20 ^{o}C, 25 mL, 1 h); [3] acetone:water (6:4, v/v) with 1% acetic acid (20 ^{o}C, 25 mL, 1 h). The 2nd and 3rd extraction steps were repeated five times each. After each extraction, the soluble material was separated from the insoluble material by centrifugation (15,000 rpm, 4 ^{o}C, 20 min) (Figure 1). Each extract and residue were frozen and freeze-dried. Regarding to ethanol, acetone:water, and soxhlet extraction, the solvent was evaporated to dryness by rotary evaporation.

Figure 1.

2.2. Sugar analysis

Neutral sugars were analysed by gas chromatography-flame ionization detection (GC-FID) after conversion to their alditol acetates. The quantification was carried out using 2-deoxyglucose as internal standard. Monosaccharides were released from polysaccharides with pre-hydrolysis of the

samples using 0.2 mL of 72% (w/w) H_2SO_4 for 3 h at room temperature followed by 2.5 h hydrolysis in 1 M H_2SO_4 at 100 ^oC. After 1 h hydrolysis, it was collected 0.5 mL for uronic acids determination. After hydrolysis, the reduction and acetylation of the monosaccharides were performed, and the alditol acetates were analysed by GC-FID using a DB-225 column (30 m, 0.25 mm, 0.25 μm) and a GC-FID PerkinElmer-Clarus 400 (Bastos et al., 2015). For the determination of free sugars composition, including sucrose, the hydrolysis step was omitted and the alditol acetates formed were analysed using a capillary column DB-1 (30 m, 0.25 mm, 0.15 μm). The oven temperature program was as follows: 100 ^oC to 350 ^oC at a rate of 20 ^oC min^{-1} (1 min). The temperature of injector was 250 ^oC and the detector was 300 ^oC. Hydrogen was used as the carrier gas. The free sugars were identified and quantified based on their retention times and response factors obtained by injection of standards. Uronic acid content was determined by 3-phenylphenol colorimetric method. The quantification was performed using galacturonic acid as standard (Bastos et al., 2015).

2.3. Size exclusion chromatography

Preparative size exclusion chromatography was carried out on a Pharmacia Biotech XK 16 chromatography column (70 cm length × 1.6 cm diameter) containing Biogel P-2, using a flow rate of 0.28 mL min^{-1}. Total (V_t) and void (V_0) volumes were calibrated with glucose (180 Da) and blue dextran (2 MDa), respectively. The calibration with stachyose (DP4) and sucrose (DP2) was also performed. The column was equilibrated with distilled water and the supernatant of the retentate sludge (1 mL) was loaded. Fractions (1.5 mL) were collected and assayed for sugars with evaporative light scattering detection (Coelho et al., 2016). Additionally, the absorbance was measured at 280 nm. Based on the chromatographic profiles, the eluate was combined in 4 fractions (A-D), evaporated, frozen, and freeze-dried.

2.4. Protein and amino acids analysis

The nitrogen content of retentate sludge, water insoluble material, and final residue were determined by elemental analysis. In order to convert the nitrogen content in protein content, a Kjeldahl factor for apple samples (5.72) was used (Sosulski and Imafidon, 1990).

The Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich) was used to determine the soluble protein in fractions A and B obtained from size exclusion chromatography.

The amino acids composition of the water insoluble material and final residue was determined after acid hydrolysis of protein and derivatization for gas chromatography analysis (Coimbra et al., 2011). 1 mL of HCl 6 M was added to the sample (5 mg) and acid hydrolysis was performed during 24 h at 110 ^{o}C, using norleucine 5.0 mM as internal standard. The derivatization was performed with: [1] HCl 3 M in isobutanol (200 μL, 120 ^{o}C, 10 + 30 min); [2] 0.2 mg mL^{-1} Butylated hydroxytoluene (BHT) prepared in ethyl acetate (200 μL); [3] heptafluorobutyric anhydride (100 μL, 150 ^{o}C, 10 min). The amino acids were separated and analysed by GC–qMS using a Shimadzu GCMS-QP2010 Ultra. The GC was equipped with a DB-1 (J&W Scientific, Folsom, CA, USA) capillary column (30 m, 0.25 mm, 0.15 μm). The sample was injected in "split" mode with the injector temperature of 250 ^{o}C, during 5 min. The temperature program used was as follows: initial temperature was 70 ^{o}C for 1 min, increasing to 170 ^{o}C at a rate of 2 ^{o}C min^{-1}, followed by a linear increase of 16 ^{o}C min^{-1} until 250 ^{o}C, then maintained at 250 ^{o}C for 5 min. The GC was connected to a mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 40–500. Calibration curves for Ala, Val, Leu, Asx, and Glx were obtained in the concentration range of 0-0.2 mg mL^{-1}; for Gly, Thr, Ser, Ile, Pro, Phe, Lys, and Tyr the concentration range was 0-0.025 mg mL^{-1}.

2.5. Lipophilic compounds analysis

To quantify free lipophilic compounds, the dried soxhlet extract (20 mg) and accurate amount of internal standard (tetracosane, 1 mg) were dissolved in 250 μL of pyridine. The compounds

144 containing hydroxyl and carboxyl groups were converted into trimethylsilyl (TMS) ethers and esters,
145 respectively, by adding 250 μL of N,O-bis(trimethylsilyl)trifluoroacetamide and 50 μL of
146 trimethylchlorosilane. The mixture was kept at 70 ^{o}C during 30 min (Vilela et al., 2014). Compounds
147 were identified as TMS derivatives by comparing their mass spectra with the GC–MS spectral
148 library and also with literature of MS fragmentation (Vilela et al., 2014). For correct quantification,
149 the chromatographic respective response factors of each compound family were calculated in
150 relation to tetracosane using (*Z*)-nonadec-10-enoic acid for fatty acids, cholesterol for sterols, decan-
151 1-ol for long chain aliphatic alcohols.

152 To quantify total lipophilic compounds (free and esterified), the alkaline hydrolysis reaction was
153 performed. Briefly, the dried soxhlet extract (30 mg) was dissolved in 10 mL of KOH 1 M in 10%
154 aqueous methanol. The mixture was kept at 100 ^{o}C during 60 min, under a nitrogen atmosphere.
155 After cool down, the mixture was acidified with HCl 1M until pH ≈ 2 and then extracted three times
156 with dichloromethane. After evaporation of organic solvent, the lipophilic compounds were
157 converted into trimethylsilyl (TMS) ethers and esters and analysed as previously described. The
158 fraction of esterified compounds was calculated considering the concentration of lipophilic
159 compounds and the amount of free lipophilic compounds.

160 To quantify fatty acid methyl esters (FAME) 0.8 mL of the heptadecanoate methyl ester (0.5 g
161 L^{-1} in *n*-hexane) was added to the dried soxhlet extract (10 mg). Then, 0.2 mL of methanolic
162 solution of KOH (2 M) was added. The mixture was shaken and 2 mL of saturated sodium chloride
163 solution was added. The sample was centrifuged at 2,000 rpm during 5 min (Aued-Pimentel et al.,
164 2004). After that, 1 mL of organic phase was collected and analysed by a GC-FID PerkinElmer-
165 Clarus 400 equipped with a capillary column DB-FFAP (30 m, 0.32 mm, 0.25 μm). The FAMEs
166 were identified based on their retention times obtained by injection of commercial FAMEs mixture
167 (C_8–C_{24}).

168

169 *2.6. Total phenolic compounds and antioxidant activity*

170 The total phenolic compounds of the ethanol and acetone:water extracts were determined by the
171 Folin-Ciocalteu method (Singleton and Rossi, 1965). Solutions of ethanol extract (6 mg mL^{-1}) and
172 acetone:water extract (1 mg mL^{-1}) were prepared using the respective extraction solvent.
173 Quantification was obtained by correlation to the calibration curve of gallic acid standard solutions
174 and the total phenolic compounds were expressed as gallic acid equivalents.

175 The antioxidant activity of ethanol and acetone:water extracts was determined by DPPH
176 (Villaño et al., 2007) and ABTS (Re et al., 1999) methods. Additionally, for comparison purposes,
177 the antioxidant activity of ascorbic acid and quercetin was determined using both methods.

178

179 *2.7. Estimation of procyanidin DPn*

180 The procyanidins fractionation was performed according to methanol/chloroform graded
181 precipitations (Passos et al., 2010). The acetone:water extract (15 mg) was dissolved in 1.5 mL of
182 water with 5% (v/v) of acetic acid and the insoluble material was separated by centrifugation (15,000
183 rpm, 4 ^{o}C, 20 min). A liquid-liquid extraction with ethyl acetate was performed to the supernatant,
184 three times. Both organic and aqueous phases were evaporated to dryness. Then, the residue from the
185 aqueous phase was redissolved in methanol (1 mL) and the insoluble material was separated by
186 centrifugation. The supernatant was submitted to successive additions of chloroform until a new
187 precipitate was formed (Supplementary Material Figure S1). The precipitate material was separated
188 by centrifugation. Each precipitate and final extract were then rotary-evaporated with several
189 additions of water to completely remove the organic solvent, frozen, and freeze-dried. The average
190 degree of polymerisation (DPn) was estimated from the calibration curve: $y = -0.0743x + 11.24$; $r^2 =$
191 0.7278 (Passos et al., 2010).

192

193 *2.8. Glycosidic-linkage analysis*

Glycosidic-linkage composition was determined by GC-qMS of the partially methylated alditol acetates, using methyl iodide in NaOH/DMSO as alkylating agent and TFA 2 M for hydrolysis (Bastos et al., 2015).

2.9. Cellulase hydrolysis

The enzymatic hydrolysis was performed based on the procedures described by Bastos et al. (2015). The final residue (30 mg) was suspended in 20 mL of 20 mM acetate buffer pH 5.0 at 37 ^{o}C and hydrolysed with cellulase (EC 3.2.1.4) from *Aspergillus niger* (Sigma-Aldrich) 1.44 U/mg. Enzymatic hydrolysis was performed during 24 h followed by 24 h, in a total of 48 h. Every 24 h, the hydrolysed material was separated from residue by centrifugation (15,000 rpm, 4 ^{o}C, 20 min) and the reaction was stopped by boiling the test tube for 10 min. The material was purified by size exclusion chromatography on Biogel P-2 and analysed by light scattering, as described in Section 2.3.

2.10. Exploitation of retentate for food and feed applications.

The retentate was used to prepare cider, allowing to develop a nutrient medium essential for yeast metabolism during fermentation, by complementing apple juice concentrate, which is deficient in amino acids and minerals. For this, an apple cider was prepared with 200 L of apple juice concentrate diluted at 12 ºBrix with the addition of 200 kg of retentate, both supplied from Indumape, S.A., Portugal. Retentate was previously macerated with malt Pilsen at 45-50ºC during 30 min, in a proportion of 10:1. Fermentation was carried out in Vadia microbrewery, Essência D'Alma Lda, Portugal, using a lager yeast (saflager w-34/70, Fermentis) in a 1,000 L bioreactor at 10-12 ºC, during one week.

A feed formulation using 15% of dried retentate water insoluble material was also prepared for racing pigeons, nutritionally adjusted for the competition period, containing approximately 3,400 kcal/kg, with 56% carbohydrates, 22% protein, 9% fat, 6% fibre, and 4% ash. The retentate used in

the new formulation replaced 20% of soymeal protein in the original formulation. The blend of the sieved raw materials (1.5 kg) was extruded using a single screw extruder (Periplast model Ø25 x 25D) and the extrudates were then oven dried at 60 ºC during 30 min until reaching a moisture content of less than 10%. After cooling, the extruded feed was stored in plastic bags at room temperature.

3. Results and Discussion

The water content of retentate (apple juice sludge) was 83% just after the ultrafiltration step, increasing to 92% in the retentate, after diafiltration, which is in accordance with the 87-89% described for apple juice sludge (Dhillon et al., 2013). When it is necessary to decolorize the juice concentrate, due to a more brownish colour than that desirable, the industry uses activated charcoal, which increases the amount of solids of the retentate, reaching 20%. In the present study, the retentates used were not submitted to this decolorizing step. On a dry weigh basis, the retentates under study were constituted by 53% of carbohydrates, of which 51% were free sugars, mainly fructose (78 mol%), glucose (16 mol%), arabinose (3 mol%), and sucrose (2 mol%), in accordance with the 49% of total carbohydrates, on a dry weigh basis, reported for the apple juice sludge (Dhillon et al., 2013). They also contained 8% protein and 3% of lipophilic compounds. Although the lipophilic compounds are in accordance to the amount of lipids reported for apple juice sludge (4%), the amount of protein was much less than the reported 25% (Dhillon et al., 2013), showing that the uncharacterized material of the retentates should be protein derived compounds.

The centrifugation of the retentate resulted in a supernatant containing 57% of the retentate total solids and 89% of free sugars. The soluble protein accounted for 4%. The washing of the residue allowed to recover an extra 6% of retentate total solids and 10% of free sugars. The remaining water insoluble material accounted for 25% of retentate total solids, containing 16% of carbohydrates and 28% of insoluble protein.

244

245 *3.1. Free sugars*

246 The retentate supernatant and washing allowed to recover 99% of the retentate monosaccharides.
247 Both fractions were rich in sugars, constituted by 79% and 88% of monosaccharides, respectively
248 (Table 1). These monosaccharides are mainly fructose (79 mol% and 75 mol% for supernatant and
249 washing) and glucose (16 mol% and 21 mol%). This sugar composition is in accordance with the
250 apple juice composition reported in literature (Thavarajah and Low, 2006), as the liquid part of the
251 retentate is apple juice not recovered through the ultrafiltration process because the ultrafiltration is
252 stopped when 40% of solids are attained in the feed tank.

253

254 **Table 1.** Carbohydrate composition of the fractions obtained by the sequential extraction of retentate**.**

Sample	Carbohydrate (mol%)										Total carbohydrate*
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Fru	Suc	UA	(mg g^{-1})
Supernatant											
Monosaccharides			3	1			16	79	1		794±20
Washing											
Monosaccharides			3	1			21	75			877±24
Water insoluble material											
Monosaccharides			16	16			24	44			14±2
Polysaccharides	1	1	9	6	5	20	44			14	142±14
Final residue											
Polysaccharides	1	1	8	7	6	18	47			12	200±7

255 *Values expressed in mg of anhydrous sugars by g of sample.
256

257 Size-exclusion chromatography of the supernatant material showed a chromatographic profile
258 (Fig 2a) that allows to observe that this material was constituted by a large amount of
259 monosaccharides (Fraction D) and a small amount of disaccharides (DP2, fraction C). These results
260 are in accordance with the high content of monosaccharides and 1% of sucrose determined by free
261 sugar analysis (Table 1). Furthermore, two fractions (A, B) with molecular weight material lower

than 2 kDa, the exclusion limit of the gel, but higher than the molecular weight of fractions C and D, were also observed, suggesting the presence of oligosaccharides with DP higher than 4. The high absorbance at 280 nm of both fractions could also indicate the presence of protein. The protein analysis showed that fraction B was composed by 59.5±0.5% of protein and fraction A was constituted by 13.1±1.4%. As apple juice is constituted also by several phenolic compounds (Spanos and Wrolstad, 1992), the absorbance at 280 nm may also be indicative for the presence of phenolic compounds in these two fractions. Nevertheless, these fractions are very rich in free sugars, which can be returned to the apple juice processing line, increasing the profitability of the process by the increase of the amount of soluble sugars and, consequently, the ºBrix of apple juice concentrate.

Figure 2.

3.2. Lipophilic compounds

The retentate lipophilic fraction was obtained by soxhlet extraction with *n*-hexane from the water insoluble material, yielding 3% on a dry weight basis of the whole retentate and 11.7% on a dry weight basis of the retentate water insoluble material. The identification and quantification of the lipophilic compounds, analysed by GC-qMS for silylated derivatives of free fatty acids, sterols and long chain alcohols, and by GC-FID for methyl esters of transesterified fatty acids, is summarised in Table 2. This approach allowed to identify 15 fatty acids, 6 sterols, 2 long chain aliphatic alcohols, and one long chain diacid. Approximately 94% of all compounds were in free form, the free fatty acids being the most predominant class (68.8%). Triacylglycerides represent only 4.1% of the lipophilic compounds, mainly constituted with linoleic (C18:2), palmitic (C16:0), oleic (C18:1), and stearic (C18:0) acids, in accordance with other reports of apple seed (Bada et al., 2014; da Silva and Jorge, 2016). The high occurrence of free fatty acids shows that the majority of apple seed triacylglycerides were deesterified. It is possible that deesterification occurs during the processing of apple juice, although a large amount of free fatty acids have been reported to be constituent of

unprocessed apple seeds (Lu and Yeap Foo, 1998). Free fatty acids have also been reported as constituents of apple skin surface waxes, which may also be an origin of retentate free fatty acids (Verardo et al., 2003). Palmitic and linoleic acids correspond to the major fatty acids identified, accounting for 42.0% and 11.5% of the lipophilic compounds and 4.7% and 1.3% of the retentate water insoluble material, respectively. These high amounts of free fatty acids should be taken into account when using this material for feed formulations, requiring their removal or dilution in other fatty sources.

Sterols accounted to approximately 24% of the retentate lipophilic compounds, the second most abundant family. β-Sitosterol is the major free sterol identified and the second major component present in the sample, accounting for 20% of dry hexane extract and 2.4% of retentate water insoluble material. Stigmasterol was the only sterol occurring in an esterified form, accounting for 2.0% of lipophilic compounds, and presenting 87% of esterification. These results are in accordance with the exclusive occurrence of fatty acid esters of phytosterols in cell membranes and the high abundance of stigmasterol esters in ripen fruits (Whitaker, 2012). Moreover, the two long chain aliphatic alcohols, which were present mainly in esterified form, may have origin from cuticle waxes (Belding et al., 1998; Verardo et al., 2003). This contrasts with the free sterols reported to be present in apple epidermal cortical tissue (Whitaker et al., 1997) and peel (Rudell et al., 2011). The reason why lipase did not act on wax-derived compounds seems to be also explained by the inhibitory activity due to the high ursenoic acid content of apple peels (McGhie et al., 2012).

Table 2. Lipophilic compounds identified in *n*-hexane extract of retentate water insoluble material (expressed on a dry weight basis).

Compounds	**Concentration (mg.g^{-1})**		
	Free	**Esterified**	**Total**
Fatty acids	**647.6±77.2**	**40.7±11.5**	**688.3**
Octanoic acid (C8:0)	5.3±1.2	0.0	5.3
Dodecanoic acid (C12:0)	0.3±0.00	0.9±0.1	1.2
Tetradecanoic acid (C14:0)	2.2±0.7	0.7±0.0	2.9
Pentadecanoic acid (C15:0)	2.6±0.2	0.2±0.05	2.8
Hexadecanoic acid (C16:0)	401.5±9.5	14.0±2.9	415.5
Heptadecanoic acid (C17:0)	4.3±2.0	0.0	4.3
Octadecanoic acid (C18:0)	53.9±20.6	3.3±0.6	57.2

Eicosanoic acid (C20:0)	26.9±8.5	1.1±0.4	28.0
Heneicosanoic acid (C21:0)	3.9±2.1	1.1±0.2	5.0
Docosanoic acid (C22:0)	8.1±3.1	0.0	8.1
Tricosanoic acid (C30:0)	3.3±3.1	0.0	3.3
Pentacosanoic acid (C25:0)	1.0±0.5	0.0	1.0
Octadec-9-enoic acid (C18:1)	24.6±7.7	3.5±1.0	28.1
Octadeca-9,12-dienoic acid (C18:2)	109.7±18.2	15.2±7.1	124.9
Octadeca-9,12,15-trienoic acid (C18:3)	0.0	0.7±0.0	0.7
Long chain diacids	**20.0±2.2**	**0.0**	**20.0**
Nonanedioic acid	20.0±2.2	0.0	20.0
Sterols	**226.2±29.3**	**16.5**	**242.7±29.9**
Campesterol	9.3±1.0	0.0	9.3±1.0
Fucosterol	2.5±0.8	0.0	2.5±0.8
Stigmasterol	2.4±1.0	16.5	18.9±0.1
β-Sitosterol	203.2±32.5	0.0	203.2±32.5
Stigmasta-3,5-dien-7-one	4.0±1.2	0.0	4.0±1.2
Lanost-8-en-3β-ol	4.8±2.0	0.0	4.8±2.0
Long chain aliphatic alcohols	**0.5±0.0**	**3.8**	**4.3±0.6**
Octadecan-1-ol (C18)	0.2±0.1	3.1	3.3±0.5
Octacosan-1-ol (C28)	0.3±0.0	0.7	1.0±0.1
Total lipophilic compounds	**894.3±70.3**	**61.0**	**955.3**

The results obtained show that the retentate is a very good source of β-sitosterol (203.2 g kg^{-1} of lipids) when compared to vegetable oils with high content in β-sitosterol, namely corn oil (3.2-14.7 g kg^{-1}), rapeseed oil (0.4-6.9 g kg^{-1}), soybean oil (0.63-2.9 g kg^{-1}), and olive oil (0.5-2.4 g kg^{-1}) (Alberici et al., 2016). Annona fruits, mainly the peel, contain 20.4-21.6 g kg^{-1} of oil, which is also reported as a good source of β-sitosterol (García-Salas et al., 2016). The 6.1 g kg^{-1} content of β-sitosterol found in apple retentate on a dry weight basis, is much higher than the yield reported for rapeseed expeller cake, 0.5-0.6 g kg^{-1}, considered as a good source of this compound (Siger et al., 2016). In addition, considering the apple juice retentate water insoluble material, easily separated by centrifugation, the content of β-sitosterol represents 23.8 g kg^{-1}, which is a much higher figure. The high availability of β-sitosterol in the retentate obtained from concentrated apple juice production allows to propose this food industry by-product as an easy and rich source of β-sitosterol, a highly valued compound as food ingredient, with known properties of cholesterol lowering agent (Gylling and Nissinen, 2015). Easily β-sitosterol, as a neutral compound, can be separated from fatty acids,

the main components of *n*-hexane extract, by the deacidification processes used in the refining process of the crude oil (Bhosle and Subramanian, 2005).

3.3. Procyanidins

The retentate phenolic compounds were sequentially extracted from the soxhlet insoluble residue with ethanol and aqueous acetone (Figure 1). The mass yield obtained was 14 and 2.5%, which correspond to 11.9 and 2.1%, respectively, of the retentate water insoluble material and 3.1 and 0.5% of the retentate sludge on a dry weigh basis.

The ethanol extracted a higher amount of mass, however contained only 1.9% of total phenolic compounds estimated as acid gallic equivalents, whereas acetone:water extract contained 51.3% of acid gallic equivalents (Figure 1). The ethanol soluble material comprised a wide range of compounds that was not quantified as phenolic compounds. Ethanolic extracts of apple peels were already described to contain mainly triterpenic compounds (McGhie et al., 2012). For the ethanolic extract, the antioxidant activity determined as EC_{50} was 577.6±65.9 µg/mL with DPPH and 273.3± 8.5 µg/mL with ABTS. However, the acetone:water extract showed much lower EC_{50} (12.1±1.5 and 8.5±0.5 µg/mL), with the same methods. These values were comparable with the EC_{50}, using DPPH and ABTS, obtained for ascorbic acid (3.7±0.7 and 6.5±0.5 µg/mL) and quercetin (3.8±0.2 and 2.1±0.1 µg/mL), showing relevant antioxidant activity. As the acetone:water extracts are usually rich in polymeric procyanidins (Ferreira et al., 2002), to determine the degree of polymerization (DP), the material was dissolved in methanol and fractionated by precipitation adding chloroform (for details, see Supplementary Material Figure S1). The insolubility of 65% of the procyanidins in methanol allowed to infer that the majority of the procyanidins of acetone:water extract had a DP higher than 8 (Passos et al., 2010) and the compounds soluble in methanol had the following DP distribution: 8 (6.7%), 7 (3.8%), 5 (7.4%), <5 (2.3%) (Supplementary Material Table S1). The low yield of the ethanol and acetone:water retentate extracts seems similar to the results obtained in dried pears,

where 86% of the procyanidins remained in final residue (Ferreira et al., 2002). The industrial process involved in the production of concentrated apple juice seems to promote modifications of phenolic compounds that could result from oxidation and from reaction with other polyphenols and polysaccharides that became irreversibly bound. They could no longer be extracted by the standard methods, using methanol or aqueous acetone solvents (Ferreira et al., 2002). It was shown that oxygenation of juice during processing resulted in a significant decrease of all classes of native polyphenols. Catechins and procyanidins were particularly affected by oxidation leading *o*-quinone derivatives catalyzed by polyphenol oxidase (Guyot et al., 2003). Moreover, the lower extraction of procyanidins may result from the association of the procyanidins with the solid part of the fruits, particularly cell-wall materials when fruits are crushed and pressed as was previously shown in a model solution (Renard et al., 2001).

3.4. Insoluble protein and polysaccharides

The insoluble material recovered after water extraction was constituted by 16.1% of carbohydrates, of which 14.2% are polysaccharides. These polysaccharides are composed by glucose (44 mol%), galactose (20 mol%) and uronic acids (14 mol%) (Table 1), suggesting glucans and pectic polysaccharides as the predominant polysaccharides. Additionally, this water insoluble material is rich in protein (28.1±0.3%) and the amino acid composition (Table 3) shows that valine (24.4 mg g^{-1}), leucine (23.6 mg g^{-1}), alanine (22.1 mg g^{-1}) and isoleucine (17.6 mg g^{-1}) were the major amino acids quantified. These amino acids were also those identified in haze-active proteins in apple juice (Wu and Siebert, 2002).

The final residue comprised 15.1% of the initial retentate mass weight and 57.9% of the water insoluble material. The final residue was composed of 20.0% polysaccharides and 35.8±0.4% of protein. The remaining 44% should be unextracted material probably containing modified procyanidins bounded to polymeric material or/and products of Maillard reaction between reducing

373 sugars and protein (Coimbra et al., 2011), both giving origin to brownish products, consistent with
374 the visual appearance of the retentate final residue. Only 30% of the proteic material was quantified
375 as amino acids after acid hydrolysis. Table 3 shows that the major amino acids were valine (31.8 mg
376 g^{-1}), alanine (26.0 mg g^{-1}), leucine (25.0 mg g^{-1}), and isoleucine (19.1 mg g^{-1}). The final residue,
377 compared with the water insoluble residue, was enriched in the majority of the amino acids, with the
378 exception of proline and hydrophilic amino acids (Table 3). It is possible that the decrease in proline
379 could be related with the association of oligopeptides rich in proline and catechin or polyflavanoids,
380 and their co-extraction with acetone:water (Hatano and Hemingway, 1996; Siebert, 1999).

381

382 Table 3. Amino acids content (mg g^{-1} on a dry weight basis) of retentate water insoluble material and final
383 residue.

Amino acids	**Amino acids content (mg g^{-1})**	
	Water insoluble material	**Final residue**
Ala	22.1±5.4	26.0±5.7
Gly	7.5±3.8	8.4±2.1
Val	24.4±1.3	31.8±7.6
Thr	0.5±0.4	0.6±0.4
Ser	1.3±0.7	1.2±0.6
Leu	23.6±3.9	25.0±2.6
Ile	17.6±1.1	19.1±3.6
Pro	4.5±1.2	0.7±0.3
Asx	2.4±1.1	
Phe	3.9±0.2	2.5±0.6
Glx	3.5±1.4	
Lys	1.5±0.9	
Tyr	0.7±0.1	
Total	**113.5±17.0**	**115.3±18.5**

384

385 The polysaccharides present in the final residue are composed mainly by glucose (47%),
386 galactose (18%), uronic acids (12%), arabinose (8%), xylose (7%), and mannose (6%) (Table 1). The

main glycosidic linkage (53.5%) was (1→4)-glucose (Figure 3). In order to check if it is (β1→4)-linked glucose, an enzymatic hydrolysis was performed with a cellulase during 24 h. The release of mainly DP3 to the supernatant, with a less extent of DP4 and DP2 (Figure 2b), allowed to infer that the polysaccharides were constituted by (β1→4)-Glucose residues. In addition, even after 48 h hydrolysis, the oligosaccharide profile was similar, with a high release of DP3 and DP4. As the abundance of DP2 oligosaccharides was very small, it can be inferred that the polysaccharide could be branched, probably indicating the presence of xyloglucan fragments. In fact, the presence of 4-Glc also with 4,6-Glc (5.8%), t-Xyl (3.9%), 2-Xyl (2.7%), t-Gal (2.6%), t-Fuc (0.3%), and 2-Gal (0.3%) are diagnostic linkages of xyloglucan-type polysaccharides. The proportions of the side chains residues (t-Xyl, 2-Xyl, t-Gal, t-Fuc and 2-Gal) to the branched glucose (4,6-Glc), also with the same proportion of 2-Gal to terminally-linked fucose and 2-Xyl to terminally-linked galactose (Figure 3) support the xyloglucan structure (Quéméner et al., 2015; Ray et al., 2014; Vincken et al., 1996). The presence of xyloglucans were already reported in apple juices produced by enzymatic pomace liquefaction with pectinases and cellulases (Mehrländer et al., 2002). The presence of xyloglucan fragments in the retentate of the apple juice clarification could be explained by the high solubility of this branched polysaccharide in the juice but, due to the high molecular weight, it is retained in the ultrafiltration membranes.

Retentate is also rich in 3-Gal (4.0%), 6-Gal (2.4%), 3,6-Gal (4.1%), t-Ara*f* (3.7%), 3-Ara*f* (2.2%) and 5-Ara*f* (0.3%). These linkages are characteristic of type II arabinogalactans, already described in apple juice (Brillouet et al., 1996; Will and Dietrich, 1992). In minor amounts, it is also present in retentate 4-Man and 4,6-Man linkages probably from galactoglucomannan, already reported in apple flesh (Nara et al., 2004; Ray et al., 2014).

Figure 3.

3.5. Food and feed applications

In order to find industrial applications for the apple juice retentate, apple cider production (10 hL) in an industrial scale was performed. The cider was fermented using the premium concentrate product from the apple juice industry, which was diluted to obtain 12º Brix, and mixing it with the retentate in a proportion of 4:1 (v/v), reconstituting the original juice. Due to the presence of protein, the retentate could supplement in amino acids the nutritional medium for the yeasts growth. For the formulation of the nutritive medium, a hydrolysis of proteins was promoted through the proteolytic enzymes present in the barley malt. The nutritive medium attained a free amino acid concentration of 1.6 g/L. After dilution in the reconstituted juice (0.6 g/L of amino acids), the concentration of the amino acids was 0.9 g/L in the fermentative broth, allowing a more efficient fermentation (Malherbe et al., 2004). The fermentation attained the stationary phase with 8 days, allowing to obtain a cider product containing 5.2% of ethanol and total soluble solids of 9.4º Brix. A consumer sensorial evaluation revealed a good acceptance of the final product that have already been commercialised under the brand Vadia. From the evaluation of 65 consumers that visit the cider spot in a local market exhibition, 91% manifested purchase intention. The cider was globally evaluated by the consumers from 1 to 10 values, where 32% attributed the score 8 and 73% of consumers attributed a score higher or equal to 7 (7-10).

Furthermore, as the retentate is composed by a large amount of monosaccharides, almost half of the dry weight, their recovery into the apple juice can increase the total soluble solids and, consequently, the ºBrix of apple juice concentrate. This can easily be performed in an industrial environment by centrifugation in order to refeed the concentrator.

The retentate water insoluble material being rich in protein can be exploited as a source of protein for the animal feed industry, that looks for alternative sources of protein as feedstock, namely in feed for pets, racing pigeons, and aquaculture. The feed is adapted to different life periods of the racing pigeons, where the animal keepers spend high financial resources in the physical preparation of the pigeons. The pigeons have meals prepared as a comparable level of a high competition athlete,

437 which require a high protein intake (20%). The extruded feeds for pigeons is a market in expansion
438 in order to improve the nutritional balance of the feed. The extrusion process allows the combination
439 of seeds with other raw materials, namely soybean meal, the elected protein source, a by-product
440 from food industry. In order to evaluate the feasibility of using the residue obtained after
441 centrifugation of the retentate, 15% was incorporated into feed formulations for racing pigeons in
442 period of competition, replacing the use of 20% of soybean meal. It was observed that the
443 formulation with retentate had the same extrusion behaviour as the control sample. However, the
444 colour observed for the formulation with retentate was dark brown, contrasting with the light brown
445 control sample. These results show that it is possible to use the retentate water insoluble material in
446 extrusion processes but formulations should include lower amounts than 15%. The decrease of the
447 incorporation to 5% will allow it to obtain an acceptable colour. The substitution of 20% soybean
448 meal with the retentate showed a different amino acid profile with an increase in molar % of
449 essential amino acids. In addition, because the retentate has a relatively high content of free fatty
450 acids (Table 2), the incorporation of 5% will be within the recommended limit for these compounds
451 without the need of a saponification pre-treatment for their removal.
452

453 ## 4. Conclusion

454 The retentate, by-product of apple juice ultrafiltration, is a sludge with 8% of dissolved and
455 suspended solids that can be exploited as a source of free sugars, protein, polysaccharides, amino
456 acids, fatty acids, sterols, triacylglycerides, and procyanidins. Free sugars represent 56% of retentate,
457 mainly fructose and glucose, able to be valorised within the industrial process. Retentate is also
458 constituted of 3% lipophilic compounds, where 20% is β-sitosterol, a valuable nutraceutical
459 ingredient, and is composed by 15% of insoluble material, rich in protein (36%) and polysaccharides
460 (20%). The use of retentate for cider and feed production show the potential for its valuation.
461

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622 ## Figure captions

623

624 **Figure 1.** Schematic representation of sequential extraction of the retentate. (Yields expressed in dry
625 weight basis, the values between brackets correspond to yields calculated in water insoluble material
626 weight basis).

627

628 **Figure 2.** Size-exclusion chromatographic profile on Bio-Gel P2 of (**a**) retentate supernatant material
629 and (**b**) oligosaccharides obtained after 24 and 48 h cellulase hydrolysis of the insoluble material
630 after the sequential extraction with *n*-hexane, ethanol, and acetone:water (final residue). V_0 - void
631 volume, DP_4 and DP_2 correspond to the elution volume of stachyose and sucrose, respectively and V_i
632 –total volume. Letters A–D refer to fractions of the retentate supernatant material.

633

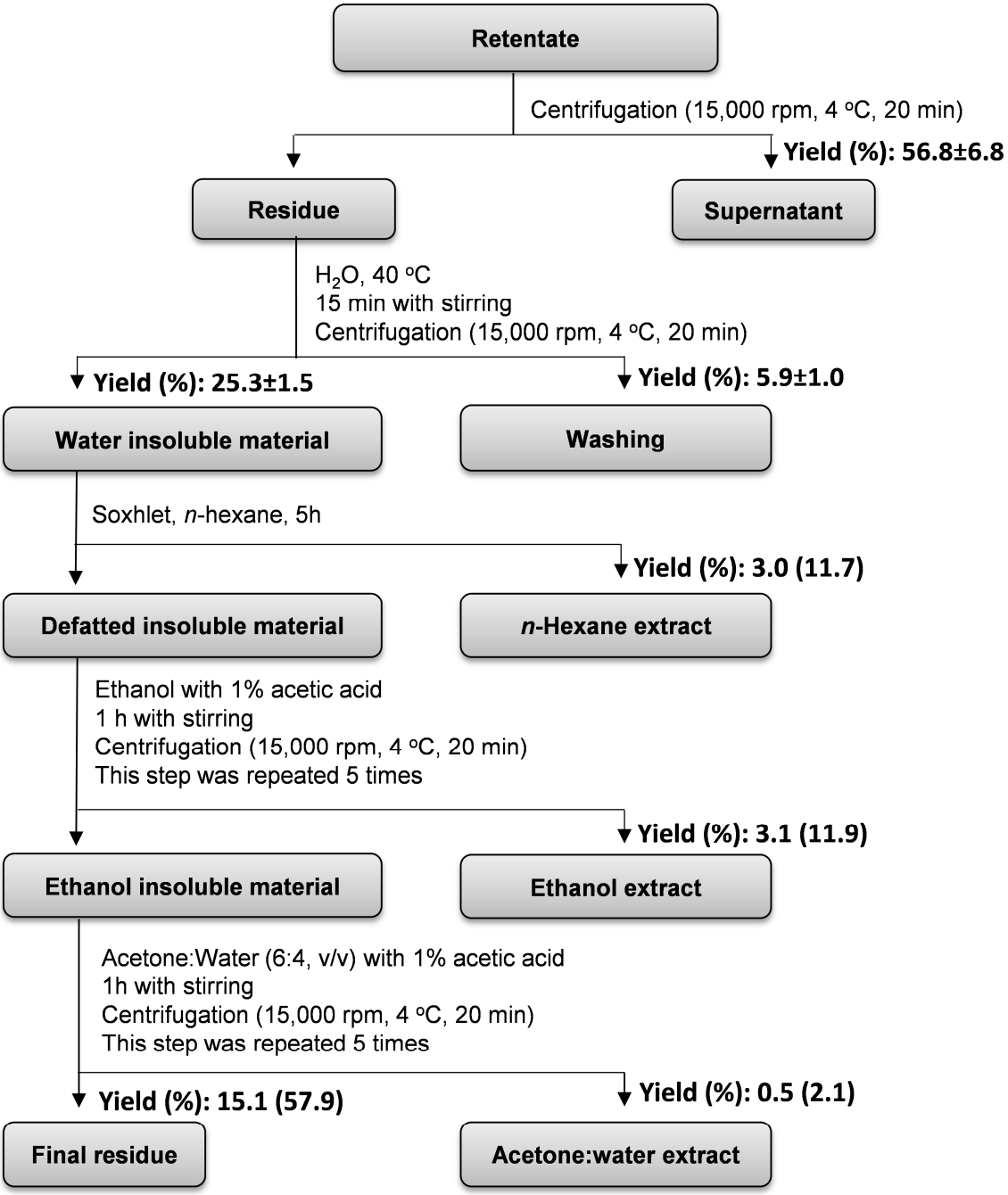
634 **Figure 3.** Glycosidic-linkage composition (mol%) of final residue.

635

636

637 # Figures

638



639
640 Figure 1

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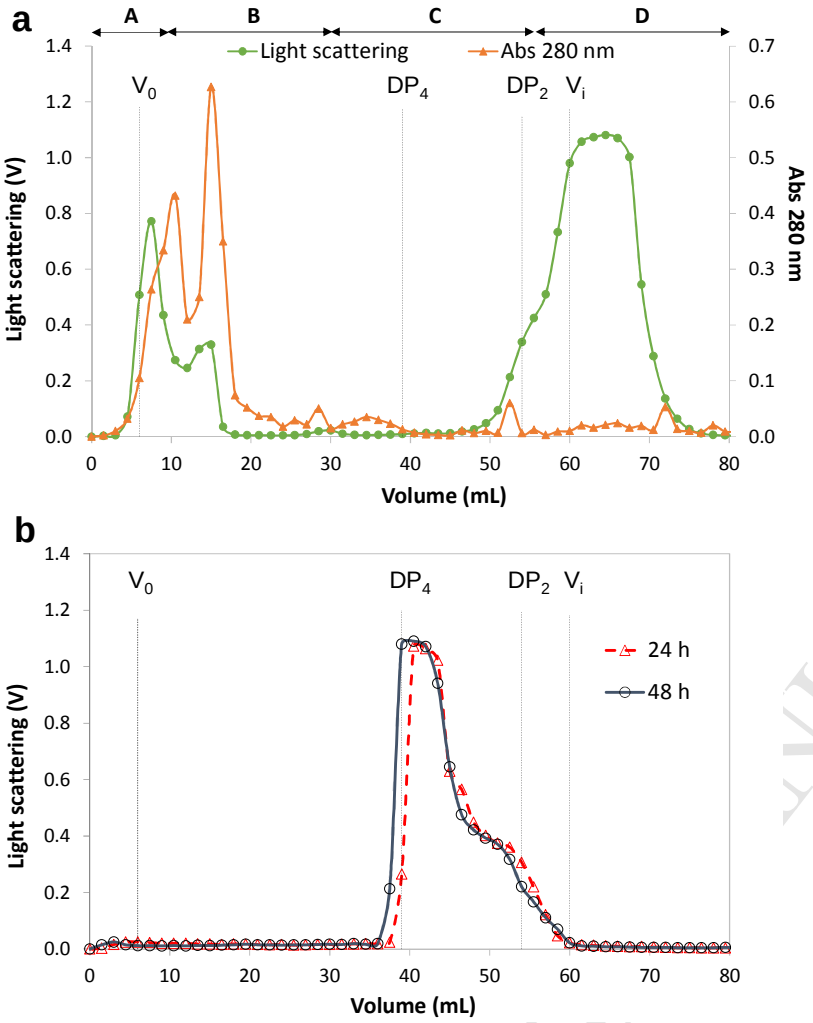

642 Figure 2

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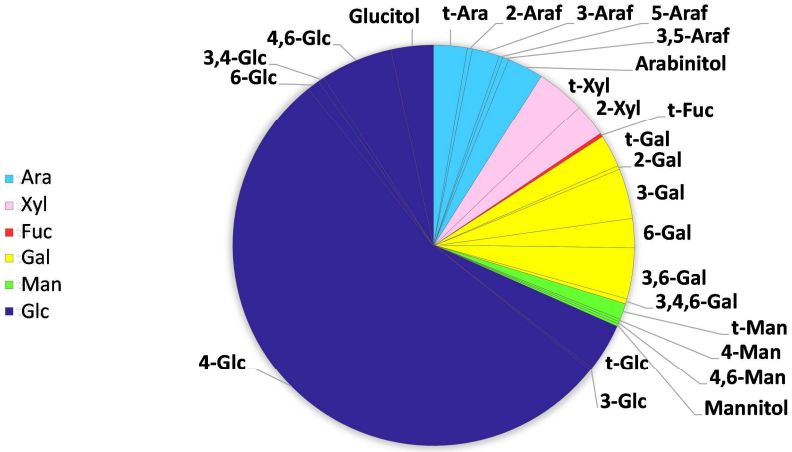

645 Figure 3

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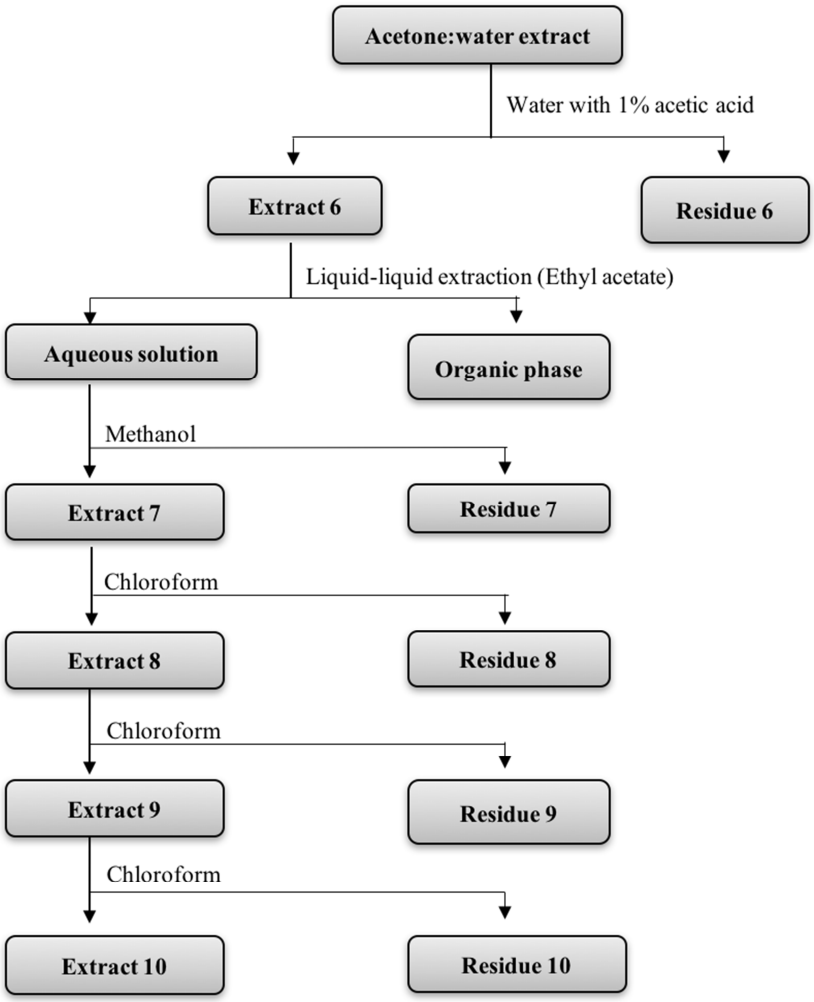
647 # Supplementary material



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649 **Figure S1.** Extraction and fractionation of procyanidin present in acetone:water extract.

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652 **Table S1.** Procyanidin composition of the fractions obtained by fractionation of acetone:water extract.

	DP*n*	**Yield (%, w/w)**	**$CHCl_3$ (%)**
Residue 6	-	45.3	-
Organic phase	-	1.9	-
Residue 7	-	17.7	-
Residue 8	8	6.7	40
Residue 9	7	3.8	60
Residue 10	5	7.4	80
Extract 10	<5	2.3	-

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Highlights

- Retentate is a source of free sugars, protein, polysaccharides, and sterols.
- Very rich in carbohydrates (53%), where 40% is fructose and 8% glucose, DW.
- An available and inexpensive source of β-sitosterol (6 g kg^{-1} of retentate, DW).
- Retentate was used as a nutritive source for production of artisanal cider.
- Water insoluble material (28% of protein) was used in feed formulations.