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Revisiting the chemistry of apple pomace polyphenols

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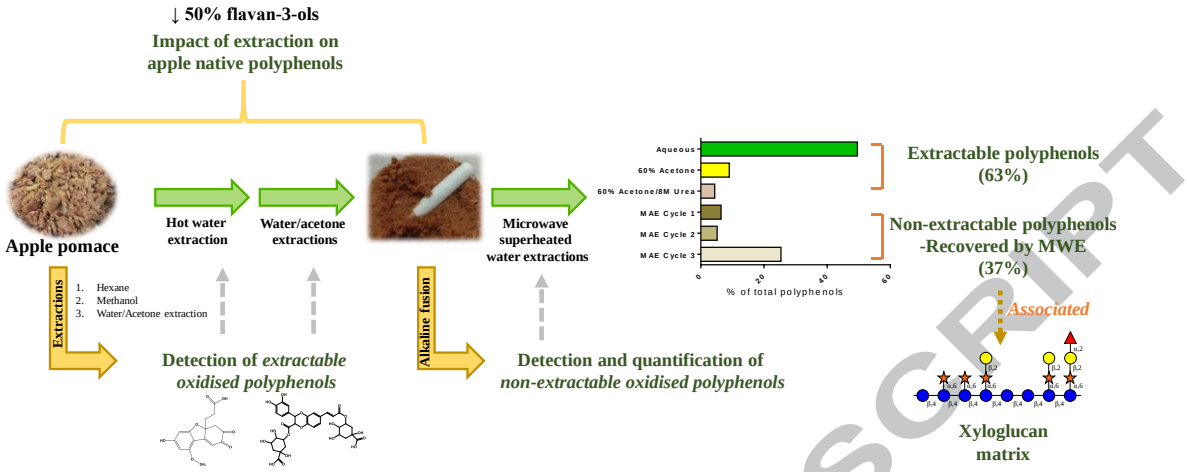
Abstract

Hot water is an easily implementable process for polyphenols extraction. To evaluate the effect of this process on apple pomace, the overall polyphenolic composition was assessed before and after hot water extraction, followed by extractions with aqueous/organic solutions. As determined by UHPLC-DAD, flavan-3-ols were the main apple native polyphenols. Their amount decreased 50% after hot water extraction, while the other classes remained unchanged. Dihydrochalcones and hydroxycinnamic acid oxidation products, were also observed, alongside with non-extractable oxidised procyanidins that represented more than 4-fold the amount of native apple polyphenols in the pomace. Microwave superheated-water extraction of the insoluble cell wall material in water/acetone solutions and the high amounts of polyphenols that were insoluble in water/ethanol solutions suggested that oxidised procyanidins could be covalently linked to polysaccharides. These complexes represented up to 40% of the available polyphenols from apple pomace, potentially valuable for agro-food waste valuation.

Keywords: oxidation, polysaccharide, microwave, hot compressed water, interaction

Graphical abstract:



1. Introduction

Apple is a widely consumed fruit all over the world, either as a fresh or processed product. Just for the latter, 11.6 million tons of apples are required to meet the global demands of, among others, the very popular apple juices and the subsidiary products such as cider and juice concentrates **(Joslyn & Deuel, 1963; Kennedy, List, Lu, Foo, Newman, Sims, et al., 1999; Rabetafika, Bchir, Blecker, & Richel, 2014)**. Notably, the industrial production of these apple-based products results in about 30% of wastes that globally may represent up to 3.5 million tons, comprising the pulp, skins, seeds and stalks of the fruit **(Kammerer, Kammerer, Valet, & Carle, 2014),** usually known as apple pomace.

Currently, simple, environmentally friendly and easily implementable industrial extraction procedures can be adopted by food industries as possible ways to promote apple pomace valuation. In fact, by performing hot water extractions under acidic pH, pectins can be obtained from apple pomace, a procedure already implemented at the industrial level **(Joslyn et al., 1963; Kennedy et al., 1999)**. These same conditions have also been suggested for polyphenol extraction **(Çam & Aaby, 2010)**, but their effect on the overall polyphenolic pool remains to be elucidated, as well as the nature of the remnant polyphenols of the apple pomace, which could be recovered and applied in diverse industrial fields**,** given their antioxidant properties **(Rabetafika et al., 2014**).

Polyphenols in apple pomace include flavan-3-ols such as (-)-epicatechin, (+)-catechin and their polymers defined as procyanidins, flavonols (quercetin glycosylated derivatives), dihydrochalcones (phloretin 2-*O*-glucoside) and hydroxycinnamic acids (mainly caffeoylquinic acids) **(Rabetafika et al., 2014; Virot, Tomao, Le Bourvellec, Renard, & Chemat, 2010)**. Nevertheless, the exposure of polyphenols to polyphenoloxidase during apple processing **(Nicolas, Richard- Forget, Goupy, Amiot,**

& Aubert, 1994) suggest that, in addition to apple native polyphenols, their oxidation products also represent a significant fraction of the overall apple pomace polyphenolic pool. Similarly, oleuropein oligomers formed by radical coupling oxidation have been reported in olive pomace **(Cardoso, Guyot, Marnet, Lopes-da-Silva, Silva, Renard, et al., 2006)**. Polyphenol oxidation reactions are of complex nature, occurring both at intra and intermolecular levels **(Bernillon, Guyot, & Renard, 2004)**, thus giving rise to a huge diversity of products with newly formed linkages. This may result in newly formed colored compounds **(Le Deun, Van der Werf, Le Bail, Le Quéré, & Guyot, 2015)** as well as impair their accurate quantification **(Mouls & Fulcrand, 2015)**.

Polyphenols also have the capacity to interact non-covalently with polysaccharides, especially pectic polysaccharides with methyl esterified groups **(Renard, Watrelot, & Le Bourvellec, 2017)**. Covalently linked polyphenols may also occur in polysaccharides, either as structures formed by biosynthesis, as described for ferulic acid in sugarbeet pectins **(Levigne, Ralet, Quéméner, Pollet, Lapierre, & Thibault, 2004)**, or, alternatively, by the reaction of the cell wall nucleophilic compounds with polyphenol quinones resulting from oxi-reduction mechanisms often associated to tissue disruption **(Le Bourvellec, Guyot, & Renard, 2009)**. In such cases, the polyphenols become non-extractable, presenting resilience to the commonly used aqueous/organic solvents **(Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2013)**. In order to overcome such non-extractability, new emerging technologies such as microwave superheated water extraction (MWE) can be performed. MWE is performed in closed vessels, which allows to reach superheated/subcritical water conditions by microwave assisted heating **(Coelho, Rocha, Saraiva, & Coimbra, 2014)**. This results in the disruption of hydrogen bonds, increased penetration of the solvent in the matrix, increased pressure that induces cell wall disintegration followed by cell rupture, and

change of the water dieletric constant towards that of some organic solvents **(Routray & Orsat, 2012)** leading to higher polyphenol **(Routray et al., 2012)** and polysaccharides **(Coelho et al., 2014)** extraction yields.

The present work aims to unveil the nature of apple polyphenols occurring in apple pomace before and after hot water, 60% acetone and 60% acetone/8 M urea extractions. For that, sugar analysis after acid hydrolysis and derivatization to the respective alditol acetates and analysis by GC-FID was performed to assess the extracts carbohydrate composition of the extracts. The polyphenols were analyzed by UHPLC-DAD after thioacidolysis. In addition, UHPLC-DAD-ESI-MS^n was also used for the detection of polyphenol oxidation products. The nature of the polyphenols that remain unextractable from the cell wall material was inferred after thioacidolysis and alkaline fusion. The possible location within the water insoluble cell wall matrix and bonding to polysaccharides were assessed by polyphenol extraction using microwave superheated water followed by gradual precipitation with ethanol.

2. Materials and Methods

2.1. Preparation of apple pomace extracts

Apples, mainly Royal Gala apple variety, were processed at industrial scale (Indumape S.A., Portugal) following a milling, enzymatic digestion and pressing process for at least 3 h. The obtained apple pomace was frozen in liquid nitrogen, milled and stored at -20 ºC until freeze-drying and further analysis. Water/acetic acid (99/1; v/v), pH 2.5, was added to the apple pomace at a ratio of 1:5 (w/v) and extracted at 100 ºC for 1 h (**Figure 1**). The resulting suspension was filtrated in a glass microfibre filter (MFV3, Filter Lab) followed by a G3 sintered funnel and submitted to three additional aqueous extractions. The water-soluble fractions were combined, concentrated on a rotary

evaporator at 40 ºC to reduce the volume of water, frozen and freeze-dried. The obtained residue was sequentially extracted following a similar procedure than before, first using five times acetone/water/acetic acid (60/39/1; v/v/v) mixture, and then acetone/8 M urea/acetic acid (60/39/1; v/v/v) mixture. The filtrates from the 60% acetone extraction were combined, evaporated under reduced pressure at 40 ºC to remove acetone and freeze dried. In turn, the acetone/8 M urea/acetic acid filtrate was rotary evaporated to remove the acetone, resuspended in water/acetic acid (98/2; v/v) and subjected to solid-phase extraction with C18 sep-pak cartridges (SPE-C18, Supelco-Discovery, 20g), to remove urea. For that, the column was preconditioned with 20 mL of methanol followed by 20 mL of water and 20 mL of water/acetic acid (98:2; v/v). The sample was loaded into the cartridge, followed by washing with 140 mL of water/acetic acid (98/2; v/v). The retained fraction was obtained using acetone/acetic acid (98/2; v/v), concentrated under reduced pressure at 40 ºC to remove acetone, frozen and freeze-dried. Urea was removed from the hot water/acetone/acetone 8 M urea insoluble cell wall material by extensive dialysis (12-14 kDa cut-off membrane, Medicell), followed by freezing and freeze-drying.

The freeze-dried hot water/acetone/acetone 8 M urea insoluble cell wall material was submitted to microwave superheated water (MWE) extraction using a MicroSYNTH Labstation (Milestone srl., Bergamo, Italy) equipment with high pressure teflon-coated vessel of 100 mL capacity. A control reactor with pressure and temperature sensors was used for monitoring the extraction experiments. 1000 W was defined as the maximum output delivery power until reaching the set point temperatures for the desired time. These included the optimal conditions for pectin extraction (liquid/solid ratio of 14:1 at 140 ºC for 5 min and continuous stirring) as determined by **Wang and Lü (2014)** (Cycle 1). The reactor was cooled at room temperature, and the retentate obtained after filtration was subjected to a second extraction under the same conditions (Cycle 2). The insoluble cell

wall material at 140 ºC was suspended in water using a liquid/solid ratio of 28:1 and subjected to microwave superheated water at 180 ºC for 2 min (Cycle 3), the optimal condition for xyloglucan extraction determined by **Coelho et al. (2014)**. After cooling, the slurry was filtrated, the final residue frozen and freeze-dried. Cycle 1, 2 and 3 freeze-dried replicates were combined and solubilized in water at a concentration of 10 mg/mL at 4 ºC. The insoluble material (Ins4C) was recovered by centrifugation at 20,000*g* at 4 ºC. To the water-soluble material, absolute ethanol was added to reach 50% (v/v) and centrifuged yielding a precipitate (Et50Pp). To the 50% (v/v) soluble material, absolute ethanol was added to reach 80% (v/v). The solution was then centrifuged yielding a precipitate (Et80Pp) and a supernatant (Et80Sn). All fractions were evaporated under reduced pressure to remove ethanol at 45 ºC, frozen and freeze dried.

2.2. Isolation of procyanidin rich-fractions from apple and apple pomace

Procyanidin were isolated following the general procedure preciously described by **Guyot, Doco, Souquet, Moutounet, and Drilleau (1997)**. Briefly, about 150 g of freeze-dried industrial apple pomace or Golden Delicious apple (90% of parenchyma and 10% of skins) were defatted with 750 mL of hexane for 15 min and filtered in a G3 funnel (**Figure 1**). The filtrate was discharged while the residue was extracted with 750 mL of methanol/acetic acid (99/1; v/v) for 15 min, followed by filtration of the suspension in a sintered glassware G3 funnel. The resulting residue was reextracted two additional times following the same procedure. At the end, the filtrates from the methanolic suspensions were combined, rotary-evaporated and freeze dried. The final residue of the methanolic extractions was extracted with 750 mL of acetone/water milliQ/acetic acid (60/39/1; v/v/v) for 15 min for three times. The obtained hydroacetonic suspensions were filtered, evaporated, frozen a freeze-dried.

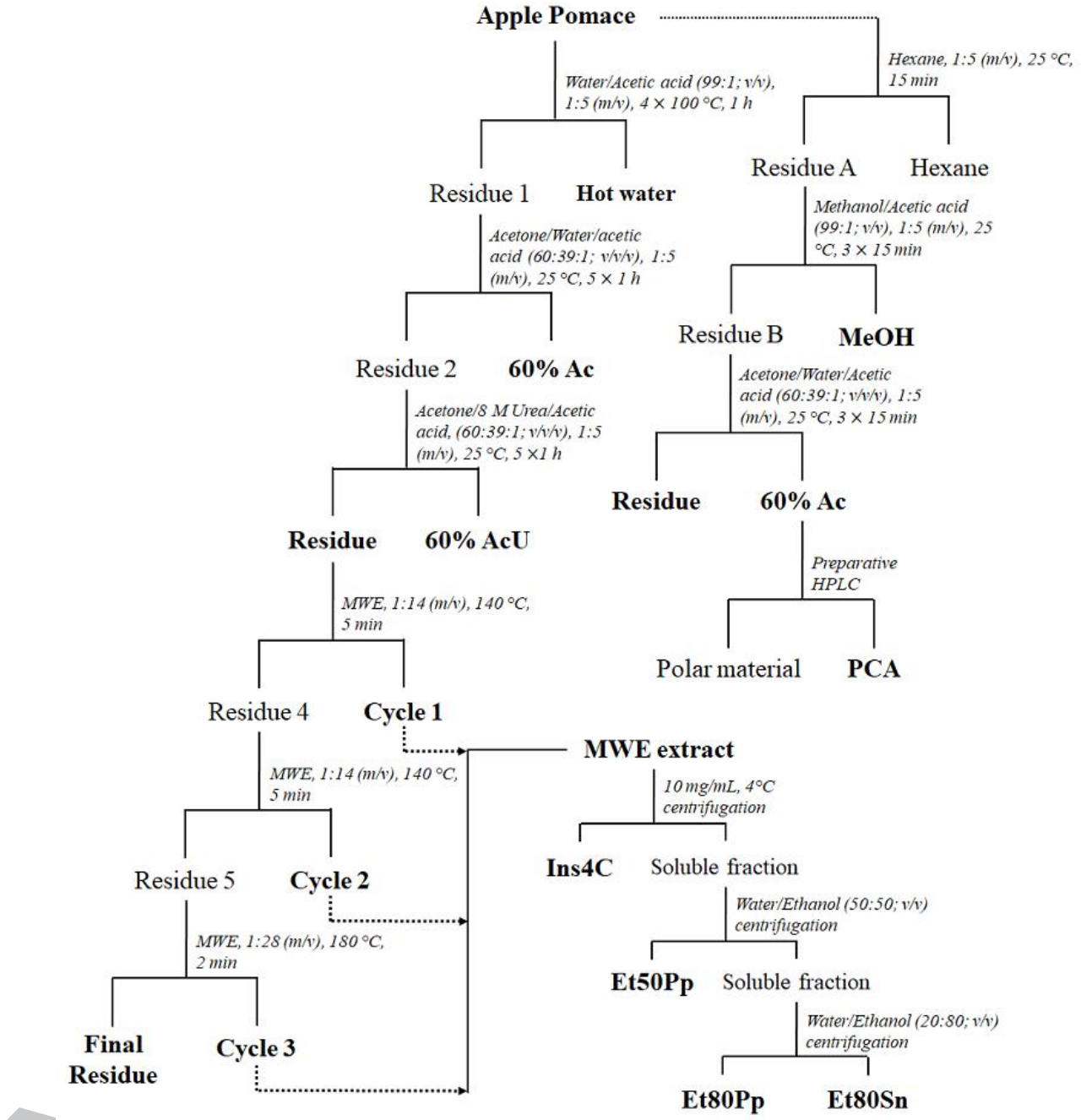


Figure 1 - Schematic representation of the extraction and fractionation procedures performed for apple pomace. In bold are assigned the fractions studied in this work.

The isolation of a procyanidin rich-fraction from the 60% acetone extract was performed by high performance liquid chromatography using a Hibar 205x25 mm Lichrospher 100 RP-18 12 µm (Merck, Darmstadt, Germany) column at preparative scale. The system was composed by two high pressure pumps PU-2077 plus, one degasser, one

interface LC-NetII/ADC, one injection valve rheodyne 3752i-038 (Rhonert Park, CA, USA) and a detector UV-visible UV-2077 plus, controlled by the software ChromNav (Jasco, Tokyo, Japan). The column was pre-conditioned with water/acetic acid (97.5/2.5; v/v) for 30 min at a flow of 35 mL/min and the detection performed at 280, 320 and 520 nm. The supernatant obtained after 60% acetone extract dissolution in 100 mL of water milliQ/acetic acid (97.5/2.5; v/v), 5 g per 100 mL, and centrifugation (16800g, 15 min), was then injected in the column and rinsed with the same solvent for 1 h for sugars removal. Procyanidins were then eluted at a flow of 25 mL/min using a gradient of water milliQ/acetic acid (97.5/2.5; v/v) (eluent A) and acetonitrile (eluent B). The gradient started with 0-30% B (0-5 min); 30-30 % B (5-20 min); 30-90% B (20-22 min); 90-90% B (22-27 min); 90-50% B (27-30 min). Polyphenols were collected by monitoring the absorbance at 280 nm and then rotary evaporated, frozen and freeze-dried.

2.3. Carbohydrate, protein and polyphenol analysis

Neutral sugars were determined after Saeman acid hydrolysis and derivatization to alditol acetates by gas chromatography with a flame ionization detector (GC-FID) following the general procedure described by **Ferreira, Passos, Cardoso, Wessel, and Coimbra (2018)**. Free sugars were determined without the hydrolysis step. Given the absence of free mannose in apple pomace **(Dhillon, Kaur, & Brar, 2013)**, fructose was quantified from the ratio that epimerized to mannitol (43%) during the reduction step **(Brunton, Gormley, & Murray, 2007)**. Free glucose was determined by difference between the total glucitol detected and the glucitol yielded by fructose reduction. Phenylphenol colorimetric method was used for uronic acids. Glycosidic-linkage analysis was performed by analysis of partially methylated alditol acetates (PMAA) using gas chromatography–mass spectrometry (GC–MS, GCMS-QP2010 Ultra, Shimadzu), as

described by **Coimbra, Delgadillo, Waldron, and Selvendran (1996)**. Briefly, glycosidic linkage analysis included two methylation steps in DMSO solution saturated with NaOH, dialysis against water/ethanol (50:50; v/v), solvent removal followed by acid hydrolysis with 2 M TFA (121 ºC for 1 h) of the dialysis retentates, reduction with deuterium borohydride and acetylation. Protein was estimated by determining total nitrogen in a Truspec 630-200-200 elemental analyzer with a thermal conductivity detector (TDC) and by using a conversion factor of 5.72 **(Sosulski & Imafidon, 1990)**. Total polyphenolic compounds were estimated by the Folin-Ciocalteu method following the general procedure described by **Singleton and Rossi (1965).** The results were expressed as phloridzin equivalents (g PLZE/kg) as representative of apple polyphenols and in gallic acid equivalents (g GAE/kg). Antioxidant activity was determined by the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) method **(Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999).** The results were expressed as µmol Trolox eq./g of sample.

Individual polyphenolic composition was determined by ultra high-performance liquid chromatography (UHPLC) coupled to a diode array detection (DAD) with or without thioacidolysis, method described by **Guyot, Marnet, Sanoner, and Drilleau (2001)**. Analyses were performed using an Ultra-Fast Liquid Chromatography Shimadzu Prominence system (Kyoto, Japan) controlled by a LC Solution software (Shimadzu, Kyoto, Japan). Separation were achieved using a Kinetex® column (100 x 4.6 mm, 2.6 µm, C_{18}, 100 Å) at 30 °C. The mobile phase consisted of water/formic acid (99:1; v/v) (eluent A) and acetonitrile (eluent B). The flow rate was 1 mL/min. The elution program was as follows: 2–2% B (0–1 min); 2–90% B (1–20 min); 90–90% B (20-25 min); 90–2% B (25–30 min); 2–2% B (30–35 min). Individual compounds were quantified by comparisons with external standards at 280 nm for (+)-catechin, (-)-epicatechin, phloretin

xyloglucoside (quantified as phloretin), phloridzin, (-)-epicatechin benzylthioether (quantified as (-)-epicatechin); at 320 nm for 5-caffeoylquinic acid, 4-*p*-coumaroylquinic acid (quantified as *p*-coumaric acid) and their methylated derivatives obtained during thioacidolysis reaction quantified as their respective non-methylated equivalents, and at 350 nm for quercetin glycosides (quantified as quercetin). The procyanidins average degree of polymerization (DPn) was determined by the molar ratio of all the flavan-3-ol units from thiolysis (thioether adducts plus terminal units) to (−)-epicatechin and (+)-catechin terminal units.

2.4. Identification of oxidation products

UHPLC-DAD-ESI-MS^n analysis of polyphenol oxidation products was performed on apple pomace procyanidins fractions using an Acquity Ultra performance LC (UHPLC) apparatus from Waters (Milford, MA, USA), equipped with a photodiode array detector (detection at 280, 320, 350 and 520 nm) coupled with a Bruker Daltonics (Bremen, Germany) HCT ultra ion trap mass spectrometer with an electrospray ionization source. Separations were achieved using a Kinetex® column (100 x 4.6 mm, 2.6 µm, C_{18}, 100 Å) operated at 30 °C, and using water/formic acid (99:1, v/v) (eluent A) and acetonitrile (eluent B) in the conditions previously described (**Section 2.3**). A capillary voltage of 2 kV was used in the negative ion mode for polyphenol analysis. Nitrogen was used as drying and nebulizing gas with a flow rate of 12 L/min. The desolvation temperature was set at 365 °C and the nebulization pressure at 0.4 MPa. The ion trap was operated in the Ultrascan mode from m/z 100 to 1000.

2.5. Alkaline fusion

Mixtures of 1 g of solid NaOH and 100 mg of zinc dust were weighed on a nickel crucible, and heated until 350 °C **(Coelho, Ribeiro, Cruz, Domingues, Coimbra, Bunzel, et al., 2014)**. After complete melting of the mixture, 10 mg of sample were added and left at 350 ºC for 10 s, followed by a rapid cooling on ice. The fusion cake was solubilized in 6 M HCl, and 200 µL of internal standard solution was added (3,4-dimethoxybenzoic acid, 1 mg/mL). After acidification to pH 1-2 with 6 M HCl, liquid/liquid extraction with 50 mL of diethyl ether was performed. The organic phase was then collected, rotary evaporated until complete dryness, redissolved in 2 mL of 50% methanol and analysed by HPLC (Dionex, Ultimate 3000) on a reversed-phase column (C18-ACE; 25 cm length, 0.45 cm internal diameter, and 5 µm particle diameter). Water/formic acid (95:5; v/v) (eluent A) and methanol (eluent B) were used as eluents. The eluent program was as follows: 5% eluent B (0−5 min); 5-40% B (5−45 min); 40-70% B (45−65 min); 70-5% B (65−75 min). The column temperature was set at 25 °C, and the flow was 0.8 mL/min. The eluent was continuously monitored from 200 to 600 nm with a photodiode array detector (PDA-100, Dionex). Standards 3,4-dihydroxybenzoic acid, catechol, gallic acid and 4-hydroxybenzoic acid were used for their identification and quantification on samples submitted to alkaline fusion in order to detect interferences from phenolic structures of any residual lignified material occurring in apple pomace.

2.6. Statistics

All chemical analyses were performed in triplicate unless otherwise stated. The reproducibility of the results is expressed as pooled standard deviation.

3. Results and Discussion

3.1. Apple pomace composition

On a dry weight basis, carbohydrates represented 670 g/kg of the apple pomace (**Table 1**), of which 50 g/kg were free sugars, mostly Fru (67 mol%) and Glc (33 mol%). Polysaccharides represented 620 g/kg, being the major monosaccharides Glc (44 mol %), GalA (25 mol%), Ara (14 mol%), Gal (7 mol%) and Xyl (7 mol%). This carbohydrate composition was in accordance with the polysaccharides reported for apple pomace, including cellulose, xyloglucans and pectic polysaccharides **(Mehrländer, Dietrich, Sembries, Dongowski, & Will, 2002)**. Protein represented 56 g/kg of apple pomace.

Polyphenols, determined by UHPLC-DAD after thioacidolysis, represented 5 g/kg of dry apple pomace and could be divided in four classes: flavan-3-ols, flavonols, dihydrochalcones and hydroxycinnamic acids. Flavan-3-ols were the major class (2.88 g/kg of dry apple pomace), with a number-average DP of 4.7 (**Table 2**), comparable to those usually found in apples **(Guyot, Le Bourvellec, Marnet, & Drilleau, 2002).** Flavonols (1.92 g/kg of dry apple pomace) were the second major class, being represented by hyperoside (58%), quercetin-pentosides (14%), quercitrin (13%) and isoquercetrin (9%). Quercetin and rutin represented less than 6% of the flavonol pool. Dihydrochalcones (0.14 g/kg of dry apple pomace) were the third polyphenolic class, only constituted by phloridzin. Flavonols and dihydrochalcones are generally abundant in apple skins and seeds, respectively **(Guyot et al., 2002)**, beyond pulp and stalks, constituting a significant part of apple pomace. Hydroxycinnamic acids, represented only by 5-caffeoylquinic acid, was the less abundant polyphenolic class with levels of 0.03 g/kg of dry apple pomace. This reflected a typical polyphenolic composition of apple pomace, although dihydrochalcones and hydroxycinnamic content were much lower than the values commonly reported **(Çam et al., 2010; Virot et al., 2010)**. Such differences can be explained by oxidation reactions catalysed by polyphenol oxidase, as 5-

caffeoylquinic acid, phloridzin and phloretin-xyloglucoside are substrates of this enzyme **(Wong-Paz, Muñiz-Márquez, Aguilar, Sotin, & Guyot, 2015)**.

3.2. Effect of hot water extraction on the polyphenolic profile of apple pomace

Hot water has been suggested for extraction of polyphenols as it is cheap, non-toxic, environmental friendly and provides an easily implementable process **(Çam et al., 2010)**. To prevent polyphenol oxidation diluted acetic acid solutions are usually used **(Ferreira, Guyot, Marnet, Delgadillo, Renard, & Coimbra, 2002)**. As represented in **Table 1**, 37% (w/w) of the apple pomace was recovered in the hot water extract which contained all free sugars and 43% of the polysaccharides present in the pomace. The occurrence of GalA (43 mol%) and Ara (23 mol%) showed that the extract was particularly rich in pectic polysaccharides. Total polyphenolic content, determined by the Folin-Ciocalteu method, was 28 g PLZE/kg (9 g GAE/kg). Such amounts were higher than those previously reported **(Çam et al., 2010)**, probably resulting from differences in the apple cultivars that originated the pomace **(Diñeiro García, Valles, & Picinelli Lobo, 2009)**. About 7 g/kg of polyphenols were detected by UHPLC-DAD after thioacidolysis (**Table 2**), represented mainly by flavonols (73%), flavan-3-ols (20%) with an average DP of 3.4, dihydrochalcones (6%), and hydroxycinnamic acids (1%). Their occurrence was associated to the observed antioxidant capacity, 33 mmol Trolox eq./kg as measured by the $ABTS^{\cdot+}$ method. Proteins corresponded to the minor component in the hot water extract, only accounting for 11 g/kg of extract.

Table 1 – Yield (%), carbohydrate composition (molar %), total carbohydrates (g/kg), protein (g/kg), total polyphenols (g phloridzin (PLZE) equivalents/kg) and antioxidant activity (mmol Trolox equivalents (TE)/kg) of the different apple pomace fractions. In parenthesis are presented that data in gallic acid equivalents (g GAE/kg) for total polyphenols. Data are expressed as Mean and pooled standard deviation (*Pooled Std*) of three replicates. n.d means for not determined.

Type of Extraction	Sample	Yield (%)	Carbohydrates (mol%)										Total Carbohydrates (g/kg)	Protein (g/kg)	Total Polyphenols (g PLZE/kg)	Antioxidant activity (mmol TE/kg)
				Rha	Fuc	Ara	Xyl	Man	Fru	Gal	Glc	GalA				
Water/acetone	Apple Pomace		Polysac.	1	1	14	7	1	-	7	44	25	622	56	n.d	n.d.
			Free Sugars	-	-	-	-	-	67	-	33	-	49			
	Hot Water	37	Polysac.	1	tr	23	3	-	-		24	43	721	11	28 (9)	33
			Free Sugars	-	-	-	-	-	68	-	32	-	116			
	60%Ac	2.4	Polysac.	tr	tr	50	tr	-	-	13	17	20	103	36	78 (29)	95
	60%AcU	2.2	Polysac.	-	-	-	-	-	-	-	-	-	-	-	42 (15)	23
	Residue	50	Polysac.	1	1	4	12	4	-	7	60	11	615	85	n.d	n.d
Microwave superheated water	Cycle 1	4.8	Polysac.	2	2	20	8	1	-	14	26	26	906	n.d.	28 (11)	n.d.
	Ins 4C	0.8	Polysac.	1	-	1	-	-	-	-	73	24	954	n.d	9 (3)	n.d
	Et50Pp	0.1	Polysac.	2	2	14	9	1	-	14	14	44	953	n.d	19 (7)	n.d
	Et80Pp	2.0	Polysac.	1	1	8	14	1	-	21	13	40	901	n.d	10 (3)	n.d
	Et80Sn	1.8	Polysac.	3	4	48	3	1	-	10	4	26	931	n.d	49 (18)	n.d
	Cycle 2	2.1	Polysac.	3	3	12	10	3	-	13	22	35	937	n.d.	53 (20)	n.d.
	Ins 4C	0.2	Polysac.	1	-	1	1	-	-	1	78	17	866	n.d	21 (8)	n.d
	Et50Pp	0.1	Polysac.	2	2	4	10	1	-	13	28	40	901	n.d	24 (8)	n.d
	Et80Pp	1.0	Polysac.	2	1	5	15	3	-	19	15	39	878	n.d	13 (5)	n.d
	Et80Sn	0.8	Polysac.	3	8	42	5	4	-	12	7	20	699	n.d	98 (39)	n.d
	Cycle 3	6.0	Polysac.	6	6	5	24	11	-	15	19	16	820	n.d.	108 (43)	n.d.
	Ins 4C	0.1	Polysac.	2	6	5	22	9	-	13	22	22	493	n.d	138 (55)	n.d
	Et50Pp	0.3	Polysac.	-	1	1	24	2	-	10	39	23	918	n.d	25 (10)	n.d
	Et80Pp	0.6	Polysac.	1	-	1	20	3	-	14	34	27	952	n.d	21 (9)	n.d
	Et80Sn	4.7	Polysac.	2	7	6	25	14	-	15	15	15	673	n.d	136 (56)	n.d
	Final Residue	35	Polysac.	-	-	-	9	3	-	3	78	6	534	n.d.	n.d.	n.d.
	Pooled std	*0.4*		*0*	*0*	*0*	*0*	*0*	*2*	*0*	*0*	*1*	*7*	*1*	*0 (0)*	*2*

Table 2 – Extraction yields (%), individual polyphenolic composition (g/kg) and total polyphenols (g/kg) of dry apple pomace and apple extracts, as determined by HPLC-DAD. Data are expressed as Mean and pooled standard deviation (Pooled Std) of three replicates.

		Sample	Yield	Flavan-3-ols				Dihydrochalcones		Hydroxycinnamic acids		Flavonols						Total
				CAT	EPI	PCA	DPn	PLZ	XPL	CQA	PCQ	Ru	Hy	Iso	Pent	Quc	Qu	
Apple Pomace	Sequential extractions	Apple Pomace	-	-	-	2.9	4.7	0.1	-	tr	-	tr	1.1	0.2	0.3	0.3	0.1	4.9
		Hot Water	37	-	-	1.3	3.4	0.4	-	0.1	-	0.1	2.6	0.4	0.4	0.8	0.6	6.7
		60% Ac	2.4	-	-	1.1	16	0.2	-	0.1	-	0.1	1.6	0.3	0.2	0.5	4.3	8.3
		60% ACU	2.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		Residue	50	-	-	1.9	6.8	-	-	-	-	-	-	-	-	-	-	1.9
	Polyphenol isolation	MeOH	12	-	-	1.4	3.3	1.0	-	0.2	-	0.2	6.2	1.2	1.6	1.6	0.5	14
		60% Ac	5.9	-	-	17.6	5.7	0.6	-	-	-	0.2	4.0	0.7	0.7	0.8	0.2	25
		AP PCA	0.1	-	-	235	5.8	11	-	0.8	-	4.1	72	8.7	12	15	2.1	360
		Residue	77	-	-	2.5	5.4	-	-	-	-	-	-	-	-	-	-	2.5
Apple	Polyphenol isolation	Apple	-	tr	1.0	11	4.4	0.4	0.2	2.5	0.2	tr	0.2	0.1	0.1	0.2	-	16
		MeOH	57	0.1	1.3	7.7	2.5	0.6	0.2	2.7	0.3	tr	0.3	0.1	0.2	0.3	-	14
		60% Ac	6.9	-	1.8	79	5.5	0.6	0.1	3.9	0.3	tr	1.2	0.3	0.3	0.3	-	88
		Apple PCA	0.4	-	16	912	7.3	4.9	-	26	2.6	0.2	12	2.4	2.7	2.5	-	965
		Residue	35	-	-	2.3	9.9	-	-	tr	tr	-	-	-	-	-	-	2.3
		Pooled std		*0.0*	*0.2*	*1.6*	*0.1*	*0.0*	*0.0*	*0.1*	*0.0*	*0.0*	*0.1*	*0.0*	*0.0*	*0.1*	*0.0*	*1.6*

CAT - (-)-Catechin; EPI - (-)-Epicatechin; PCA – Procyanidins. DPn – Flavan-3-ols average degree of polymerization; PLZ – Phloridzin; XPL – Phloretin-xyloglucoside; CQA - 5-cafeoylquinic acid; PCQ - Para-coumaroylquinic acid; RU – Rutin; HY – Hyperoside; ISO – Isoquercitrin; PENT – Quercetin pentoside (Reynoutrin+Guajaverin+Avicularin); QUC – Quercitrin; QU -Quercetin; AP PCA – Apple pomace procyanidins; Apple PCA – Apple procyanidins.

To determine the polyphenols still retained in the water-insoluble cell wall material, 60% acetone extractions were performed. The material recovered represented 2.4% (w/w) of the initial apple pomace, composed by a low amount of carbohydrates 103 g/kg, particularly rich in Ara (50 mol%). Total polyphenols analysis revealed the additional presence of 78 g PLZE/kg (29 g GAE/kg) of polyphenols, of which 8 g/kg were identified by UHPLC-DAD. Flavonols represented 83% of the total identified polyphenols while flavan-3-ols, dihydrochalcones and hydroxycinnamic acid represented 14, 2 and 1%, respectively. In contrast, the following extraction with 60% acetone/8 M urea, did not allow to recover any carbohydrates nor polyphenols as determined by GC-FID and UHPLC after thioacidolysis, respectively. However, the observed 42 g PLZE/kg (15 g GAE/kg) by the Folin-Ciocalteu method as well as the measured antioxidant activity by the $ABTS^{\cdot+}$ method (23 mmol TE/kg) suggested the presence of polyphenols. This allows to infer the occurrence of oxidised structures with bonds resistant to thioacidolysis **(Le Deun et al., 2015; Mouls et al., 2015)**.

Carbohydrate analysis of the residue obtained after the extraction with 60% acetone/8 M urea, which represented 50% of the initial apple pomace, demonstrated a high prevalence of Glc (60 mol%), Xyl (12 mol%), GalA (11 mol %) and some Gal (7 mol%), Ara (4 mol%) and Man (4 mol%). Glycosidic linkages analysis (**Figure S1** and **Table S1**) revealed the occurrence of (1→4)-Glc*p* (54 mol%), typical of cellulose. Furthermore, (1→4)-Glc*p*, together with (1→4,6)-Glc*p* (12%), (1→2)-Xyl*p* (3.6%), (1→2)-Gal*p* (1.3%), t-Xyl*p* (5.7%), t-Gal*p* (2.4%) and t-Fuc*p* (1.1%), were diagnostic linkages of xyloglucan. In fact, the proportion the side chains residues ((1→2)-Xyl*p*, (1→2)-Gal*p*, t-Xyl*p*, t-Gal*p* and t-Fuc*p*) explained 9.3 out of 11.7% of the (1→4,6)-Glc*p*, supporting the xyloglucan structure **(Mehrländer et al., 2002; Stevens & Selvendran, 1984)**. The remaining 2.4% of (1→4,6)-Glc*p* could be explained by the t-Glc*p*, indicative

that some residual starch might be present. Galactose, represented by (1→4)-Gal*p* (3.1%) and (1→3,4)-Gal*p* (0.5%), pointed out also to the possible occurrence of galactans branched to a small extent at C-3 **(Stevens et al., 1984)**. The (1→5)-Ara*f* (2.4%), (1→3,5)-Ara*f* (1.5%), (1→2,5)-Ara*f* (0.4%) and t-Ara*f* (2.1%), characteristic of side chain arabinans of pectic polysaccharides indicated that these polysaccharides were also relevant in the residue. The proportion of (1→5)-Ara*f* to (1→3,5)-Ara*f*, (1→2,5)-Ara*f* and t-Ara*f* supported the occurrence of a highly branched arabinan structure **(Renard, Voragen, Thibault, & Pilnik, 1991)**. UHPLC-DAD after thioacidolysis also demonstrated the presence of flavan-3-ols, 2 g/kg with a DP of 6.7, that were not able to be extracted.

The mass balance performed to the sequential extraction demonstrated a decrease in about 49% of the procyanidins (**Figure 2 a**) without impact on their average DP (4.7). A similar decrease is observed for pear procyanidins submitted to 95 ºC for 480 min **(Le Bourvellec, Gouble, Bureau, Loonis, Plé, & Renard, 2013)**, attributed to procyanidin depolymerization followed by thermal degradation caused by the hot acidic conditions. The levels of quercetin-glycosylated derivatives also presented an average loss of 15%. Nevertheless, as is described to occur with flavonol-glucosides of onion submitted to 180 ºC **(Rohn, Buchner, Driemel, Rauser, & Kroh, 2007)**, they were mainly converted to their aglycone form, as shown by the 5-fold increase in the amount of recovered quercetin, when compared to that initially present in apple pomace. Their molar balance demonstrated that they remained non-degraded alongside with dihydrochalcones and hydroxycinnamic acids (**Figure 2 a**). This resulted in an overall balance of 74% of the polyphenols in apple pomace and a shift of the polyphenolic pool towards the prevalence of flavonols (**Figures 2 b**).

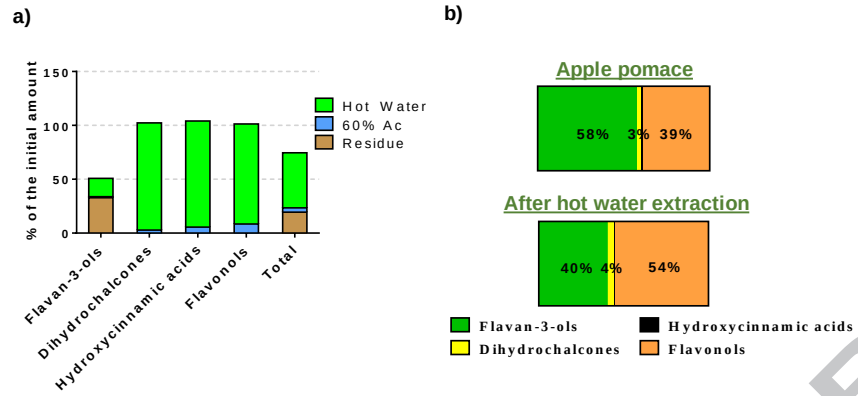


Figure 2 – Relative a) amount of the different polyphenolic classes after hot water/60%acetone/60%Acetone 8M urea extractions in relation to their initial amount in apple pomace, and b) proportion of apple pomace polyphenolic classes before and after the sequential extractions.

The difference in the polyphenolic content as determined by the Folin-Ciocalteu method and the UHPLC-DAD analysis, alongside the very low amounts of dihydrochalcones and hydroxycinnamic acids, suggested that oxidised polyphenolic structures could be present in the different fractions. To test this hypothesis, UHPLC-DAD-ESI-MS^n of purified polyphenol fractions was performed. Furthermore, as procyanidins oxidation may result in the formation of new linkages with resilience to thioacidolysis, alkaline fusion, a technique shown to be useful in the analysis of polyphenolic condensed structures in coffee melanoidins **(Coelho et al., 2014)** was performed to better estimate the polyphenols remaining in the residue obtained after hot water/acetone/acetone 8 M urea sequential extractions.

3.3. Occurrence of extractable polyphenol oxidation products

To study the existence of extractable polyphenol oxidation products, apple pomace was extracted with hexane, to remove fats and oils, methanol to remove most of the sugars and organic acids and the 60% acetone (**Figure 1**). The latter fraction was then submitted to semi-preparative HPLC to remove remnant sugars and organic acids, yielding an extract composed of 360 g/kg of polyphenols of which 65% corresponded to procyanidins

with and average DP of 5.8 (**Table 2**). Since oxidation reactions of polyphenol occur within and between polyphenol classes, leading to the formation of multiple oxidation products, each in very low concentration, HPLC-DAD-ESI-MS^n analysis of the purified fraction was performed in SIM mode, at *m/z* of known oxidation products formed in model solutions and known to occur in oxidised juice samples **(Bernillon et al., 2004)**.

The first eluting detected oxidation product, at RT of 5.8, presented a $[M-H]^-$ at *m/z* 597 (**Figure S2 a**), forming three major fragments (**Table 3**), at *m/z* 553, 303 and 259. The first fragment corresponded to the loss of 44 Da, justified by the elimination of a CO_2 molecule of a carboxylic group, while the second corresponded to the loss of 294, a typical loss of the Xyl-Glc moiety of phloretin xyloglucoside found in apples **(Le Deun et al., 2015)**. The latter can be attributed to the loss of 338, outcoming from both CO_2 and Xyl-Glc moieties. This compound was identified as an oxidation product of phloretin-xyloglucoside (**Figure S1 d**), present in apple juices submitted to oxidative conditions **(Le Deun et al., 2015).** At RT of 5.9 min (**Figure S1 b**), the $[M-H]^-$ at *m/z* 465 was detected. The ESI-MS^2 spectrum of that ion (**Table 3**) revealed a similar fragmentation pattern with the one detected at 5.8 min. Namely, the main signal was obtained from the loss of 44 Da (ion at *m/z* 421), followed by the loss of 162 (ion at *m/z* 303) attributed to a hexose, probably the Glc unit of phloridzin **(Guyot, Serrand, Le Quéré, Sanoner, & Renard, 2007)**. A loss of 206 (ion at *m/z* 259) was also observed, attributed to the loss of both molecules. These results were in agreement with the fragmentation patterns of a final oxidation product of phloretin-glucoside (**Figure S1 e**) reported by **Guyot et al. (2007)**. Both these molecules represent coloured oxidation products, being one of the contributors to yellowish/brownish colour of apple pomace and its extracts.

Four additional oxidation products were detected at RT 6.3, 6.4, 6.8 and 7.3 min (**Figure S1 c**), with a $[M-H]^-$ at *m/z* 705. Their ESI-MS^2 fragmentation pattern showed a

strong ion signal at *m/z* 513, corresponding to the loss of 192 generally attributed to the loss of quinic acid (**Table 3**). ESI-MS^3 analysis showed that these four components could be divided in two groups according with their fragmentation pattern. ESI-MS^3 analysis of the compounds eluting at 6.3 and 6.4 min, group A, showed major ions at *m/z* 339, 495, 321 and 295. These corresponded to a loss of 174, 18, 192 and 218, attributed to the loss of deoxyquinic acid, H_2O, quinic acid and deoxyquinic acid jointly with CO_2, respectively. This fragmentation pattern was indicative that they represent isomers with probable structural similarities of previously identified dihydrobenzofuran type oxidation (**Figure S2 f**) products from 5-cafeoylquinic acid **(Bernillon et al., 2004)**. Compounds eluting at 6.8 and 7.3 min showed also similar fragmentation patterns yielding ions at *m/z* 339 and 311, corresponding to a loss of 174 attributed to deoxyquinic acid and 202 attributed to the loss of both deoxyquinic acid and CO. An ion at *m/z* 161, was also observed, attributed to the caffeic acid moiety after loss of quinic acid **(Bernillon, 2005)**. This fragmentation pattern was indicative that these components represent 5-caffeoylquinic acid oxidation products of dihydrobenzodioxan types (**Figure S2 g**) **(Bernillon, 2005; Bernillon et al., 2004)**. Their formation is proposed to result from a two electrons oxidative coupling with the simultaneous loss of two hydrogen atoms **(Wong-Paz et al., 2015)**.

3.4. Occurrence of non-extractable oxidised polyphenols

Alkaline fusion is a technique that can be used to provide structural features of unknown polyphenolic fractions **(Gramshaw, 1968)**. This is achieved by the capability of the strong alkaline medium to induce flavonoids C-ring opening and cleavage with formation of phloroglucinol (A-ring) and catechol (B-ring). In the latter, for instance, 3,4-dihydroxybenzoic acid and catechol might be formed **(Coelho et al., 2014; Gramshaw,**

1968). These products, alongside with gallic acid and 4-hydroxybenzoic acid can also be formed by alkaline fusion of lignin due to the presence of syringyl, p-hydroxyphenyl and guaiacyl units found in lignin **(Batistic & Mayaudon, 1970)**. However, as most of the so-called apple pomace lignin (measured as "Klason lignin" i.e. material insoluble in concentrated sulfuric acid) is represented by polyphenol oxidation products formed during juice extraction **(Renard, Lemeunier, & Thibault, 1995)**, 3,4-dihydroxybenzoic acid and catechol can be used as fingerprints for identification and quantification of non-extractable polyphenols.

Table 3 – Retention time (RT) and mass spectrum (MS) of extractable polyphenol oxidation products found in apple pomace procyanidin rich fraction (AP PCA). MS^n fragments are expressed in decreasing order in relation to their intensity.

Nº	RT (min)	*m/z* $[M-H]^-$	MS^n	Tentatively identified as
1	5.8	597	MS^2 [597]: **553**, 303, 259	Phloretin-xyloglucoside oxidation product
2	5.9	465	MS^2 [465]: **421**, 303, 259	Phloridzin oxidation product
3	6.3	705	MS^2 [705]: **513** MS^3 [513]: **339**, 495, 321, 295	Caffeoylquinic acid oxidation product A_1
4	6.4	705	MS^2 [705]: **513** MS^3 [513]: **339**, 495, 321, 295	Caffeoylquinic acid oxidation product A_2
5	6.8	705	MS^2 [705]: **513** MS^3 [513]: **339**, 311, 161	Caffeoylquinic acid oxidation product B_1
6	7.3	705	MS^2 [705]: **513** MS^3 [513]: **339**, 311, 161	Caffeoylquinic acid oxidation product B_2

Since (-)-epicatechin is known to be a precursor of coloured oxidation products **(Nicolas et al., 1994)** and the main monomeric unit of apple procyanidins, this compound was used as standard for assessment of the products resulting from alkaline fusion. Furthermore, as procyanidins were the major polyphenolic family present in the apple pomace final extraction residue, procyanidins from fresh fruits, structurally equivalent to those found in Royal Gala, were obtained and tested as representative of native, non-

oxidized procyanidins. This fraction was particularly rich in procyanidins (930 g/kg), with a DP of 8, and residual amounts of hydroxycinnamic acids (3%), flavonols (2%) and dihydrochalcones (1%). Isolated apple pomace procyanidins were also tested as representative of oxidized procyanidin fractions given the detection of oxidation products already shown in this study. In this extract, procyanidins only accounted 235 g/kg, a much lower content than the one obtained from fresh Golden Delicious. This was attributed to polyphenol oxidation that impairs procyanidin quantification **(Mouls et al., 2015)**.

As represented in **Figure 3 a** and **Table S2**, alkaline fusion of (-)-epicatechin and apple procyanidins produced 3,4-dihydroxybenzoic acid (0.67 mol/kg) and catechol (0.15 mol/kg) in similar amounts. As the formation of 3,4-dihydroxybenzoic acid and catechol is equimolar to the amount of (-)-epicatechin, it was estimated a yield of about 24% (w/w), possibly resulting from side reactions during alkaline fusion. In contrast, 0.12 mol/kg for both 3,4-dihydroxybenzoic acid and catechol were formed when performing alkaline fusion of apple pomace procyanidins. This represented a yield of 7% (w/w), indicative that during procyanidins oxidation new bonds and structural rearrangements occur, turning them less prone to yield 3,4-dihydroxybenzoic acid and catechol when submitted to alkaline fusion. This is supported by the formation of oxidation products of procyanidins involving the catechol B-ring **(Mouls et al., 2015)**, as well as by the capability of the A-ring of one (-)-epicatechin unit to react with the B-ring via condensation reactions when submitted to extensive oxidative conditions **(Guyot, Vercauteren, & Cheynier, 1996)**.

Alkaline fusion of the residue after hot water/acetone/acetone 8 M urea sequential extractions demonstrated the formation 3,4-dihydroxybenzoic acid and catechol, 0.04 and 0.08 mol/kg, respectively. This represented a yield of 3.7% (w/w), 19-fold higher than the amount observed by UHPLC-DAD after thioacidolysis. Additionally, extrapolating

these non-extractable polyphenols to the initial apple pomace samples, they corresponded to at least 1.8% of the by-product, more than 4-fold than those extracted with aqueous organic solvents and 17-fold than the non-extractable amount determined by UHPLC-DAD after thioacidolysis. This amount might be underestimated, given the yields obtained after alkaline fusion of (-)-epicatechin and procyanidins.

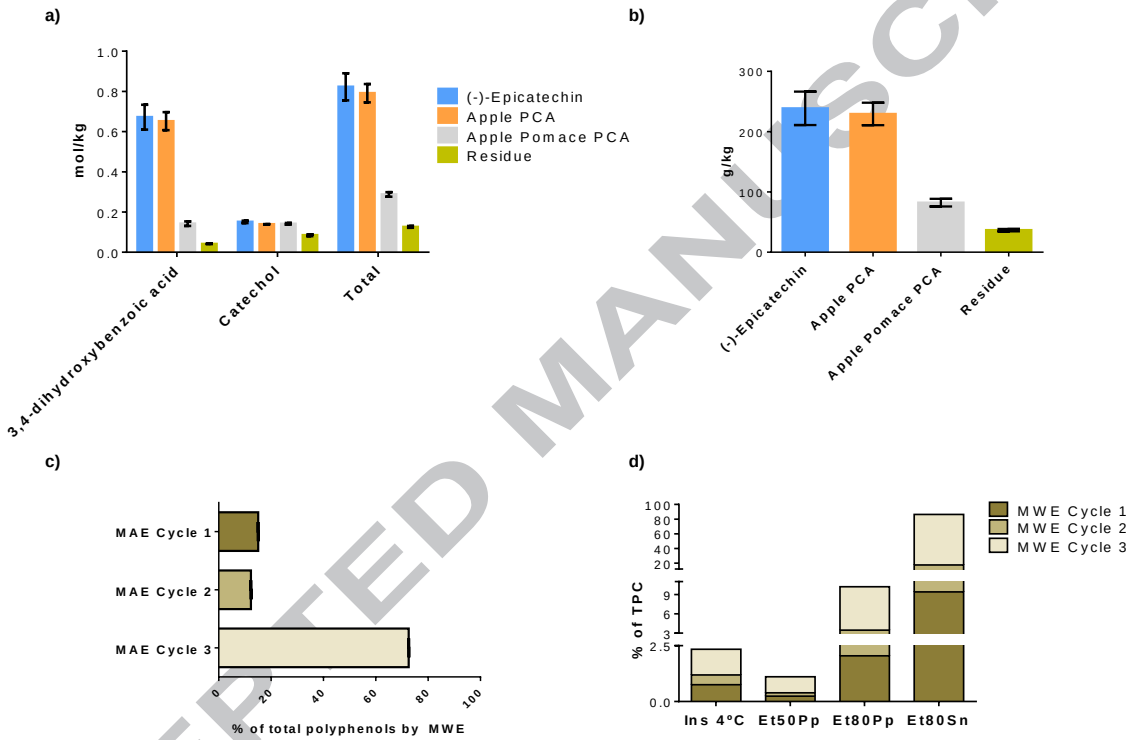


Figure 3 - Estimated non-extractable polyphenols a) in mol/kg dry weight and b) g/kg dry weight as determined by alkaline fusion of (-)-epicatechin, apple procyanidins (Apple PCA), apple pomace procyanidins (Apple pomace PCA) and hot water/acetone/acetone 8 M urea extraction residue. Relative distribution of non-extractable polyphenols among the different fractions c) recovered by superheated hot water extraction d) and their ethanol precipitates.

The ratio between the amount of 3,4-dihydroxybenzoic acid and catechol formed for (-)-epicatechin and apple procyanidins was about 4.4 while this was close to 1.0 and 0.5 for apple pomace procyanidins and residue, respectively. This, complemented by the data obtained by HPLC-DAD after thioacidolysis, suggested that the polyphenols remaining in the residue are more closely related to oxidised procyanidins than to native

apple (-)-epicatechin and procyanidins. However, it is important to note that such nature and amount may depend on the extent and processing conditions, more precisely the extension in which the pomace is exposed to oxygen involved in polyphenol oxidation reactions catalysed by polyphenoloxidase. Such conditions results in the formation of structures responsible for the observed brownish coloration **(Le Deun et al., 2015)** and that were found to be present in the pomace and its residue obtained after hot water extraction.

3.5. Carbohydrate structures associated to non-extractable oxidised polyphenols

Cellulose, xyloglucans, pectic polysaccharides and protein have been shown to interact with procyanidins by hydrogen bonding or hydrophobic interaction **(Renard et al., 2017)**. Their non-extractability was indicative that polyphenols were irreversibly bound to the polysaccharide and/or protein matrix that composes the residue, very probably by covalent linkages **(Pérez-Jiménez et al., 2013)**. In order to estimate to which polysaccharides, the non-extractable oxidised procyanidins were associated, microwave superheated water extraction (MWE) was performed on the hot water/acetone/acetone 8 M urea insoluble cell wall material in a three-cycles microwave sequential extraction. Two cycles were performed at 140 ºC for 5 minutes, the optimal conditions estimated for pectin extraction **(Wang et al., 2014)**, while the third cycle was performed at 180 ºC for 2 minutes, the optimal extraction conditions for xyloglucans **(Coelho et al., 2014)**. Polyphenols were assessed by the Folin-Ciocalteu method in order to account both native and oxidized procyanidins.

As represented in **Table 1**, the two cycles of MAE at 140 ºC extracted additional 5 and 2% (w/w) of the apple pomace. The extracts were composed for more than 900 g/kg of carbohydrates and the high prevalence of GalA (26-35 mol%), Ara (12-20 mol%) and

Gal (13-14 mol%) pointed out to the prevalence of pectic polysaccharides. In the MWE at 180 ºC, 6% of the initial pomace were obtained, composed mainly of carbohydrates (820 g/kg). The high prevalence of Xyl (24 mol%), Glc (19 mol%), Gal (15 mol%) and Fuc (6 mol%) indicated that xyloglucans corresponded to the main polysaccharides. The observed differences between the microwave extracts in terms of their polysaccharide composition is explained by the higher solubility of xyloglucans at temperatures above 150 ºC, through promotion of hemicellulose network degradation **(Wang, Gao, Liu, Wang, Yin, & Lü, 2018)**. In all these fractions, significant amounts of polyphenols were detected, ranging from 28 to 108 g PLZE/kg. Of the total amount of recovered polyphenols, 77% were obtained in the xyloglucan rich-fractions, against the 23% recovered in the pectic-rich fractions (**Figure 3 c** and **Table S3)** suggesting that a relevant amount of non-extractable oxidised procyanidins were strongly associated to the xyloglucan matrix. In order to consolidate the occurrence of such carbohydrate/polyphenol complexes, the different microwave extracts were subject to graded ethanol precipitation with the principle that if they were not bond to polysaccharides, polyphenols would remain soluble in the ethanol supernatants.

As shown in **Table 1**, ethanol precipitation of each of the MWE extracts yielded four distinct fractions corresponding to material insoluble in water at 4 ºC (Ins4C), insoluble in 50% ethanol (Et50Pp), insoluble in 80% ethanol (Et80Pp) and soluble in 80% ethanol (Et80Sn). Overall, these fractions presented a carbohydrate content ranging from 500-950 g/kg. Only 14% of the polyphenols were detected in the precipitates (**Figure 3 d** and **Table S3)**, mainly in the Et80Pp (10.2%), followed by the Ins4C (2.3%) and Et50Pp (1.1%), supporting the hypothesis that polyphenols were attached to the carbohydrate structures. Of these, it can be highlighted the Ins4C derived from the extract obtained at 180 ºC for 2 min that presented 138 g PLZE/kg of polyphenols along with Xyl (22 mol%),

Glc (22 mol %), Gal (13 mol%) and Fuc (6 mol%), supporting that the polyphenols were attached to xyloglucans. The additional occurrence of GalA (22 mol%) in this fraction indicated that it may correspond to a pectic-xyloglucan complex, described to occur in apple **(Stevens et al., 1984)**, in which polyphenols may act as the linking bridges between pectic polysaccharides and xyloglucans, as suggested by **Le Bourvellec et al. (2009)**.

Most of the polyphenols, alongside with a significant fraction of the extracted polysaccharides, remained soluble in 80% ethanol. Given their non-extractability with the aqueous/acetone solutions, it is very likely that they also occurred as covalent polysaccharide/polyphenol complexes. The suggested capability of procyanidins to reinforce the interactions between pectic polysaccharides and cellulose **(Le Bourvellec et al., 2009)**, as well as the occurrence of polyphenols in the Glc-rich fractions obtained by MWE and Ins4C, indicated that in the final residue, composed of 534 g/kg of carbohydrates, mainly Glc (78 mol%) and residual Xyl (9 mol%) and GalA (6 mol%), some polyphenols may still remain to be extracted as a result of their involvement as cross-linkers between cell wall polymers.

So far, only indirect evidences of covalent adducts between polyphenols and polysaccharides, with exception for those occurring by biosynthesis as for ferulic acid in sugar-beet cell walls **(Levigne et al., 2004)**, have been demonstrated by irreversible polyphenol/polysaccharide complexes, as in sun-dried pear **(Ferreira et al., 2002)** or wine polymeric material **(Gonçalves, Fernandes, Wessel, Cardoso, Rocha, & Coimbra, 2018).** These covalent adducts might be mediated by coupled oxidation/reduction reactions between procyanidins and caffeoylquinic acid *o*-quinones, in which the newly formed procyanidin *o*-quinones may nucleophilically react with other compounds, forming covalent adducts, as observed for proteins **(Trombley, Loegel, Danielson, & Hagerman, 2011)**. This mechanism seems also to occur with apple cell

wall polysaccharides **(Le Bourvellec et al., 2009).** The type of linkages involved in these carbohydrate/polyphenol complexes still remains unknown but the increase in the yield of pectic material extracted from oxalate-depectinized apple, kiwi and onions by chlorite/acetic acid treatment, in comparison to alkali saponification, suggests also the presence of phenolic ether linkages involved in carbohydrate/polyphenol complexes **(Stevens et al., 1984)**. The apple pomace carbohydrate/polyphenol complexes may represent up to 40% of the total polyphenols (**Figure S3**) that can be obtained. It is expected that they present distinct properties of the compounds alone. For instance, the polyphenol/carbohydrate complexes present higher *in vitro* fermentability, as measured by the increased short-chain fatty acid production, as well as polyphenol derived metabolites rather than free occurring polyphenols **(Pérez-Jiménez et al., 2013)**.

4. Conclusion

In this work it was shown that hot water extraction allowed to recover apple native polyphenols together with oxidized polyphenols derived from dihydrochalcones and hydroxycinnamic acids. Non-extractable oxidized procyanidins were also present, partially recovered by MWE. This non-extractability can be attributed to their possible linkage to apple xyloglucans and pectic polysaccharides, as suggested by MWE of the hot water/60%acetone/60%acetone 8M urea insoluble material and ethanol fractionation of the obtained extracts. This work paves the way to a more detailed structural characterization considering the level of oxidation of the pomace, able to identify the structures of these complexes. Alongside with the understanding of the potential biological and functional properties of the complexes, this knowledge will allow to design more diversified solutions for agro-food waste valuation.

5. Acknowledgements

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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:

No conflict of interests

Highlights:

- Flavan-3-ols were the main apple native polyphenols in apple pomace;
- Hot water extraction reduced flavan-3-ols in 50%;
- Oxidation products of polyphenols were part of the polyphenolic pool;
- Non-extractable oxidized polyphenols were strongly attached to polysaccharide