Valorization of apple tree wood residues by polyphenols extraction: Comparison between conventional and microwave-assisted extraction

Manuela M.Moreira

M. Fátima Barroso

Annick Boeykens

Hannes Withouck

Simone Morais

Cristina Delerue-Matos

Abstract

For the first time, the characterization of antioxidant activity and phenolic profile of apple tree (Malus domestica) bark, core and roots was carried out. Phenolic compounds were extracted from the Belgium apple tree wood residues collected at two seasons, namely summer 2015 and winter 2016, using conventional (CE) and microwave-assisted extraction (MAE) techniques. For each extraction technique, the influence of the most important operational parameters, namely solvent composition, extraction time and temperature, on the total phenolic and flavonoid content, and antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH-RSA) and ferric reducing activity power (FRAP) assays were optimized. The phenolic profile from the obtained extracts was also characterized by high-performance liquid chromatography with photodiode array detection (HPLC-PDA). Optimum conditions were: 20 mL ethanol:water 60:40 v/v, 20 min, 100 °C, sample weight 0.1 g for MAE and 20 mL ethanol:water 50:50 v/v, 2 h, 55 °C, sample weight 0.5 g for CE. Root extracts obtained by MAE (the most efficient technique) presented the highest phenolic (47.7 ± 0.9 mg gallic acid equivalents/g dry weight) and flavonoid (17.1 ± 0.8 mg epicatechin equivalents/g dry weight) content, and antioxidant activity (28.4 ± 2.0 mg trolox equivalents/g dry weight and 36.1 ± 2.7 mg ascorbic acid equivalents/g dry weight for DPPH-RSA and FRAP assays, respectively), followed by bark and core wood extracts. HPLC-PDA analysis revealed that phloridzin was the main contributor to the phenolic composition representing 52%–87% of the total amount of phenolic compounds quantified, while phenolic acids represents less than 10%. This study reveals the potential of apple tree wood residues valorization through the recovery of phenolic compounds for food, pharmaceutical and cosmetic applications.

Keywords

Apple tree wood; Microwave-assisted extraction; Conventional extraction; Antioxidant activity; Phenolic compounds; HPLC analysis

1.Introduction

Polyphenols are one of the main groups of secondary plant metabolites, essentially for their normal growth and defense against infection and injury, and their health benefits have been extensively described (El Gharras, 2009, Ghitescu et al., 2015, Stevanovic et al., 2009). In the last few years, search of inexpensive and renewable sources of polyphenols has been attracting researchers interest. For that reason, the number of publications concerning the extraction of these compounds from biomass has been increasing (Bouras et al., 2015, Ghitescu et al., 2015, Hofmann et al., 2015, Lazar et al., 2016).

Every year, Belgium apple farmers renew 6% of the apple plantation, which reflects in the annual production of 30,000 ton of woods residues (FAOSTAT, 2015). Traditionally, these apple tree residues are used in low added value applications, such as firewood or dispersed (Dedrie et al., 2015, Ghitescu et al., 2015). Still, in last few years, tree materials, such as bark, have been emerging as possible sources of valuable compounds (Table 1) (Ghitescu et al., 2015, Hofmann et al., 2015). To this regard, the recovery of phenolic compounds from these wood wastes is gaining considerable attention, especially ascribable to the antioxidant properties that these compounds exert (Kammerer et al., 2014, Stevanovic et al., 2009).

Table 1
Summary of the published reports on the extraction of phenolic compounds from tree wood residues.

Sample	Extraction technique and conditions	TPC[a]	TPC units	Reference
Birch (*Betula pendula*) and pine (*Pinus sylvestris*) bark varieties	Stirring: 500 mg, 2 × 10 mL of methanol:water 80:20 v/v, 1 min	2.0 ± 0.1–76.0 ± 2.9	mg GAE[b]/g DW[c]	Kähkönen et al. (1999)
Port Orford cedar (*Chamaecyparis lawsoniana* (A. Murr.) Parl.) bark	SE[d]: 100 g, 1 L of methanol	77.7–88.8	mg GAE/g DW	Gao et al. (2007)
Pine (*Pinus rigida* × *taeda* and *Pinus kesiensis*) bark	10 g, 100 mL boiling water, 1 h	111–862	mg CE[e]/g extract	Ku et al. (2007)
Bark of *Phyllanthus emblica* L.	MAE: 3 g, 75% aqueous ethanol, 25 min, 45 °C	19.8	%	Yang et al. (2009)
Oak (*Prunus avium* and *Quercus robur*) wood from different countries	500 mg of sawdust, 30 mL methanol, 30 min, RT[f]	6.95 ± 0.37–101.2 ± 1.2	μg GAE/g oak wood	Alañón et al. (2011)
Pinus radiata bark	Maceration: 20 g, 200 mL acetone:water 70:30 v/v, 40 °C	412 ± 0	mg CE/g bark	Aspé and Fernández (2011)
	MAE[g]: 20 g, 200 mL acetone:water 70:30 v/v, 900 W, 2450 MHz	479 ± 49		
	UAE[h]: 20 g, 200 mL acetone:water 70:30 v/v, 35 kHz, 85 W, RT	388 ± 7		
	SE: 20 g, 200 mL acetone:water 70:30 v/v, 82 °C	622 ± 40		
Pinus radiata bark	100 g, 2 L ethanol:water 3:1 w/w, 120 min, 120 °C	0.55 ± 0.01	g GAE/g extract	Bocalandro et al. (2012)
Bark of *Maclura tinctoria* (L.)	Stirring: acetone:water 70:30 v/v, 30 min, RT + 10 min at 85 °C	43.2 ± 1.2	mg GAE/g	Lamounier et al. (2012)
Quercus (*Q. robur L.*) bark	Maceration: 5 g, 100 mL water, 120 min, 25 °C	3.7 ± 0.6	mg GAE/g bark	Bouras et al. (2015)
	Heat reflux: 5 g, 100 mL water, 120 min, 100 °C	11.7 ± 0.5		
	MAE: 5 g, 100 mL water, 120 min, 100 °C, 400 W	16.50 ± 0.07		
French maritime pine bark (*Pinus pinaster*)	Maceration: 1 g, 9 mL of water + 1% NaOH + 0.25% Na_2SO_3 + 0.25% $NaHSO_3$, 2 h, 70 °C	54.2 ± 19.4	mg GAE/g bark	Chupin et al. (2015)
	MAE: 1 g, 10 mL ethanol:water 80:20 v/v, 3 min, 100 W	28.3 ± 2.9		
	Hydrodistillation: 100 g, 1 L water, 180 min	14.3		Mellouk et al. (2015)
	Solvent free MAE: 100 g, 92.4 min, 803.5 W	139.2		
Oak bark *Quercus robur* L. and *Quercus petraea*	SE: 1 g, 70 mL with 2 successive extractions with water and ethanol, 6 h	5.0 ± 0.3–13.4 ± 0.2	mg GAE/g DW	Dedrie et al. (2015)
Bark of *Solidago canadensis* L.	Stirring: 1 g, 20 mL 50% aqueous ethanol, 25 °C, 30 min	0.601 ± 0.041–1.584 ± 0.020	mg GAE/g	Deng et al. (2015)
	UAE: 5 g, 100 mL 50% aqueous ethanol, 30 min			
	HPE[i]: 1 g, 20 mL 50% aqueous ethanol, 25 °C, 500 MPa, 10 min			
Spruce bark	UAE: 10 g, 100 mL ethanol:water 70:30 v/v, 54 °C, 60 min, 35 kHz, 320 W	13.2	mg GAE/g	Ghitescu et al. (2015)
Beech bark (*Fagus sylvatica* L.)	Stirring: 0.15 g, 15 mL ethanol:water 80:20 v/v, 5 h, RT	48.3 ± 1.2	mg QE[j]/g DW	Hofmann et al. (2015)
	Sonication: 0.15 g, 15 mL ethanol:water 80:20 v/v, 10 min, RT	49.9 ± 1.1		
	MAE: 0.15 g, 15 mL ethanol:water 80:20 v/v, 20 min, 120 °C	65.2 ± 5.6		
Moroccan Acacia mollissima bark	Stirring: 1.0 g, 20 mL methanol:water 80:20 v/v, 2 h, 20 °C	353.6 ± 0.5	mg GAE/g bark	Naima et al. (2015)
	Infusion: 1.0 g, 20 mL methanol:water 80:20 v/v, 2 h, 60 °C	258.4 ± 3.6		
	MAE: 1.0 g, 20 mL methanol:water 80:20 v/v, 5 min, 150 W	441.6 ± 0.3		
	MAE: 1.0 g, 20 mL ethanol, 5 min, 150 W	279.7 ± 8.1		
	MAE: 1.0 g, 20 mL water, 5 min, 150 W	298.8 ± 0.6		
Spruce bark (*Picea abies*)	UAE: 5 g, 50 mL ethanol:water 70:30 v/v, 60 °C, 35 kHz, 320 W	43.1	mg GAE/g	Lazar et al. (2016)

[a] TPC: total phenolic content.
[b] GAE: gallic acid equivalents.
[c] DW: dry weight.
[d] SE: Soxhlet extraction.
[e] CE: catechin equivalents.
[f] RT: room temperature.
[g] MAE: microwave assisted extraction.
[h] UAE: ultrasound assisted extraction.
[i] HPE: high hydrostatic pressure-assisted extraction.
[j] QE: quercetin equivalents.

The extraction technique applied in polyphenols recovery is the key step for a high-yield and efficient process. Despite of conventional extraction (CE) techniques, such as maceration and Soxhlet extraction (SE), still being widely used, there is an increasing demand for more sustainable extraction methods. In recent years, new green extraction techniques, such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), have been developed and used for the extraction of phenolic compounds from tree woods (Aspé and Fernández, 2011, Khoddami et al., 2013). Regarding polyphenols extraction from apple tree woods, only one report for phloretin extraction was published until now. Xü et al. (2010) separated and purify phloretin from Fuji apple tree bark by high-speed counter-current chromatography (HSCCC) on a preparative scale. Phloretin was extracted by reflux with ethanol as extracting solvent, and 39.2 mg (98.2% purity) of phloretin was obtained from 767.3 mg of the crude phloretin extract. This lack of information on phenolic composition characterization from apple tree wood residues represents an interesting research area to create a more profitable valorization of these residues.

The principal aim of the present work was the phenolic and antioxidant characterization of Malus domestica 'King Jonagold' bark, core and roots collected at two different seasons, namely in summer and winter. To achieve this goal, CE and MAE were carried out. The most important parameters in each extraction technique were optimized. Extraction efficiency of both techniques was evaluated by the total phenolic content (TPC) and total flavonoid content (TFC), as well as by the antioxidant activity assays, namely, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH-RSA) and ferric reducing activity power (FRAP). Moreover, characterization of phenolic composition from apple tree bark, core and roots by high-performance liquid chromatography with photodiode array detection (HPLC-PDA) was also carried out to see which individual phenolic compound is contributing the most to the antioxidant properties of the produced extracts.

2. Materials and methods

2.1. Chemicals and reagents

Gallic acid (GA, ≥98%), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid, 98%) and (−)-epicatechin (≥97%) standards were obtained from Sigma-Aldrich (Madrid, Spain). Other products purchased from Sigma-Aldrich or Fluka were: Folin-Ciocalteu reagent, sodium-carbonate (≥99%), DPPH reagent, TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine, 99%) and aluminum chloride hexahydrate (99%). Ascorbic Acid (AA, 99.7%) was purchased from Riedel-de Haën and sodium nitrite (≥97%) from Merck. Ethanol absolute anhydrous (p.a.) was bought from Carlo Erba (Peypin, France), methanol and formic acid (HPLC grade) were obtained from Merck (Darmstadt, Germany), and ultrapure water (18.2 MΩ cm; Simplicity 185 apparatus; Millipore, Molsheim, France) was used during the experiments.

For HPLC analysis reference standards of phenolic compounds purchased from Sigma-Aldrich (Steinheim, Germany) were used in this study, namely the phenolic acids: protocatechuic acid (99.63%), vanillic acid (≥97%), syringic acid (≥98%), β-resorcylic acid (≥97%), 4-hydroxybenzoic acid (≥99%), 4-hydroxybenzaldehyde (98%), ferulic acid (≥99%), sinapic acid (≥99%), cinnamic acid (≥99%), caffeic acid (≥98%), p-coumaric acid (≥98%), chlorogenic acid (>95%), 4-hydroxyphenylacetic acid (98%); flavonoids: (+)-catechin (≥98%), naringin (≥95%), naringenin (98%), rutin hydrate (≥94%), quercetin (95%), kaempferol (≥98%), myricetin (≥96%), pinocembrin (95%), tiliroside (≥98%), quercetin-3-O-glucopyranoside (≥99%), kaempferol-3-O-rutinoside (≥98%), kaempferol-3-O-glucoside (≥95%); tiliroside (≥98%); chalcones: phloridzin dehydrate (99%) and phloretin (≥98.5%); and the stilbenoid resveratrol (≥99%). Individual stock solutions of the above polyphenols were prepared in methanol at concentrations ranging from 1 to 5 g/L and stored at −20 °C.

2.2. Sample preparation

Apple tree wood (Malus domestica 'King Jonagold') was collected during September of 2015 and February 2016 in Herk-de-Stad, Belgium. Bark, core and roots were separated and dried first at 50 °C for 22 h, followed by 4 h drying at 103 °C. Moisture content was measured using a Moisture Analyser (Kern MLS 50-3IR160) until a constant weight of the sample was attained. The moisture content ranged from 10.1 ± 0.4 to 15.2 ± 0.8%. After drying, apple tree woods were ground in a mill (Retsch ZM200) and sieved to select particles smaller than 0.08 mm. The ground bark, core and roots were kept in sealed bags at room temperature. All analytical results were presented per dry weight of sample (DW).

2.3. Extraction procedures

2.3.1. Microwave-assisted extraction

MAE was performed with a MARS-X 1500 W (Microwave Accelerated Reaction System for Extraction and Digestion, CEM, Mathews, NC, USA) using 14 Teflon extraction vessels.

In a first stage, some preliminary assays were performed in order to analyze the influence of extraction solvent (water, methanol and ethanol, and mixtures of these solvents in several proportions), temperature (60, 100 and 120 °C), and mass/solvent ratio (0.1/20, 0.5/20 and 1.0/20, w/v) on the amount of phenolic compounds recovered from bark and core collected at 2015. In a second stage, based on the previous results, MAE optimization was planned according to a 23 factorial experimental design (Table 2) as described in Section 2.7. After centrifugation (Heraeus™ Megafuge™ 16R Centrifuge, Thermo Scientific) at 4000 rpm for 10 min, MAE wood extracts were collected and stored in the freezer at −20 °C until further analysis.

Table 2. Real values and coded levels for the experimental design 2^3 (X_1 – extraction time; X_2 – sample weight; X_3 – temperature), experimental results (mean of four replicates for each run) and predicted values for the total phenolic content (mean ± standard deviation, $n = 3$). Conditions: 20 mL of ethanol:water (60:40; v/v) and medium stirring speed.

Run	Extraction time (X_1, min)	Sample weight (X_2, g DW[a])	Temperature (X_3, °C)	$TPC^{b}_{experimental}$ ± SD (mg GAE/g DW)[c]	$TPC_{predicted}$ ± SD (mg GAE/g DW)
1	(−1) 10	(−1) 0.25	(−1) 80	31.0 ± 1.1	30.3 ± 1.1
2	(1) 30	(−1) 0.25	(−1) 80	32.4 ± 1.6	32.9 ± 1.6
3	(−1) 10	(1) 0.75	(−1) 80	27.2 ± 0.3	26.4 ± 0.3
4	(1) 30	(1) 0.75	(−1) 80	31.0 ± 3.4	30.6 ± 3.4
5	(−1) 10	(−1) 0.25	(1) 120	37.0 ± 2.9	37.4 ± 2.9
6	(1) 30	(−1) 0.25	(1) 120	36.5 ± 2.5	37.2 ± 2.5
7	(−1) 10	(1) 0.75	(1) 120	31.4 ± 0.4	30.9 ± 0.4
8	(1) 30	(1) 0.75	(1) 120	31.6 ± 2.3	32.3 ± 2.4
9	(−1.682) 3	(0) 0.50	(0) 100	27.0 ± 1.0	27.9 ± 1.0
10	(1.682) 37	(0) 0.50	(0) 100	32.2 ± 3.0	31.2 ± 2.9
11	(0) 20	(−1.682) 0.0795	(0) 100	39.6 ± 0.7	39.0 ± 0.7
12	(0) 20	(1.682) 0.9205	(0) 100	31.2 ± 2.3	31.7 ± 2.3
13	(0) 20	(0) 0.50	(−1.682) 66	27.1 ± 1.1	27.9 ± 1.1
14	(0) 20	(0) 0.50	(1.682) 134	36.2 ± 3.4	35.3 ± 3.3
15	(0) 20	(0) 0.50	(0) 100	28.5 ± 0.3	30.6 ± 0.3
16	(0) 20	(0) 0.50	(0) 100	32.0 ± 0.1	30.6 ± 0.1
17	(0) 20	(0) 0.50	(0) 100	30.8 ± 0.3	30.6 ± 0.3
18	(0) 20	(0) 0.50	(0) 100	31.0 ± 0.4	30.6 ± 0.4

a DW: dry weight.

b TPC: total phenolic content.

c mg GAE/g DW: mg gallic acid equivalents/g dry weight of sample.

2.3.2. Conventional extraction

CE optimization was carried out using 0.5 g of bark sample (collected at 2015) and 20.0 mL of solvent in glass Erlenmeyer flasks (protected with plastic paraffin film to prevent solvent evaporation and covered with aluminum foil to preserve phenolic compounds against reactions with light) placed in a water bath shaker (model BSC127E, C from OVAN) at 100 rpm. The parameters studied were the solvent composition (1:4, 1:1, 4:1 v/v; ethanol:water), extraction time (2, 6 and 24 h) and temperature (20 and 55 °C), and their range was defined taking into consideration our previous results from MAE and the results reported by Hofmann et al. (2015). Extracts were centrifuged at 4000 rpm for 10 min and stored in the freezer at −20 °C until further TPC analysis.

2.4. Total phenolic and flavonoid content

TPC was measured using the Folin-Ciocalteau method (Paz et al., 2015) using GA as the standard, and the absorbance was measured at 765 nm in a microplate reader (black 96-well plates, Nunc™ black microwell, Denmark) after 90 min. Results were expressed as mg of GA equivalents per g of dry weight of sample (mg GAE/g DW).

TFC was determined by the aluminum chloride method described in detail by Paz et al. (2015). (−)-Epicatechin was used as standard, and the results were expressed as mg of epicatechin equivalents per g dry weight of sample (mg EE/g DW). All measurements were performed in triplicate.

2.5. Antioxidant capacity

FRAP assay was performed according to the procedure developed by Paz et al. (2015) in a microplate reader at 593 nm at 37 °C. The calculations were made using a standard curve of AA, and the results were expressed as mg AA equivalents per g dry weight of sample (mg AAE/g DW).

The free radical-scavenging activity of the extracts was determined spectrophotometrically using the stable radical DPPH following the procedure described before (Paz et al., 2015). A trolox standard was used to make the calibration curve and the results were expressed in mg trolox equivalents per g dry weight of sample (mg TE/g DW). All the analyses were performed in triplicate.

2.6. High performance liquid chromatography analysis of phenolic profile

A Shimadzu HPLC system equipped with a LC-20AD prominence pump, a DGU-20AS prominence degasser, a CTO-10AS VP column oven, a SIL-20A HT prominence autosampler, and a SPD-M20A photodiode array detector (Kyoto, Japan) was used to analyze the apple wood extracts according to Carvalho et al. (2016) and Rubilar et al. (2007). A Phenomenex Gemini C18 column (250 mm × 4.6 mm, 5 µm) and a guard column with the same characteristics maintained at 25 °C with a gradient program were used for the separation of phenolic compounds. Mobile phase A (methanol) and mobile phase B (water) both with 0.1% formic acid were used for elution at a flow rate of 1.0 mL/min. The following gradient was applied: 0–13 min: 20–26.5%A; 13–18 min: 26.5% A; 18–25 min: 26.5–30% A; 25–50 min: 30–45%A; 50–60 min: 45–50%A; 60–70 min: 50–55% A; 70–90 min: 55–70% A; 90–100 min: 70–100% A, followed by 100% A for 5 min and back to 20% A in 10 min and 5 min of reconditioning before the next injection. The identification and quantification of the phenolic compounds in apple wood extracts was carried out by the comparison of the retention time and UV–vis spectra of detected peaks with those obtained for their pure standards. UV spectra was recorded in a range of 190–600 nm, and the quantification was made at 280, 320 and 360 nm depending of the maximum absorption from the phenolic compound. Before injection, apple tree extracts were filtered through a 0.2 µm PTFE syringe filter. Calibration curves from the previously mentioned standards in the concentration range of 1–200 mg/L, prepared in a mixture of methanol–water (50:50, v/v) by dilution of appropriate amounts of the stock solutions, were obtained. Relevant analytical data, namely limit of detection (LOD) and quantification (LOQ), intra and inter-day method precision, are shown in Table 1S (Supplementary material). For the apple wood extracts, the concentrations were calculated based on triplicate injections and the results were expressed as mg/100 g of DW of sample.

2.7. Experimental design

Response surface methodology (RSM) coupled to a 23 full-factorial central composite design was employed to analyze the influence of MAE variables on the extraction of phenolic compounds from apple wood bark. Extraction time (X1, min.), sample weight (X2, g) and extraction temperature (X3, °C) were chosen as independent variables, and their effect on polyphenols yield was evaluated by the TPC. The complete design consisted of 18 experiments, including 4 runs of the central point and two axial points on the axis of each variable at a distance of α = 1.682 from the center. Coded and real values of the MAE variables and their ranges of variation, which were defined taking into consideration the results from preliminary tests, are listed in Table 2.

The relationship between the extraction yield and the independent variables were fitted with a second-order response surface model expressed in Eq. (1) (Montgomery, 1991):

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y is the experimental response (expressed as TPC), β0, β j, βjj, βij are the constant coefficients of interception, linear, quadratic, and interaction terms, respectively; Xì and Xj are the coded independent variables, and ε is the experimental error. Statistical analysis and response surface 3D plots (generated by using the function of two factors, and keeping the other constant) were performed with Statistica (v. 7, StatSoft Inc., USA) and used to evaluate the interaction among the independent variables and their effect on the polyphenols yield.

2.8. Statistical analysis

All the assays were conducted in triplicate and the results were expressed as mean ± standard deviation (SD). The comparison of the results was made by the software SPSS (IBM SPSS Statistics 20) applying the nonparametric Mann-Whitney U test at the significance level of $p \leq 0.05$.

3. Results and discussion

3.1. Preliminary assays

Taking in consideration our previous knowledge in MAE (Mendes et al., 2016, Moreira et al., 2012, Paíga et al., 2013), some preliminary experiments were carried out in order to choose the relevant variables and their experimental range in phenolic compounds recovery from apple tree woods. TPC of the obtained extracts was used to evaluate the effect of each variable.

One of the most important and investigated parameters in polyphenols extraction efficiency is the solvent composition. Tree woods are composed by different compounds with varied chemical characteristics and polarities which requires the selection of a suitable solvent (Bouras et al., 2015, Ghitescu et al., 2015, Muñiz-Márquez et al., 2013, Naima et al., 2015). Different pure solvents, i.e. water, methanol and ethanol, and mixtures of these solvents in several ratios were tested using a sample weight of 0.50 g and a solvent volume of 20 mL at 100 °C during 15 min with medium stirring. Table 3 shows the impact of solvent choice in TPC of bark and core (from samples collected during 2015). For both samples, it was observed that a mixture of organic solvent and water is more efficient in extracting phenolic compounds than pure solvents (Bouras et al., 2015, Lazar et al., 2016) and that, whatever the type of alcohol used, the trend of the results was similar. The highest TPC was obtained using mixtures of solvents with concentrations of 60% and 80% of organic solvent in water, with no significant differences between both compositions. Recent investigations in matrices similar to apple tree woods are in agreement with these results, where the yield of phenolic compounds was improved till 80% of ethanol or methanol (Ghitescu et al., 2015, Hofmann et al., 2015, Lazar et al., 2016, Naima et al., 2015). This phenomenon can be attributed to the properties of both solvents, where water is altering the sample structure acting as a swelling agent enabling the solvent to easily penetrate inside bark and core, while the organic solvent is disrupting the bonding between the solutes and sample (Bouras et al., 2015, Ghitescu et al., 2015, Muñiz-Márquez et al., 2013). Another explanation for the obtained results can be associated to the high dielectric constant of water, which is a very important parameter in MAE (Morais, 2013). The efficiency and selectivity of MAE strongly depend from the dielectric constant of the solvent mixture; therefore, the addition of water

increases the polarity indices of organic solvents, which reflects in an easier absorption of microwave energy increasing the temperature inside the sample leading to the rupture of cells and an easier release of phenolic compounds (Bouras et al., 2015, Chan et al., 2011, Drosou et al., 2015, Morais, 2013).

Table 3. Evaluation of microwave-assisted extraction solvent effect on the total phenolic content (mean ± standard deviation, $n = 3$) of bark and core samples collected during 2015. Other operational conditions: 20 mL of solvent, 0.5 g sample, 100 °C, 15 min and medium stirring speed.

Solvent Composition (v/v)	**TPC**[a] **(mg GAE/g DW)**[b]	
	Bark	**Core**
Water	29.7 ± 2.4	10.7 ± 0.4
Methanol:water (20:80)	34.0 ± 3.2	15.0 ± 1.3
Methanol:water (40:60)	38.1 ± 3.7	19.7 ± 1.0
Methanol:water (60:40)	39.2 ± 3.1	20.7 ± 1.8
Methanol:water (80:20)	39.4 ± 3.2	19.9 ± 1.9
Methanol	17.5 ± 1.7	13.4 ± 1.1
Ethanol:water (20:80)	35.7 ± 0.5	17.1 ± 0.7
Ethanol:water (40:60)	39.3 ± 3.2	20.2 ± 1.2
Ethanol:water (60:40)	41.1 ± 1.7	23.0 ± 1.9
Ethanol:water (80:20)	41.3 ± 3.5	23.6 ± 2.2
Ethanol	18.2 ± 0.8	10.4 ± 0.9

a TPC: total phenolic content.

b mg GAE/g DW: mg gallic acid equivalents/g dry weight.

Extraction temperature is another crucial parameter in the recovery of polyphenols. In this study, three temperatures, namely 60, 100 and 120 °C, were tested on bark and core samples for pure water, 60% aqueous ethanol and 60% aqueous methanol. The selection of these temperatures was based on previous data from the literature (Liazid et al., 2007, Tsubaki et al., 2010). According to the results from Liazid et al. (2007), the majority of phenolic compounds can be extracted without degradation at temperatures up to 125 °C for an extraction time of 20 min. TPC for each tested condition is presented in Table 4. The obtained results revealed that the increase of extraction temperature from 60 °C to 120 °C improved the polyphenols yields. In fact, TPC values were very similar for 60% aqueous ethanol or methanol at 100 °C or 120 °C, which mean that no significant polyphenols degradation occurred. These results are also in accordance with the results previous obtained for solvent composition (Table 3). Therefore, as ethanol is a food grade and eco-friendly solvent (Dai and Mumper, 2010, Lazar et al., 2016), for the subsequent RSM optimization studies, 60% aqueous ethanol was selected as the optimum extraction solvent.

Table 4. Evaluation of microwave-assisted extraction temperature effect on the total phenolic content (mean ± standard deviation, $n = 3$) of bark and core samples collected during 2015. Other operational conditions: 20 mL of solvent, 0.5 g sample, 15 min and medium stirring speed.

Solvent Composition (v/v)	Temperature (°C)	TPC[a] (mg GAE/g DW)[b]	
		Bark	Core
Water	60	18.0 ± 1.6	4.9 ± 0.4
Methanol:water (60:40)		28.8 ± 1.3	16.1 ± 1.1
Ethanol:water (60:40)		32.0 ± 2.4	11.7 ± 1.1
Water	100	29.7 ± 2.4	10.7 ± 0.4
Methanol:water (60:40)		39.2 ± 3.1	20.7 ± 1.8
Ethanol:water (60:40)		41.1 ± 1.7	23.0 ± 1.9
Water	120	27.2 ± 1.3	10.4 ± 0.5
Methanol:water (60:40)		41.8 ± 1.8	18.7 ± 1.2
Ethanol:water (60:40)		37.7 ± 2.8	21.8 ± 1.1

a TPC: total phenolic content.

b mg GAE/g DW: mg gallic acid equivalents/g dry weight.

Since the extraction yield can also be affected by the accessibility of the solvent to the sample (Chan et al., 2011), the efficiency of MAE of phenolic compounds by 60% aqueous ethanol using different sample weight:solvent volume ratios (0.10:20–1.0:20, w/v) for two temperatures (100 and 120 °C) was also compared. It can be seen in Table 5 that, when the solid/liquid ratio decreased the TPC increased. These results agree with those reported by Yang et al. (2009), and can be explained by the presence of a larger volume of solvent which can accelerate the diffusion of compounds increasing the polyphenols yield (Muñiz-Márquez et al., 2013). Also, the increase of temperature for the lower solid/liquid ratios

(0.1:20 and 0.5:20, w/v) decreased slightly the yield of polyphenols extracted. This behavior was observed for both samples analyzed. Overall, the best results were reached using the lowest solid/liquid ratio (0.1:20, w/v) at 100 °C.

Table 5. Evaluation of microwave-assisted extraction sample weight:solvent volume ratio effect on the total phenolic content (mean ± standard deviation, $n = 3$) of bark and core samples collected during 2015. Other operational conditions: 20 mL of ethanol:water (60:40), 15 min and medium stirring speed.

Sample weight:solvent volume ratio (g/mL)	Temperature (°C)	TPC[a] (mg GAE/g DW)[b]	
		Bark	Core
0.1:20	100	42.0 ± 3.9	26.7 ± 0.9
0.5:20		41.1 ± 1.7	23.0 ± 1.9
1.0:20		32.4 ± 1.6	22.0 ± 0.7
0.1:20	120	37.1 ± 2.3	24.1 ± 2.3
0.5:20		37.7 ± 2.8	21.8 ± 1.1
1.0:20		34.7 ± 3.2	24.2 ± 1.8

a TPC: total phenolic content.

b mg GAE/g DW: mg gallic acid equivalents/g dry weight.

3.2. Extraction optimization

The impact of extraction technique, namely the CE and MAE, on the yield of phenolic compounds recovered from apple tree woods was evaluated in this study. Taking in consideration the results obtained in the preliminary assays (Section 3.1), independent variables for the both optimized extraction techniques were defined. Moreover, as the preliminary results revealed that TPC obtained for bark was 2-fold higher than for core extracts (Table 3–5), and that the influence of variables in bark and core TPC levels was similar, the optimization was performed with the bark samples.

3.2.1. Microwave assisted extraction – response surface methodology

The full optimization of MAE of polyphenols from apple tree bark (Table 2) was made applying a 23 full-factorial central composite design coupled with RSM (Fig. 1S, supplementary material), allowing us to study the impact of all parameters combined on TPC. A response surface regression was performed to fit a mathematical model to the experimental data and the following model equation for the recoded factor values was created:

(2)

$$Y = 30.60 + 1.00X_1 - 2.19X_2 + 2.21X_3 - 0.37X_1^2 + 1.68X_2^2 + 0.35X_3^2 + 0.40X_1X_2 - 0.68X_1X_3 - 0.66X_2X_3$$

By eliminating the non-significant parameters ($p > 0.05$) the second order polynomial model was redefined as:

(3)

$$Y = 30.60 + 1.00X_1 - 2.19X_2 + 2.21X_3 + 1.68X_2^2$$

Analysis of variance (ANOVA) showed that TPC was significantly influenced ($p < 0.05$) by all the linear terms, as well as by the quadratic effect of sample weight (X2). As desired, yield of second order model (Eqs. (2) and (3)) reached high statistical significance ($p < 0.05$) and the attained second order model quadratic correlation coefficient (0.85) can be considered acceptable for data of chemical nature, advocating a good correlation between observed and predicted values (Table 2). The optimum conditions predicted by the model were 0.1 g of sample, 100 °C, 33.5 min and a polyphenol content of 39.8 mg GAE/g DW. 3D surface plots (Fig. 1S, Supplementary material) showed that maximum recovery was attained by using a sample weight < 0.2 g, temperature > 110–120 °C and time < 40 min. Thus, by analyzing all the attained results (including experimental data; Table 2), the optimum microwave conditions for maximizing the extraction efficiency (TPC) and energy

and time savings were selected as being a low amount of sample 0.1 g, combined with medium stirring speed for 20 min using 20 mL of 60% aqueous ethanol at a temperature of 100 °C. Applying these selected optimum conditions, the attained TPC was 39.6 mg GAE/g DW, being not statistically different from the one predicted. Observing the RSM plots, slightly higher TPC can be obtained if the extraction is performed at higher temperature. However, since there is a risk of degradation of the most thermo-sensitive compounds (Liazid et al., 2007) causing the loss of valuable polyphenols, 100 °C was chosen.

3.2.2. Conventional extraction

Based on previously published data for other matrices (Dai and Mumper, 2010, Hofmann et al., 2015, Naima et al., 2015) and since no significant differences were found between methanol and ethanol for the yield of phenolic compounds, the effect of organic solvent composition was only studied for ethanol (0, 20, 50 and 80%) in CE. The achieved results (Table 6) show that the TPC of bark increases significantly (2-fold higher) up to 50%, being not statistically different for 80% ($p = 0.057$). These results agree with the reported by Yang et al. (2009), that optimized the ethanol concentration for the recovery of polyphenols from the bark of Phyllanthus emblica L. These authors reported an increase in the extraction rate of polyphenols from 30 to 70%, and also concluded that an ethanol concentration higher than 70% extracts a higher amount of liposoluble materials, which difficult the purification process. Thus, in this study, 50% of ethanol was selected as the optimum solvent.

Table 6. Evaluation of conventional extraction operational conditions on the total phenolic content (mean ± standard deviation, $n = 3$) of bark samples collected during 2015. Other parameters: 20 mL of solvent and 0.5 g sample.

Extraction conditions	TPC[a] (mg GAE/g DW)[b]
Water, 2 h, 20 °C	17.0 ± 0.8
Ethanol 20%, 2 h, 20 °C	24.4 ± 1.4
Ethanol 50%, 2 h, 20 °C	34.4 ± 0.7
Ethanol 80%, 2 h, 20 °C	32.8 ± 1.4
Ethanol 50%, 6 h, 20 °C	34.7 ± 1.5
Ethanol 50%, 24 h, 20 °C	37.2 ± 2.1
Ethanol 50%, 2 h, 55 °C	43.2 ± 1.4

a TPC: total phenolic content.

b mg GAE/g DW: mg gallic acid equivalents/g dry weight.

Another parameter optimized for the CE was the extraction time, which was studied for 2, 6 and 24 h, while the other extraction conditions were defined as solid/liquid ratio of 0.5/20 (g/mL), 50% aqueous ethanol and the water bath temperature of 20 °C. For the tested times, CE performed for 24 h give a higher TPC when compared to the other extraction times studied (34.4 ± 0.7, 34.7 ± 1.5, and 37.2 ± 2.1 mg GAE/g DW for 2, 6 and 24 h, respectively). Although, no significant differences were achieved in the TPC for the extraction times tested ($p > 0.114$), thus 2 h was selected as the optimum value. Moreover, longer extraction times can induce more chemical reactions and cause oxidative conversion of phenolic compounds, which can influence the real levels (Yang et al., 2009). Hofmamn et al. (2015) investigated the influence of extraction time (2, 5 and 24 h) in the polyphenols recovery from the beech bark, and they found significant differences ($p < 0.001$) for the tested times, with the highest phenolic content obtained for an extraction time of 5 h (48.3 ± 1.2 mg quercetin/g dry bark). In another study, Naima et al. (2015) also reported that the highest polyphenol yield was obtained at lower extraction time (2 h), which decrease at 6 h

and increased again after 24 h of extraction. They reported that the decrease may be due to the degradation of polyphenols with progressing extraction time, but on the other hand additional substances, such as lignanes, can be extracted increasing the TPC for the 24 h of extraction.

Taking in consideration possible degradation of phenolic compounds and concerning the literature information (Ghitescu et al., 2015, Lazar et al., 2016, Naima et al., 2015, Yang et al., 2009), tests were conducted at two temperatures (20 and 55 °C) using 50% aqueous ethanol and 2 h as extraction time (Table 6). The yield of polyphenols extracted from bark was highest when using 55 °C instead of 20 °C. This increase in the extraction yield may be related with the cell wall integrity, which could be weaken by the increase of temperature resulting in a higher contact of the solvent and polyphenols present in sample which reflects in the TPC (Yang et al., 2009). According to the presented results, it was also observed that TPC obtained at 55 °C during 2 h was higher than the value obtained at 20 °C for 24 h, indicating that the temperature has a greater impact on the polyphenols yield than the extraction time in CE. Ghitescu et al. (2015) investigated the influence of temperature (40, 50 and 60 °C) for different extraction time (30, 45 and 60 min) on polyphenol yield from spruce wood bark, and they also demonstrated that TPC was lower at 40 °C and 60 min (8.3 mg GAE/g dry bark) than at 50 °C and 30 min (9.5 mg GAE/g dry bark). In fact, they also showed that for the same extraction time, TPC of spruce bark increased with temperature. Some authors defended that extraction temperature should not be higher than 50 °C due to the loss of solvent and possible polyphenols oxidation (Lazar et al., 2016, Yang et al., 2009). Although, recently Naima et al. (2015) reported that an increase from 40 to 80 °C improved the yield of polyphenols at least 3-fold (76.2 ± 4.0–279.3 ± 3.1 mg GAE/g bark) from Maroccan bark of Acacia mollissima. According to the obtained experimental results, in the present study the optimal conditions for CE were considered as being 50% aqueous ethanol, an extraction time of 2 h at 55 °C.

3.3. Characterization of apple tree extracts

3.3.1. Phenolic content and antioxidant activity

The MAE and CE extracts (obtained applying the optimal extraction conditions) from apple tree woods collected during September 2015 and February 2016 were characterized by TPC, TFC, DPPH-RSA and FRAP assays (Fig. 1). As it can be seen (Fig. 1), MAE technique enabled to obtain root extracts collected at 2016 with higher phenolic (44.4 ± 1.1 mg GAE/g DW) and flavonoid (20.3 ± 1.2 mg EE/g DW) contents, as well as with the highest antioxidant capacity (29.6 ± 1.3 mg TE/g DW and 39.8 ± 2.0 mg AAE/g DW by DPPH-RSA and FRAP assays, respectively) compared to the CE technique for all the samples studied. This behavior was expected since several authors reported the higher efficiency of MAE against the CE technique for wood residues (Aspé and Fernández, 2011, Bouras et al., 2015, Naima et al., 2015). Aspé and Fernández (2011) evaluated the performance of four extraction techniques, namely maceration, Soxhlet extraction, MAE and UAE, for the recovery of phenolic compounds from Pinus radiata bark. The obtained results showed the same trend, with the MAE extracts presenting higher amount of phenolics, tannins and antioxidant capacity, demonstrating that MAE technique was an efficient method for extraction of P. radiata bark polyphenols. This difference in results may be related with the main MAE principle (volumetric heating- heat is originated inside the material). MAE is considered as a green extraction process as the time and amount of solvents used are lower in comparison with the CE. Moreover, one of the greater advantages of MAE technique is that the extraction solvent penetrates more easily inside the cell walls and break those linkages between the phenolics and cell walls increasing the amount of phenolic compounds recovered (Drosou et al., 2015). In fact, in the study performed by Aspé and Fernández (2011), the bark surface morphology was examined after the extractions with a scanning electron microscope and they reported that MAE produced a cell destruction, while maceration only resulted in slightly ruptured cell pores, which could explain its low extraction efficiency. Despite of higher yield achieved with MAE, to employ this extraction technique, a more expensive equipment is necessary in comparison to the requirements of CE. Although, in the case of apple tree residues, CE requires 2 h and MAE takes only 20 min, which means that the extraction time was reduced in 83.3%. Therefore, the application of MAE technique despite of a high initial investment can become more profitable in terms of yield, and time and money savings.

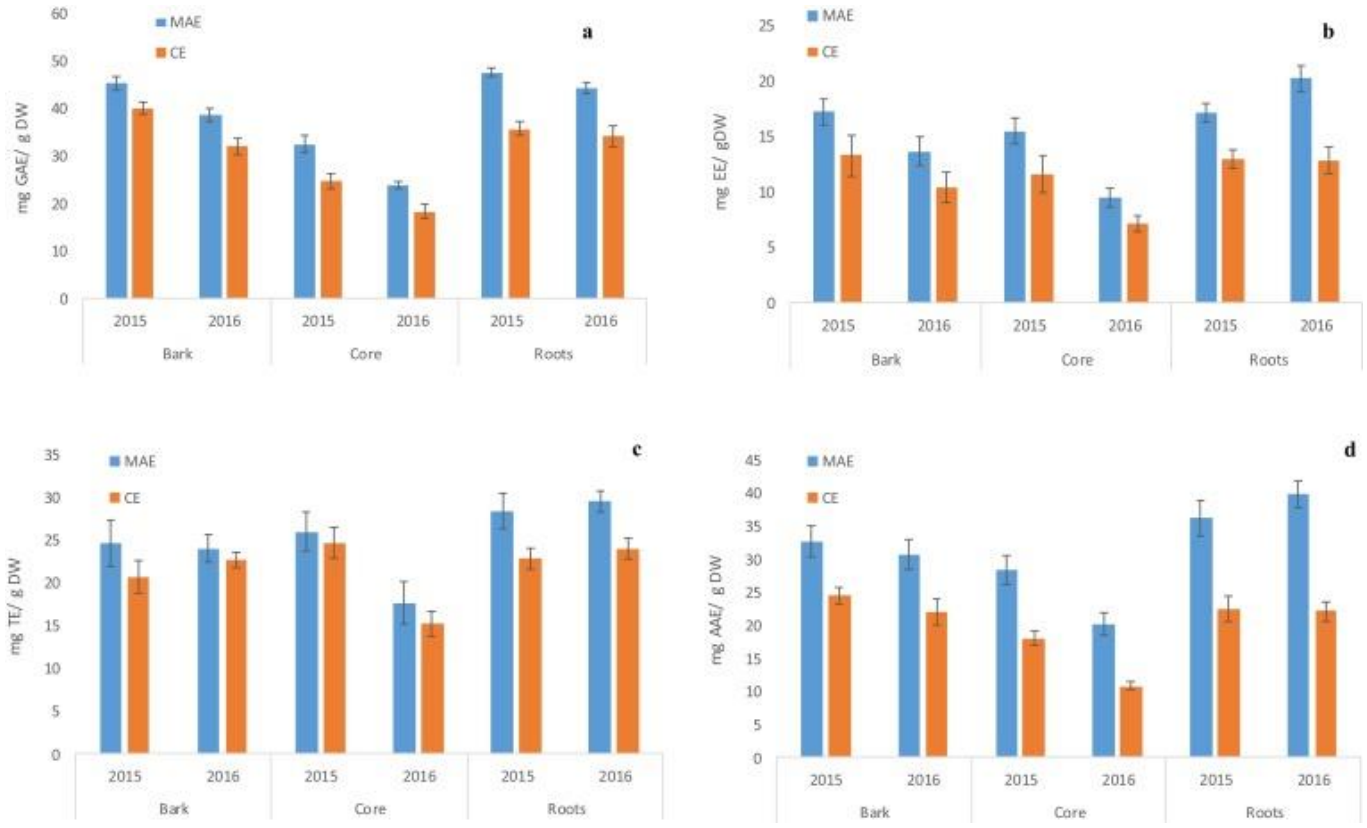

Fig. 1. Comparison of microwave-assisted extraction (MAE) with conventional extraction (CE) for (a) total phenolic content (mg gallic acid equivalent/g dry weight), (b) total flavonoid content (mg epicatechin equivalent/g dry weight), (c) DPPH-RSA, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (mg trolox equivalents/g dry weight) and (d) FRAP, ferric reduction activity power (mg ascorbic acid equivalents/g dry weight) of different apple wood residues; results are expressed as mean ± standard deviation, $n = 3$.

Concerning the differences between the samples (Fig. 1), there is an ongoing trend for all the applied assays in the characterization of apple tree extracts with roots presenting the highest phenolic and flavonoid content, as well as the highest antioxidant capacity followed by bark, and core samples. This variation may be explained by the ability of phenolic compounds, which can be found in soil, to bind to organic nitrogen sources, which are useful for the tree (El Gharras, 2009, Hofmann et al., 2015, Kähkönen et al., 1999). Thus, these compounds can be absorbed by the roots and transported further to the apple tree. Roots function is the absorption of the necessary water and minerals for the tree, but on the other hand, roots are highly exposed to diverse atmospheric conditions, insects, and bacteria. Therefore, apple tree roots need to behave as a strong agent to protect the tree from threats existing in soil, which means that they need to absorb higher amount of phenolics to prevent these threats. As bark shields the core, it will protect it from several elements such as wind, rain, insects, and possible infections, thus bark needs more

phenolic compounds than core. This explanation can justify the distinction between roots, bark and core extracts. For the TPC and TFC assays, there are significant differences between the analyzed samples, with root extracts presenting the highest phenolic and flavonoid content in comparison to bark or core extracts. In fact, for samples collected during 2016, this variation is more pronounced, as TPC is at least 2-fold higher for bark (38.7 ± 1.4 mg GAE/g DW) or roots (44.4 ± 2.2 mg GAE/g DW) in comparison to core (23.8 ± 0.8 mg GAE/g DW). The same tendency was found for the flavonoid content. Regarding the assays for the measurement of antioxidant capacity, no significant differences between the samples or the season of collection from samples were detected. Significant changes were only detected for core collected at 2016, which in comparison with the other samples presented the lowest antioxidant capacity. Several studies indicated that the antioxidant capacity is related to the phenolic content (Aspé and Fernández, 2011, Lamounier et al., 2012). Therefore, this result was expected since, in the total phenolic and total flavonoid assays, it was already the sample with the lowest content. As previously mentioned, this significant distinction between the bark or roots and core samples may be related to the function that this apple tree residues exerts accumulating phenolics and flavonoids in vacuoles, which contribute to the development and protection against infection and injury of plants (El Gharras, 2009, Hofmann et al., 2015). Regarding the influence of the collection season (Fig. 1), there is not a marked trend. Although, it may be noticed that samples collected during Summer 2015 presented the highest values in comparison with samples from Winter 2016. This slight variation may be caused by the atmospheric conditions that apple trees are subjected before the collection of the sample, which can result in dissimilar phenolic composition and antioxidant properties (Zhang et al., 2015). In fact, the results obtained by Chupin et al. (2013) agreed with these findings, demonstrating that trees subjected to higher sun exposure (namely the samples collected in September of 2015) have an higher phenolic content comparing with the shaded trees.

This is the first paper evaluating the phenolic composition and the antioxidant properties from the apple tree residues, namely from bark, core and roots. According to the reported data in literature (Table 1), different types of tree and distinct parts of the tree enable to obtain diverse phenolic contents. The results achieved in this work agree with some of the results found in the studies reported in Table 1. Using a similar CE technique in bark from the Maroccan Acacia mollissima, the TPC values found were 8–20 times higher than any of

the samples used in this work. In this study, the TPC from CE extracts ranged from 18.4 ± 1.6 to 40.2 ± 1.4 mg GAE/g DW, while the value reported by Naima et al. (2015) was 353.6 ± 0.5 mg GAE/g bark. In the opposite way, the TPC obtained for oak wood from different countries was at least 360 times lower (TPC values ranged from 25.3 ± 0.4 to 51.0 ± 1.6 μg GAE/g oak wood) (Alañón et al., 2011) than the values found for samples from this work. Other CE techniques reported in literature for samples similar to the ones investigated in this work are infusion (Naima et al., 2015) and Soxhlet extraction (Gao et al., 2007). For both studies, the attained TPC values were higher than the ones found in this work (258.4 ± 3.6 mg GAE/g bark (Naima et al., 2015) and 77.7–88.8 mg GAE/g bark (Gao et al., 2007) vs 18.4 ± 1.6–40.2 ± 1.4 mg GAE/g bark). Concerning the application of recent extraction techniques, such as MAE, in this type of wood residues, the majority of published studies are from the last two years (Table 1) demonstrating the novelty of this study. Comparing the results obtained in the present study for bark with the values reported in Table 1, the differences within the values obtained for the TPC could be related not only with the matrix studied, but they are also dependent of the applied extraction conditions (Drosou et al., 2015).

3.3.2. Phenolic composition by HPLC-PDA analysis

Apple tree wood extracts obtained by MAE and CE techniques were also characterized by HPLC-PDA to find which phenolic compounds were contributing to their antioxidant properties. Results are exhibited in Table 7 while representative chromatograms are presented in Fig. 2. According to the obtained results, flavonoid compounds were the main contributors to the phenolic profile, and phenolic acids represents less than 10% of all quantified compounds. HPLC analysis revealed that phloridzin was the main contributor to the phenolic composition for all the analyzed extracts representing 52% to 87% of the total amount of phenolic compounds quantified. Despite of the lack of studies regarding these apple tree residues, other authors (Liaudanskas et al., 2014, Rana et al., 2016, Walia et al., 2016) also reported that apple tree leaves accumulate high amounts of phloridzin, a dihydrochalcone, which health benefits have been widely studied specially in glucose uptake and diabetes. Therefore, it is not surprising that apple tree residues were mostly composed by this phenolic compound. In the present study, the highest content of phloridzin was 0.40 mg/g DW, which is close to the value reported in apple tree leaves by Rana et al. (2016) (0.15 ± 0.05 mg/g), whereas lower than the content reported by Liaudanskas et al. (2014) (1.40 ± 0.0–2.40 ± 0.09 mg/g DW). Besides phloridzin, another

four flavonoids were identified in the majority of the extracts as the most abundant, namely myricetin, kaempferol-3-O-glucoside, naringin and quercetin-3-O-glucopyranoside. In the case of myricetin and kaempferol-3-O-glucoside, the higher contribution to the total amount of phenolic compounds quantified corresponds to approximately 16% and 12%, respectively, for core wood collected during 2015 for both extraction techniques applied. The contribution of these compounds, and from naringin and quercetin-3-O-glucopyranoside compounds, to the other apple residues extracts were less than 3%. None of these compounds were identified in apple tree leaves (Liaudanskas et al., 2014, Rana et al., 2016, Walia et al., 2016).

Table 7. Content (mean ± standard deviation, n = 3) of the identified phenolic compounds in apple tree wood extracts obtained by microwave-assisted extraction (MAE) and conventional extraction (CE) techniques.

Phenolic compound	Bark_2015 (mg/100 g DW[a])		Bark_2016 (mg/100 g DW)		Core_2015 (mg/100 g DW)		Core_2016 (mg/100 g DW)		Roots_2015 (mg/100 g DW)		Roots_2016 (mg/100 g DW)	
	MAE	CE	MAE	CE	MAE	CE	MAE	CE	MAE	CE	MAE	CE
Gallic acid	27.1 ± 1.0	21.3 ± 1.1	3.1 ± 0.1	0.49 ± 0.02	ND	1.83 ± 0.09	4.31 ± 0.22	1.92 ± 0.10	49.8 ± 0.5	29.9 ± 1.5	< LOD	1.98 ± 0.10
Protocatechuic acid	7.2 ± 0.4	7.5 ± 0.4	20.8 ± 1.0	2.6 ± 0.1	26.9 ± 1.4	4.67 ± 0.23	18.3 ± 0.9	3.51 ± 0.18	23.2 ± 1.2	8.71 ± 0.44	18.6 ± 0.9	3.94 ± 0.20
(+)-Catechin	30.7 ± 1.5	18.9 ± 0.9	91.7 ± 4.6	8.7 ± 0.4	30.1 ± 1.5	15.9 ± 0.8	10.3 ± 0.5	8.41 ± 0.42	51.2 ± 2.6	21.9 ± 1.1	63.2 ± 3.2	10.9 ± 0.5
4-hydroxyphenilacetic acid	< LOD[b]	< LOD	< LOD	< LOD	67.7 ± 3.4	47.7 ± 2.4	39.5 ± 1.9	27.4 ± 1.4	< LOD	< LOD	< LOD	< LOD
4-hydroxybenzoic acid	45.3 ± 2.3	43.2 ± 2.2	40.3 ± 2.0	37.2 ± 1.9	< LOD	< LOD	< LOD	< LOD	49.1 ± 2.5	47.3 ± 2.4	42.6 ± 2.1	39.9 ± 1.9
Chlorogenic acid	18.9 ± 0.9	17.5 ± 0.9	51.3 ± 2.6	41.7 ± 2.1	< LOD	< LOD	18.5 ± 0.9	7.64 ± 0.38	31.9 ± 1.6	19.6 ± 0.9	36.3 ± 1.8	23.0 ± 1.2
Vanillic acid	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	3.6 ± 0.2	< LOD	< LOD	< LOD
Caffeic acid	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	7.8 ± 0.4	< LOD	8.34 ± 0.42	< LOD
Syringic acid	15.0 ± 0.8	14.5 ± 0.7	28.0 ± 1.4	41.7 ± 2.1	11.3 ± 0.6	10.2 ± 0.5	< LOD	15.4 ± 0.8	< LOD	4.26 ± 0.21	< LOD	32.5 ± 1.6
(−)-Epicatechin	24.5 ± 0.2	15.2 ± 0.3	< LOD	1.46 ± 0.07	< LOD	< LOD	< LOD	1.46 ± 0.07	< LOD	10.7 ± 0.5	< LOD	1.46 ± 0.07
β- Resorcylic acid	52.2 ± 2.6	< LOD	56.6 ± 2.8	< LOD	< LOD	< LOD	< LOD	< LOD	65.6 ± 3.3	< LOD	55.5 ± 2.8	< LOD
Ferulic acid	12.1 ± 0.6	12.5 ± 0.6	8.7 ± 0.4	0.63 ± 0.03	8.9 ± 0.4	3.01 ± 0.15	4.09 ± 0.20	1.81 ± 0.09	6.67 ± 0.33	18.2 ± 0.9	4.41 ± 0.22	1.55 ± 0.08
Sinapic acid	17.6 ± 0.9	19.2 ± 0.9	6.3 ± 0.3	0.51 ± 0.03	< LOD	7.58 ± 0.38	6.30 ± 0.32	4.17 ± 0.21	48.6 ± 0.4	66.2 ± 3.3	4.89 ± 0.24	2.64 ± 0.13
Naringin	106.7 ± 5.3	121.2 ± 6.1	63.5 ± 3.2	59.4 ± 2.9	18.2 ± 0.9	2.92 ± 0.15	< LOD	2.37 ± 0.12	129 ± 6	115 ± 6	74.5 ± 0.7	61.3 ± 0.6
Rutin	72.7 ± 0.6	64.8 ± 3.2	8.0 ± 0.4	9.19 ± 0.46	9.84 ± 0.49	10.9 ± 0.6	6.83 ± 0.34	8.19 ± 0.41	56.1 ± 0.3	40.4 ± 2.0	6.66 ± 0.33	6.24 ± 0.31
Resveratrol	25.1 ± 1.2	23.0 ± 1.1	20.1 ± 1.0	16.0 ± 0.8	32.0 ± 1.6	20.0 ± 1.0	15.7 ± 0.8	6.76 ± 0.34	< LOD	< LOD	< LOD	< LOD
Quercetin-3-*O*-glucopyranoside	134.8 ± 6.7	111.8 ± 5.6	98.4 ± 4.9	95.7 ± 4.8	< LOD	< LOD	< LOD	< LOD	111.6 ± 5.6	91.9 ± 4.6	72.4 ± 3.6	51.7 ± 2.6
Phlorizin	2916 ± 146	2891 ± 87	2092 ± 109	2166 ± 108	877 ± 55	870 ± 18	689 ± 55	745 ± 18	2936 ± 147	2336 ± 117	2521 ± 126	2340 ± 117
Cinnamic acid	53.2 ± 2.7	51.2 ± 2.6	50.2 ± 2.5	43.2 ± 2.2	81.5 ± 4.1	61.5 ± 3.1	40.3 ± 2.0	20.6 ± 1.0	58.4 ± 2.9	50.44 ± 2.52	48.7 ± 2.4	42.3 ± 2.1
Myricetin	97.9 ± 4.9	92.9 ± 4.6	92.9 ± 4.6	82.9 ± 4.2	259 ± 13	237 ± 12	193 ± 10	106 ± 5	79.9 ± 4.0	66.49 ± 3.32	69.8 ± 3.5	50.6 ± 2.5
Kaempferol-3-*O*-glucoside	22.9 ± 1.1	18.9 ± 0.9	18.9 ± 0.9	12.9 ± 0.6	196 ± 9	174 ± 9	150 ± 8	101 ± 5	22.7 ± 1.1	18.10 ± 0.91	15.9 ± 0.8	10.9 ± 0.5
Kaempferol-3-*O*-rutinoside	35.8 ± 1.8	32.8 ± 1.6	32.8 ± 1.6	25.8 ± 1.3	16.1 ± 0.8	12.1 ± 0.6	7.69 ± 0.38	< LOD	< LOD	< LOD	< LOD	< LOD
Naringenin	7.7 ± 0.4	< LOD	1.6 ± 0.1	3.94 ± 0.20	< LOD	< LOD	3.10 ± 0.16	7.07 ± 0.35	< LOD	< LOD	10.7 ± 0.5	13.3 ± 0.7
Quercetin	21.6 ± 1.1	20.7 ± 1.0	21.4 ± 1.1	2.63 ± 0.13	22.9 ± 1.2	9.12 ± 0.46	21.4 ± 1.1	7.12 ± 0.36	48.7 ± 2.4	20.75 ± 1.04	25.7 ± 1.3	7.54 ± 0.38
Phloretin	21.2 ± 0.6	15.5 ± 0.5	27.5 ± 0.6	12.1 ± 0.9	16.7 ± 0.6	11.6 ± 0.9	19.5 ± 0.6	8.27 ± 0.50	26.2 ± 1.3	26.17 ± 1.31	39.9 ± 2.0	27.5 ± 0.9
Kaempferol	30.1 ± 1.5	18.5 ± 0.9	25.1 ± 1.3	12.5 ± 0.6	21.9 ± 1.1	17.9 ± 0.9	12.3 ± 0.6	6.87 ± 0.34	37.6 ± 1.9	17.61 ± 0.88	12.4 ± 0.6	7.89 ± 0.39
SAP[c] (mg/100 g DW)	3796 ± 190	3632 ± 182	2859 ± 143	2677 ± 134	1696 ± 85	1518 ± 76	1261 ± 63	1090 ± 55	3843 ± 192	3009 ± 150	3132 ± 157	2737 ± 137

[a] DW: dry weight.

[b] < LOD: below the limit of detection of the method.

[c] SAP: sum of analyzed phenolics = sum of individual phenolic compounds quantified.

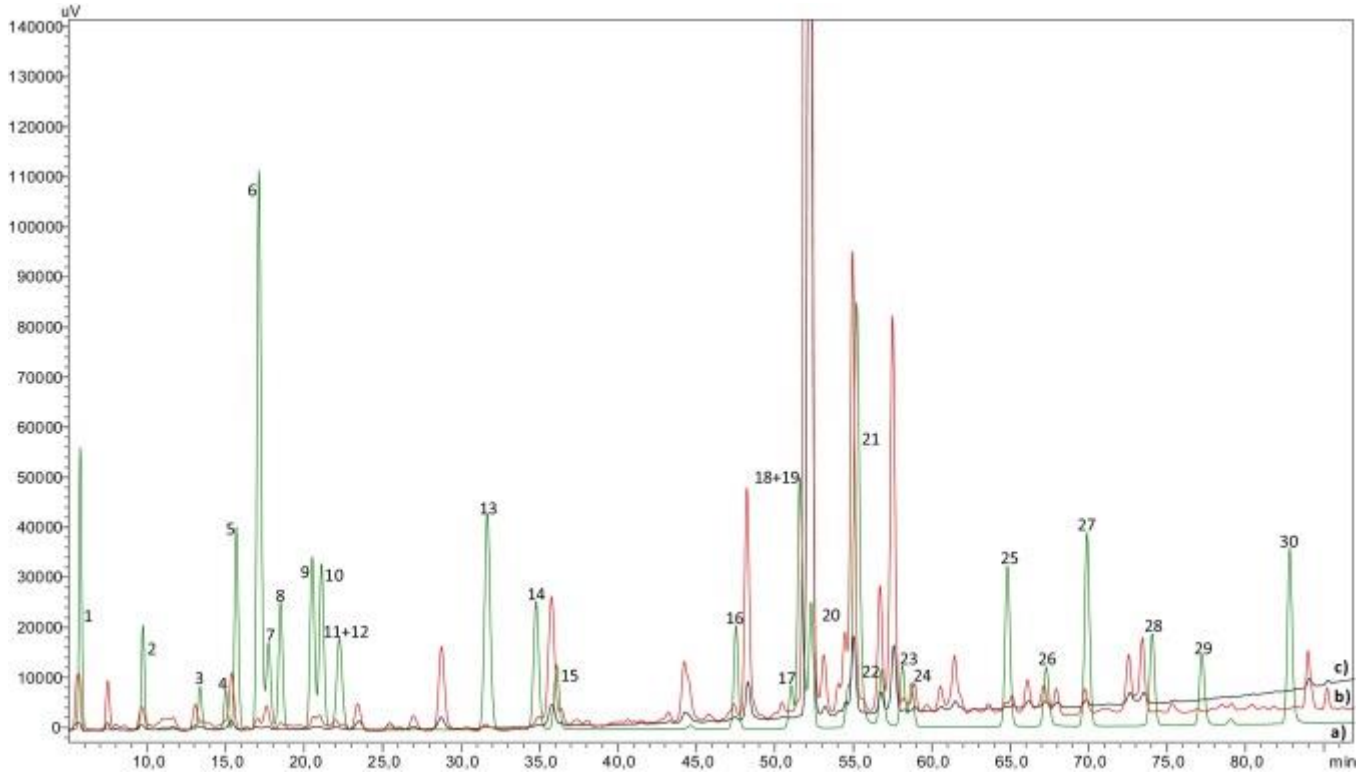

Fig. 2. HPLC chromatograms at 280 nm for a) polyphenols standard mixture of 5 mg/L, and roots extract collected at 2015 obtained after the application of b) MAE (conditions: 20 mL of 60% aqueous ethanol, 100 °C, 20 min, 0.08 g, and medium stirring speed) and c) CE (conditions: 20 mL of 50% aqueous ethanol, 55 °C, 2 h, 0.5 g); (1) gallic acid, (2) protocatechuic acid, (3) (+)-catechin, (4) 4-hydroxyphenilacetic acid, (5) 4-hydroxybenzoic acid, (6) 4-hydroxybenzaldehyde, (7) chlorogenic acid, (8) vanillic acid, (9) caffeic acid, (10) syringic acid, (11) (−)-epicatechin, (12) β-resorcylic acid, (13) p-coumaric acid, (14) ferulic acid, (15) sinapic acid, (16) naringin, (17) rutin, (18) resveratrol, (19) quercetin-3-O-glucopyranoside, (20) phloridzin, (21) cinnamic acid, (22) myricetin, (23) kaempferol-3-O-glucoside, (24) kaempferol-3-O-rutinoside, (25) naringenin, (26) quercetin, (27) phloretin, (28) tiliroside, (29) kaempferol and (30) pinocenbrim.

The highest content of phenolic acids identified and quantified by the HPLC-PDA analysis (344.6 mg/100 g DW) was reported for roots sampled during 2015 and obtained by MAE (Table 7). Concerning the apple tree wood composition in phenolic acids, an interesting finding was the presence of 4-hydroxybenzoic acid in the extracts of bark and roots, and its non-detection in core. On the other hand, the acid 4-hydroxyphenylacetic was only reported for core extracts. The presence of β-resorcylic acid was only reported in the bark and root extracts obtained through the MAE technique and not in the CE extracts. These phenolic composition differences in apple tree residues reflect not only the composition of the studied samples, but also the applied extraction techniques. Several investigations have reported that MAE can prevent some polyphenols degradation, which can occur in CE due to the longer extraction times used (Azmir et al., 2013, Hofmann et al., 2015). Moreover, changes in sample collection dates (September 2015 and February 2016) could also affect the

phenolic profiles, as Belgium climatic conditions were very different at the two dates of sample collection. Comparing these results with the literature available, none of these phenolic acids were detected in apple tree leaves (Liaudanskas et al., 2014, Rana et al., 2016, Walia et al., 2016).

Regarding the comparison of the total amount of phenolic compounds quantified by the HPLC analysis (SAP, sum of analyzed phenolics) and the content obtained through the FC-method, the same trend for both extraction techniques was followed, with extracts collected during summer and obtained by the MAE technique with higher SAP and TPC values. From the apple tree residues studied, roots and bark represent the wood residues with the highest TPC and SAP. The slight difference between the results obtained by the HPLC analysis and FC-method could be related to the interferences that TPC assay is subjected to, such as sugars or proteins, which could cause overestimated results (Kähkönen et al., 1999). Moreover, the HPLC quantification was made only with the phenolic standards available, which could result in a lower amount than the real value. As can be seen in Fig. 2, there are some compounds that have not been identified and quantified, which could also justify these differences in the amount achieved. In a future work, it will be interesting to analyze apple tree residues by HPLC with mass spectrometry detection in order to identify and quantify the unknown compounds, which may correspond to phenolics and thereby contribute to the antioxidant properties of the produced extracts.

4. Conclusions

The results of this study demonstrated for the first time that Belgium apple tree residues, especially bark and roots, can be a potential source for recovery of phenolic compounds for further application in food, cosmetic, pharmaceutical and other related industries. CE and MAE techniques were optimized to achieve the highest phenolic and flavonoid content, as well as the highest antioxidant activity, for bark, core and roots extracts. From the analyzed samples, root extracts obtained after the application of optimal conditions of MAE (20 mL ethanol:water 60:40 v/v, 20 min, 100 °C, sample weight 0.1 g) and CE (20 mL ethanol:water 50:50 v/v, 2 h, 55 °C, sample weight 0.5 g) and collected during summer 2015 presented the highest polyphenols yields (47.7 ± 0.9 and 35.8 ± 1.4 mg GAE/g DW, respectively), followed by bark and core extracts. Furthermore, HPLC-PDA analysis enables to conclude

that apple tree residues were mainly composed by phloridzin, followed by quercetin-3-O-glucopyranoside, naringin, myricetin and kaempferol-3-O-glucoside.

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