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Dedicated to Professor Mihaela Hillebrand on the occasion of her 85th anniversary

ENZYMATIC AND HYDROALCOHOLIC TWO-STEP CASCADE EXTRACTION FOR VALORIZATION OF ANTIOXIDANT BIOMOLECULES FROM APPLE POMACE

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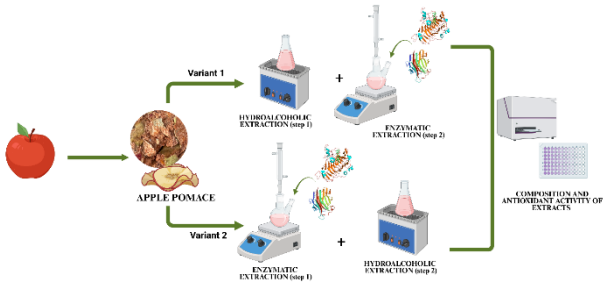
Apple pomace is an agro-industrial by-product known to be rich in phenolic compounds. Instead of being discarded, it could be capitalized into novel food ingredients and products. The aim of this study was to evaluate the optimal processing order in a sequential two-step eco-friendly extraction consisting of one hydroalcoholic (ethanolic) step and one enzymatic step. The highest content of phenolic compounds, as measured by TPC, TFC, and THA was obtained in the case of the order – hydroalcoholic extraction (HE) followed by the enzymatic extraction (EE). High positive correlations were observed between the phenolic content and the antioxidant activity, especially THA with FRAP, and negative correlations with most polyphenol-normalized AOA. The proposed method for recovery of antioxidant compounds from apple pomace stands out as a green, environmentally friendly method for valorization of this agro-industrial by-product and each step can be further optimized in order to maximize the content of polyphenols. The generalizability of the results for other apple cultivars and economic feasibility will have to be established in future studies.



INTRODUCTION

The agri-food industry generates large quantities of various by-products such as apple pomace that are rich in valuable compounds.[1] Apple pomace is generated in tens of millions of tons each year and it is currently mainly used for animal feed or as fertilizer, when it is not burned or disposed of in

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open-air landfills. In the last decade, there is a growing interest in valorizing apple pomace, either as bioactive (carbohydrates, polyphenols, etc.) extraction or used as it is in a circular bioeconomy approach. The main domains that could benefit from the valorization of apple pomace are the food, pharmaceutical, agricultural, and energetic industries.[2] Therefore, apple pomace could be integrated in the processes of a biorefinery and valorized by a cascade route considering the value pyramid principles, in which the sub-product from one process becomes the raw material for the next process.[2] One example for value pyramid implementation will imply extracting first value-added compounds such as polyphenols and vitamins, followed by polysaccharide recovery from the resulting substrate and/or production of fermentable sugars, enzymes, biogas, biofuel, fertilizer etc. For the recovery of polyphenols, several chemical methods have been investigated, *e.g.*, aqueous, organic solvent (methanol, ethanol, acetone, etc.) – which extract the free/soluble compounds, and enzymatic, basic, acidic – which can extract bound compounds as well, etc. in combination with physical methods, *e.g.*, hydrothermal, ultrasonic, microwave, etc. [3–7] For efficient extraction of free and/or bound polyphenols, sequential extractions were applied, for example aqueous extraction followed by organic solvent extraction[8] or aqueous/solvent followed by basic and/or acidic extractions.[4] To the best of our knowledge, there is no information on cascade extractions involving the combination of hydroalcoholic and enzymatic treatments, in particular on the optimal order of steps in order to maximize the total polyphenolic extraction and antioxidant activity. In this study we investigated a two-step cascade extraction from apple pomace consisting of a hydroalcoholic (ethanol), ultrasound-assisted extraction (HE) and an enzymatic extraction (EE) – both eco-friendly methods, each of them as the first or the second step and discussed context-dependent best extraction variant.

RESULTS AND DISCUSSION

Figure 1 shows stereomicroscope images of the apple pomace particles after grinding and sieving through a 1 mm mesh seize sieve. The average size of the larger particles was 0.7 ± 0.1 mm.

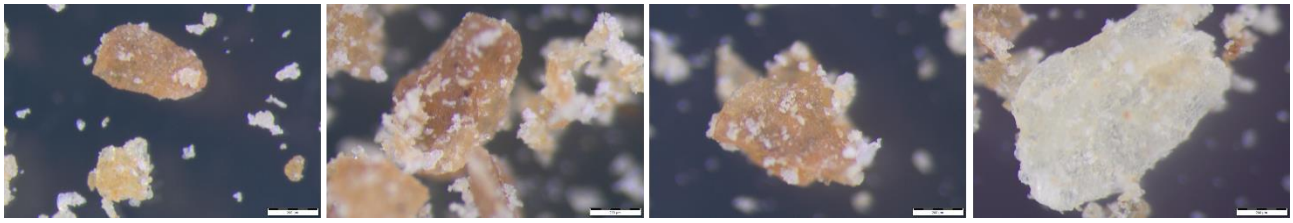


Fig. 1 – Stereomicroscope images of apple pomace particles after grinding and sieving through a 1 mm mesh seize sieve.

The extraction yield, expressed as substrate depletion, was 55.5 ± 6.0% w/w and 12.6 ± 0.5% (5.6 ± 0.6% of the initial mass) after HE as the first step and EE as the second step, respectively (variant 1 of extraction). In the case of variant 2 of extraction (EE – first step, HE – second step), the extraction yield of EE as the first step was 52 ± 5%. The extraction yield of HE as the second step was 24.5 ± 3.8% (11.7 ± 0.6% of the initial mass). The total extraction yield reported as depletion of the initial substrate was 61.1 ± 6.0% and 63.7 ± 5.0% for variant 1 and variant 2, respectively.

The highest antioxidant activity (AOA) was given by the hydroalcoholic extraction, for all the three assays tested, *i.e.*, antioxidant capacity of copper ion reduction (CUPRAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) (Figure 2). The AOA cumulative for the two steps was slightly higher in the case of variant 1 than AOA of variant 2 in the case of FRAP (Table 1). In the case of CUPRAC and DPPH, variant 2 was slightly higher than variant 1, but only CUPRAC had statistically significant differences. Applying EE as the first step (variant 2) increased AOA compared with EE applied as second step (variant 1), and did not result in a higher, but a lower AOA of HE used as the second step, especially in the case of FRAP (4.18 ± 0.03 μmol TE/g dry sample) compared to HE as the first step (12.4 ± 1.3 μmol TE/g dry sample).

The antioxidant activities were as expected for the TPC extracted, and in the same range as in other studies with similar polyphenol content, although some variations could be observed, depending on the antioxidant assay, including higher AOA despite lower TPC values than other studies.[5, 9]

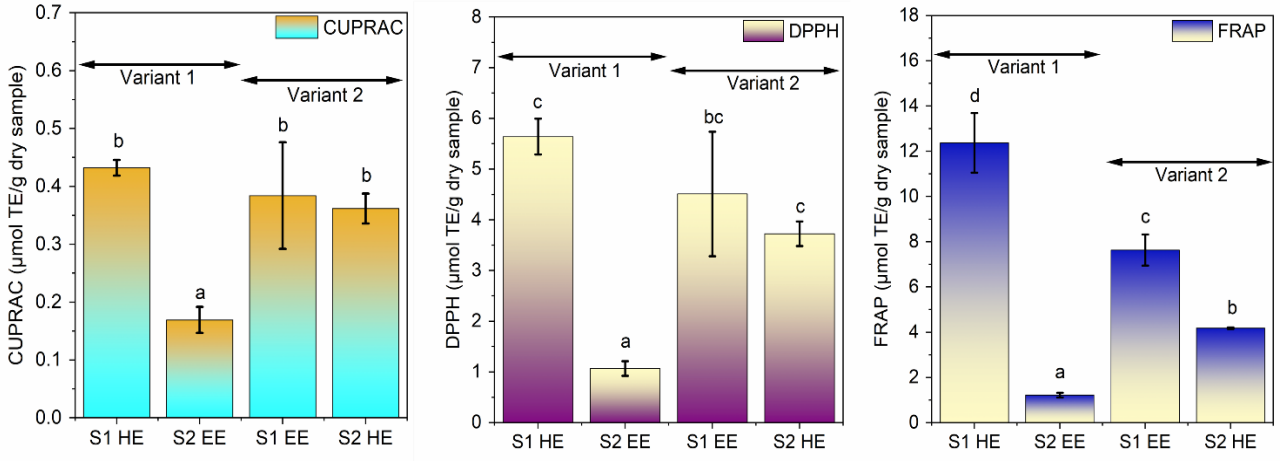


Fig. 2 – Antioxidant activity of the extractions (means ± standard deviations); TE – Trolox equivalents; S1 – step 1; S2- step 2; HE – hydroalcoholic extraction; EE – enzymatic extraction. Different letters represent statistically significant differences ($p < 0.05$, $n = 3$).

The polyphenolic content was higher in the case of variant 1 than in the case of variant 2 (Fig. 3). The total polyphenol content (TPC), cumulative from the two steps in the first and second variant was 351 ± 17 and 269 ± 8 μg gallic acid equivalents (GAE)/g dry sample, respectively (Table 1).

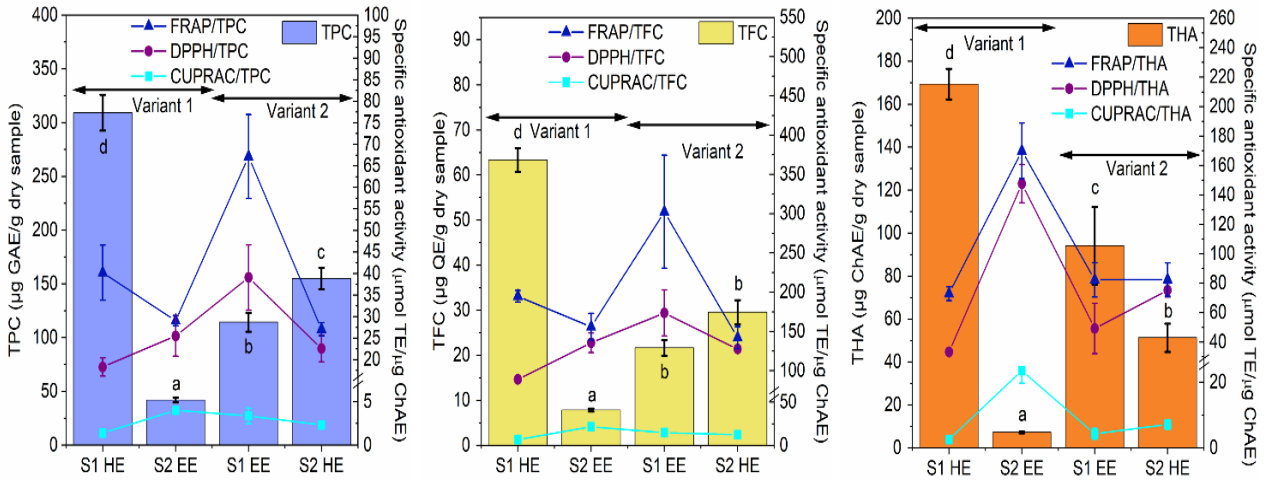


Fig. 3 – Polyphenol composition of the extractions (means ± standard deviations); TPC – total polyphenolic content; TFC – total flavonoid content; THA – total hydroxycinnamic acid content; GAE – gallic acid equivalents; QE – quercetin equivalents; ChAE – chlorogenic acid equivalents; S1 – step 1; S2- step 2; HE – hydroalcoholic extraction; EE – enzymatic extraction. Different letters represent statistically significant differences ($p < 0.05$, $n = 3$).

The values were lower than reported in some studies and in the same range as values reported in other studies, see[3–6,9,10] and the references within. The TPC values found in the literature ranged between 68 μg/g dry sample for Golden Delicious and 155 μg/g dry sample for Panaia-red (lower than in this study) extracted with methanol[10] to 15.8 mg/g dry sample for Red Delicious under microwave optimized conditions[6] (over 50× higher than in this study), *i.e.*, a range of more than two orders of magnitude. There are several aspects that influence the level of extracted polyphenols, such as the apple

cultivar, geographical and meteorological conditions, and the processing parameters, including the apple-based drink production.[7,11] Therefore, a direct comparison with other studies is in fact difficult to assess. The pomace studied in this work was an industrially obtained pomace after presser I and presser II (1:1), by which the juice is efficiently extracted, therefore there are probably less polyphenols remained in the resulting pomace. Moreover, the pomace was a mix of three cultivars, Golden, Gala, and Jonathan, and the TPC values was a mean of all cultivars. Drying apple pomace under heat, although faster and more convenient, was reported to reduce slightly the content of extracted antioxidants, probably due to partial degradation and oxidation of the compounds.[12] Polyphenol oxidase, which was not inhibited by blanching in our study, could partially oxidize polyphenols to quinones and affect polyphenol extraction and reaction. Another parameter that could contribute to lower phenolic content compared with other studies are the extraction methods proposed, which are less intensive, but have the advantage to be eco-friendly. Nevertheless, the purpose of the study, *i.e.*, to compare the optimal order of hydroalcoholic and enzymatic extractions of apple pomace was not affected, as both variants were performed under similar conditions. Additionally, the two steps applied in this study can be each further optimized in order to obtain higher extracted polyphenol content. The TPC values were significantly higher in the case of HE than in the case of EE, independent of the order of extraction, particularly when HE was the first step. TPC was higher if HE and EE were each the first step (309 ± 17 and 114 ± 9, respectively, μg GAE/g dry sample) than if it was the second step (155 ± 10 and 42 ± 2, respectively, μg GAE/g dry sample). It should be emphasized that the TPC method, although largely used for polyphenolic content, is not specific to phenolic compounds, as the reagent interacts with other reducing compounds, such as proteins and vitamins. Therefore, the TPC values should be interpreted as an estimation of the total antioxidant activity rather than phenolic content.

The total flavonoid content (TFC) and hydroxycinnamic content (THA) assays indicated 71 ± 5 μg quercetin equivalents (QE)/g dry sample and 177 ± 8 μg chlorogenic acid equivalents (ChAE)/g dry sample, respectively, for the first variant, which were higher than for the second variant (Table 1). The differences in TPC, TFC, and THA values between the two variants were statistically significant. The TFC values were significantly higher in the case of HE than in the case of EE, independent of the order of extraction, but particularly when HE was the first step. TFC of HE and EE was higher if they were applied as the first step (63 ± 4 and 26 ± 4, respectively, μg QE/g dry sample) than if it was the second step (30 ± 3 and 8 ± 0.35, respectively, μg QE/g dry sample). The THA values were significantly higher in the case of HE (169 ± 7 μg ChAE/g dry sample) than in the case of EE (7 ± 0.34 μg ChAE/g dry sample) for the first variant, but lower for the second variant (51 ± 7 μg ChAE/g dry sample for HE and 94 ± 18 μg ChAE/g dry sample for EE). THA of HE was significantly higher if HE was the first step (169 ± 7 μg ChAE/g dry sample) than if it was the second step (51 ± 7 μg ChAE/g dry sample). Similar trend was observed for the THA from EE (94 ± 18 μg ChAE/g dry sample for EE as the first step and 7 ± 0.34 μg ChAE/g dry sample for EE as the second step). In conclusion, the enzymatic treatment (EE) as first step increased only THA compared with EE as second step and did not result in higher polyphenol content of HE as second step compared with HE as first step. The TPC, TFC, and THA data indicated that the enzymatic treatment applied as the first step did not result in significant additional antioxidant compounds from apple pomace compared with S1 HE alone, but the cumulative AOA of S1 EE and S2 HE was higher than that of S1 HE alone. This suggests that the enzymatic pre-treatment with VinoTaste enhances the total AOA, possibly by modifying molecular structures of polyphenols and/or by releasing other molecules, such as oligosaccharides, which were shown to stimulate AOA.[13–15] The THA content was higher than the TFC content for the first step, in the case of both HE and EE. A close similitude was observed between the trends of THA and FRAP.

Table 1

Parameter cumulative values for each of the two extraction variants

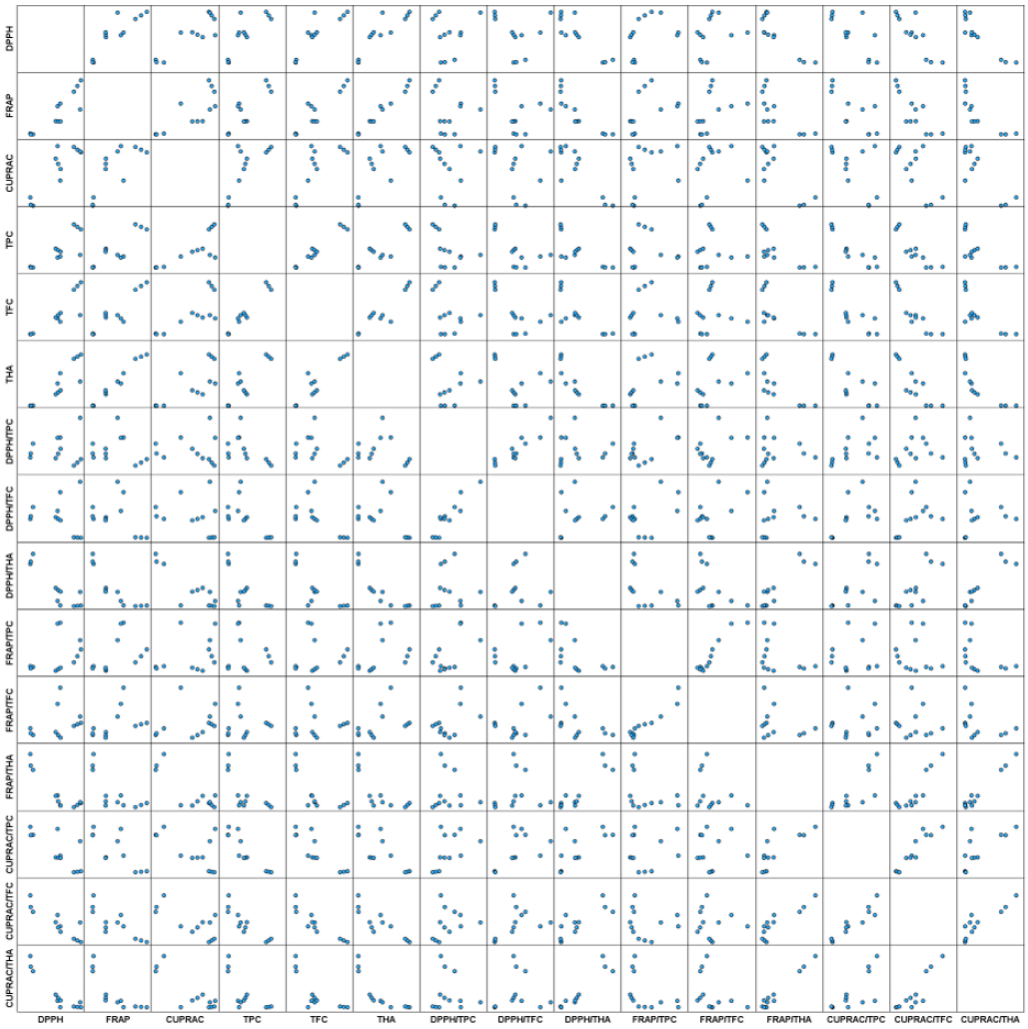
Variant	TPC[a]	TFC[a]	THA[a]	CUPRAC[a]	DPPH[a]	FRAP[a]
1 (HE+EE)	351 ± 17** (p = 0.002)	71 ± 5* (p=0.026)	177 ± 8* (p = 0.026)	0.60 ± 0.02	6.71 ± 0.48	13.6 ± 1.3 (p = 0.112)
2 (EE+HE)	269 ± 8	55 ± 6	145 ± 14	0.75 ± 0.07* (p = 0.033)	8.24 ± 1.43 (p = 0.154)	11.8 ± 0.7

[a]TPC: total polyphenolic content; TFC: total flavonoid content; THA: total hydroxycinnamic acid content; expressed as: µg gallic acid equivalents/g dry sample (TPC); µg quercetin equivalent/g dry sample (TFC); µg chlorogenic acid equivalents/g dry sample (THA); µmol Trolox equivalent/g dry sample (CUPRAC, DPPH, FRAP); * 0.01<p<0.05; ** 0.001<p<0.01 (n = 3).

We noticed that the pectin precipitation from EE resulted in the decrease of all measured parameters, which suggests that part of the polyphenols precipitated with pectin. Nevertheless, the values for EE were higher than the water extraction without enzymes, even after pectin precipitation (by 110 ± 23%, 28 ± 5%, 41 ± 2%, 94 ± 18%, 24 ± 3%, 61 ± 5% for TPC, TFC, THA, CUPRAC, DPPH, and FRAP, respectively). The higher values suggest that the enzymes cleaved covalent bonds of polysaccharides within the pomace matrix, and released polyphenols as well. The enzymatic activity was additionally confirmed by a more turbid solution after incubation compared with water extraction, due to pectin release.

We have additionally assessed the theoretical specific antioxidant capacity (SAC) of the various polyphenol categories for each extraction, assuming major contribution (Fig. 3). It was observed that TPC followed the trend of TFC, with maximum SAC for S1 EE in the case of DPPH and FRAP and S2 EE in the case of CUPRAC. THA had the maximum SAC for S2 EE in the case of all AOA assays. One relevant aspect is that CUPRAC and DPPH, for which SAC of S1 HE was lower than for the other steps had a reverse variant trend than the polyphenols (variant 2 slightly higher than variant 1). For these two AOA, and partially for FRAP, the enzymatic extractions seem to have a higher SAC compared to hydroalcoholic extractions. One possible explanation could be the amplification / synergism induced by saccharides present in the enzymatic extracts, but also the polyphenolic composition of the extracts. It is known that the polyphenols, including those from apple pomace have different antioxidant capacities that depend on various parameters. Quantum mechanical approaches such as Density Functional Theory (DFT) and Valence Bond Theory (VB), as well as QSAR models showed that AOA depends on the number and position of the hydroxyl groups and methoxy substitutions, steric effects, glycosylation, double bonds, polarizability, Log*P*, E_{HOMO} and E_{LUMO}, bond length, thermochemical parameters, etc.[16–24] Moreover, intra- and inter-hydrogen bonds and synergic / antagonistic effects can occur in complex systems such as biomass extracts. Therefore, despite the high polyphenolic content present in S1 HE, the antioxidant activity was not much higher compared with the steps from variant 2, especially in the case of CUPRAC and DPPH, which determined the variant trend observed. This shows the complex behavior of our extracts.

High and statistically significant levels of positive correlation were observed between all AOA and polyphenol parameters (Fig. 4). High correlations between polyphenols and AOA have been reported in numerous other studies, including for apple pomace.[25–27] The correlations depend on the composition of the samples, therefore on the plant species as well, and on the specificity of the assays. High correlations indicate that AOA, as measured by the respective assays, is mainly given by phenolic compounds, *i.e.*, the latter represent the majority of antioxidant compounds in the samples.[25] It is worth mentioning the previous high correlation reported for TPC with CUPRAC for various samples, which is in line with the results obtained in this study,[26] although in our case CUPRAC had the lowest correlation among the AOA with the polyphenols.



(A)

Pearson Correlations

		DPPH	FRAP	CUPRAC	TPC	TFC	THA	DPPH/ TPC	DPPH/ TFC	DPPH/ THA	FRAP/ TPC	FRAP/ TFC	FRAP/ THA	CUPRAC/ TPC	CUPRAC/ TFC	CUPRAC/ THA
DPPH	Corr.	1														
	Sig.															
FRAP	Corr.	0,877**	1													
	Sig.	0,000														
CUPRAC	Corr.	0,887**	0,773**	1												
	Sig.	0,000	0,003													
TPC	Corr.	0,815**	0,874**	0,757**	1											
	Sig.	0,001	0,000	0,004												
TFC	Corr.	0,863**	0,919**	0,777**	0,978**	1										
	Sig.	0,000	0,000	0,003	0,000											
THA	Corr.	0,879**	0,991**	0,744**	0,899**	0,929**	1									
	Sig.	0,000	0,000	0,006	0,000	0,000										
DPPH/TPC	Corr.	0,072	-0,171	-0,012	-0,498	-0,398	-0,204	1								
	Sig.	0,823	0,595	0,971	0,100	0,201	0,526									
DPPH/TFC	Corr.	-0,166	-0,395	-0,247	-0,664*	-0,624*	-0,409	0,919**	1							
	Sig.	0,607	0,204	0,439	0,019	0,030	0,187	0,000								
DPPH/THA	Corr.	-0,885**	-0,879**	-0,870**	-0,770**	-0,790**	-0,876**	0,042	0,220	1						
	Sig.	0,000	0,000	0,000	0,003	0,002	0,000	0,896	0,493							
FRAP/TPC	Corr.	0,379	0,464	0,346	0,005	0,100	0,410	0,598*	0,463	-0,532	1					
	Sig.	0,225	0,128	0,270	0,987	0,756	0,186	0,040	0,130	0,075						
FRAP/TFC	Corr.	0,285	0,419	0,233	-0,007	0,039	0,385	0,478	0,454	-0,508	0,950**	1				
	Sig.	0,370	0,176	0,465	0,983	0,905	0,216	0,116	0,138	0,092	0,000					
FRAP/THA	Corr.	-0,891**	-0,734**	-0,826**	-0,709**	-0,722**	-0,752**	-0,079	0,093	0,905**	-0,367	-0,314	1			
	Sig.	0,000	0,007	0,001	0,010	0,008	0,005	0,806	0,773	0,000	0,240	0,320				
CUPRAC/TPC	Corr.	-0,729**	-0,728**	-0,574	-0,901**	-0,860**	-0,782**	0,494	0,564	0,704*	0,111	0,060	0,754*	1		
	Sig.	0,007	0,007	0,051	0,000	0,000	0,003	0,102	0,056	0,011	0,732	0,852	0,005			
CUPRAC/TFC	Corr.	-0,868**	-0,826**	-0,717**	-0,893**	-0,905**	-0,860**	0,247	0,433	0,811**	-0,115	-0,072	0,888**	0,934**	1	
	Sig.	0,000	0,001	0,009	0,000	0,000	0,000	0,438	0,160	0,001	0,722	0,823	0,000	0,000		
CUPRAC/THA	Corr.	-0,913**	-0,810**	-0,852**	-0,726**	-0,753**	-0,814**	-0,097	0,097	0,940**	-0,475	-0,413	0,987**	0,728**	0,881**	1
	Sig.	0,000	0,001	0,000	0,008	0,005	0,001	0,764	0,764	0,000	0,119	0,182	0,000	0,007	0,000	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

(B)

Fig. 4 – Pearson correlations between the parameters of the extracts: (A) Matrix Scatter Plot; (B) heatmap.

Most of the specific AOA had either negative correlations or a tendency towards negative correlations, except FRAP/TPC and FRAP/TFC, which had a weak, statistically insignificant positive correlation with the polyphenolic content and AOA. FRAP was also the only AOA slightly higher for variant 1 than for variant 2, although not statistically significant. This trend might indicate that, in some cases, at high polyphenolic content, there are some antagonistic or inhibitory effects, which could be determined, for example, by pro-oxidant, auto-oxidant effects and/or intermolecular H-bonds.[28-33] As the sample size used for correlations was not very high (n = 12), the correlations, especially the less significant ones ($p > 0.05$ and $r < 0.8$), should be treated with cautious and confirmed with larger sample sizes. Nevertheless, the agreement with previously reported data suggests that most of the positive correlations are probably real and not due to chance. In conclusion, though we expected that the better order of extraction would be enzymatic followed by hydroalcoholic, due to additional release of polyphenols by the enzymes, apparently the reverse order gives higher polyphenolic content. Nonetheless, the result is in line with the considerations from other studies that claim a more commonly used sequential procedure of extracting first hydroalcoholic-soluble phenolics and in the next step, enzymatically-released phenolic compounds.[4] The enzymatic treatment, at least with the cocktail used in this study, does not favor the release of more polyphenols soluble in hydroalcoholic solution and insoluble in water. Moreover, the existing polyphenols in apple pomace seem to be more soluble in hydroalcoholic solutions than in water, which is expected, considering the rather amphiphilic nature of polyphenols. In the same time, a higher polyphenol content obtained by the first variant did not result in higher total AOA activity. The reason could be related to various factors such as the composition of the extracts, antagonist / synergic effects, crowd effects etc. that can influence the antioxidant capacity. In this respect, some specific AOA, especially CUPRAC, and especially in the case of THA, were negatively correlated with the polyphenolic content. As mentioned, total CUPRAC also had the lowest positive correlation with polyphenols. The high polyphenolic content in S1 HE did not help to ensure an equally high AOA for this step and, in consequence, for variant 1. Future studies should identify the exact causes for reduced AOA capacity and the differences between the AOA assays. The choice of the first or second variant or the usefulness of both steps should consider the purpose and the costs as well, therefore future studies should focus on techno-economic analysis, which will need the quantification and characterization of the extracted polysaccharides as well. If the purpose is to obtain only polyphenols, the first variant might be the choice, but probably without S2 EE, as S2 EE had much lower values than S1 HE and might not justify additional costs with the enzymes. If the purpose is to obtain both polyphenols and polysaccharides (pectin) by green methods, and to maximize AOA, variant 2 might be the better choice, as S1 EE and S2 HE had close values. The remaining substrate (after the second step) could be further valorized within a cascade approach for further pectin extraction, bioethanol production, etc.

Some limitations of the study should be emphasized. First, the results were obtained under one set of experimental parameters only, without optimization of individual and/or sequential steps. It is possible that optimal / different parameters, as well as other enzyme cocktails would give different outcome. One particular parameter is the ethanol concentration. The enzyme mix VinoTaste Pro was previously shown to deglycosylate polyphenol glycosides into aglycones, which are soluble in less polar or high concentrations of organic solvents. In the case of alcohol, concentrations higher than 70% favor the solubilization of aglycones. In this study, the 50% ethanol concentration used in the second step might have been too low to efficiently extract the aglycones produced by enzymes in the first step. A higher ethanol concentration might favor aglycones and increase the polyphenol recovery in variant 2. These aspects will need to be elucidated in future studies. Another factor that could influence the results is the apple cultivar, as the composition can differ significantly between cultivars. A comparative study between various cultivars and their mixes will be useful to establish the generalizability of the results from this study. Upon establishing the optimal

conditions and the specificities for various cultivars, a techno-economic analysis will allow to assess the economic feasibility of applying two-step extractions.

EXPERIMENTAL

1. Materials

Apple pomace, a mix of Gala, Golden, and Jonathan cultivar, from presser I and presser II after juice extraction in 2023, was acquired fresh from Răureni (www.raureni.ro), a Roumanian national producer of fruit-based products, and kept at -20°C until processing. The enzyme cocktail was VinoTaste Pro (Novozymes A/S, Bagsvaerd, Denmark). The following substances were used to determine the antioxidant and phenolic profile of the apple pomace extracts: gallic acid, 2,2-diphenyl-1-picrylhydrazyl, quercetin dihydrate, and ascorbic acid (Sigma-Aldrich, Merck Group, Darmstadt, Germany), Trolox with a purity of 97%, (Acros Organics, Thermo Fisher Scientific, Pittsburgh, PA, USA), Folin Ciocalteu's phenol reagent, iron(III) chloride with a purity of 98%, 2,9-dimethyl-1,10-phenanthroline with a purity of 98%, and 2,4,6-tri(2-pyridyl-1,3,5-triazine) with a purity of 98%, aluminium chloride (Alfa Aesar, Kandel, Germany), hydrochloric acid, acetic acid, 96% pharmaceutical ethanol, copper sulfate (Chimopar Srl, Bucharest, Roumania), sodium acetate, sodium nitrite, sodium molybdate, sodium hydroxide, disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, sodium carbonate (Scharlau, Barcelona, Spain), chlorogenic acid, sodium chloride (ChimReactiv, Neamt, Roumania), hydrochloric acid 1N (Scharlau, Barcelona, Spain), glacial acetic acid (Chimreactiv, Bucharest, Roumania).

2. Apple pomace characterization and extraction methodology

Apple pomace from presser I and, respectively, presser II was dried at 50°C in an oven FD-S 115 (Binder, Tuttlingen, Germany), mixed 1:1, and next ground in a planetary ground mill (PM 100, Retsch GmbH, Haan, Germany) and sieved through a 1 mm mesh seize sieve. Stereomicroscope images of apple pomace particles were recorded with an SZX16 Stereo Microscope (Olympus, Tokyo, Japan). The initial moisture (83.7 ± 0.2%) and moisture after drying at 50°C (11.5 ± 1%) of apple pomace was determined by drying at 105°C until constant weight. The water activity after sieving (0.3236 ± 0.0017) was determined in triplicate at 23°C by using a LabMaster-aw neo equipment (Novasina AG, Lachen, Switzerland), according to the manufacturer's instructions. Two extraction steps, *i.e.*, one hydroalcoholic extraction and one enzymatic extraction, were applied in cascade to extract biomolecules from apple pomace. In a first experimental variant, the first step (S1 HE) involved the hydroalcoholic extraction (50% ethanol solution) of polyphenols from apple pomace using a Substrate/Liquid (S/L) ratio of 1:10 (w/v), 30 min. ultrasound treatment in an ultrasonic bath Elmasonic P (580 W, 37 Hz, Elma Schmidbauer GmbH, Singen, Germany) at a temperature below 30°C. Afterwards, the extract was centrifuged at 8420 rcf, 4°C, for 30 min, in a Universal 32 centrifuge (Hettich, Tuttlingen, Germany). The supernatant was concentrated in a rotary evaporator Rotavapor R-300 (BUCHI Corporation, New Castle, DE, USA), frozen with liquid nitrogen and stored at –80°C in a freezer TwinGuard MDF-DU302VX-PE (PHCBi, Tokyo, Japan) for further polyphenol analysis. The sediment was washed twice with double-distilled water and centrifuged at 8420 rcf, 4°C, for 10 min. in order to remove the residual ethanol. The sediment was dried at 50°C in an oven. The second step (S2 EE) consisted of an enzymatic treatment with 10 g/hL blend of β-glucanase(exo-1,3-)/pectinase/protease/arabinanase (VinoTaste Pro, Novozymes A/S, Bagsvaerd, Denmark, with main enzyme activities Polygalacturonase 2500 PGNU/g and β-glucanase (exo-1,3-) 75 BGXU/g), as recommended by the producer. A S/L ratio of 1:10 (w/v) apple pomace/enzyme mixture in double distilled water (ddH_2O) was used. The reaction was performed in a round flask with condenser and slow magnetic stirring at 50°C for 4 h. The pH of the sample that resulted in ddH_2O (4.5) was not adjusted, as VinoTaste is recommended to be used at slightly acidic pH, characteristic to wine. These parameters were chosen based on previous studies with pectinases as well.[34–36] The extract was then centrifuged at 8420 rcf, 4°C, for 30 min. The supernatant and the sediment were processed as mentioned above. A second experimental variant

was applied, in which the experiment was reversely performed, *i.e*., the enzymatic extraction (S1 EE) was applied before the hydroalcoholic extraction (S2 HE). The treatment between the two steps was the same as in the case of the first variant. Due to the simultaneous extraction of pectin by the enzymes, which induced a turbid aspect to the extracts, the pectin in EE was precipitated by mixing 1:1 with ethanol. In order to confirm the enzymatic contribution, aqueous extracts without enzyme were performed for comparison with the enzymatic extract applied as first step.

The extraction yield was calculated as substrate depletion: $\eta = \frac{mi-mf}{mi} \times 100$, where *mi* = initial mass, before each extraction, *mf* = final mass of substrate, after the extraction.

3. Composition characterization of extracts

3.1. Total polyphenol content

TPC was determined using the Folin-Ciocalteu test. 10 µL of each sample or standard solution was mixed with 10 µL of Folin-Ciocalteu reagent and 90 µL of ddH_2O. After 5 min. of reaction, 100 µL of 7% (w/v) Na_2CO_3 solution and 40 µL of ddH_2O were added. The reaction mixture was incubated at room temperature for 60 min., after which the absorbance was read at 765 nm using a microplate reader (CLARIOstar, BMG LABTECH, Ortenberg, Germany). A calibration curve was prepared with gallic acid standards at concentrations ranging from 5 to 30 µg/mL in 70% ethanol, which was then used to quantify TPC in the samples.[37] TPC was expressed as µg GAE/g dry sample of apple pomace.

3.2. Total flavonoid content

TFC was determined using the aluminum chloride/sodium acetate method with some modifications. To assess TFC, 0.1 mL of sample/standard was mixed with 0.1 mL of 10% sodium acetate, followed by the addition of 0.12 mL of 2.5% aluminum chloride and 0.68 mL of ddH_2O to the mixture. Absorbance was read at λ = 430 nm after 45 min. of incubation at room temperature and TFC was calculated based on a calibration curve with quercetin in the interval 0-30 µg/mL.[37] TFC was expressed as µg QE/g dry sample of apple pomace.

3.3. Total hydroxycinnamic content

THA was determined through an adapted approach from the European Pharmacopoeia. Specifically, 25 µL of the sample/standard was mixed with 50 µL of 0.5 M HCl, followed by the addition of 50 µL of a solution composed of 1% $NaNO_2$ and 1% Na_2MoO_4. Subsequently, 50 µL of 8.5% NaOH and 75 µL of ddH_2O were added to the mixture. Absorbance was measured at λ = 524 nm. To quantify hydroxycinnamic acids, a calibration curve was established using chlorogenic acid at concentrations ranging from 0 to 50 µg/mL in 70% ethanol (v/v).[38] THA was expressed as µg ChAE/g dry sample of apple pomace.

4. Antioxidant activity of extracts

4.1. CUPRAC Method for Antioxidant Activity Determination

CUPRAC was performed according to an adapted method as follows: 10 µL of sample/standard solutions were mixed with 30 µL of 5 mM $CuSO_4$, 30 µL of 3.75 mM neocuproine, and 280 µL of ddH_2O, reaching a final volume of 350 µL. After 30 min., absorbance was measured at λ=450 nm. A Trolox calibration curve was calculated as a standard substance based on multiple tested Trolox concentrations. Standard solutions started from a 10 mM Trolox stock solution in 70% ethanol (v/v) and were used for the calibration curve in the concentration range of 0–2 mM Trolox.[39] Results were expressed in µmol Trolox equivalents (TE/g) dry sample of apple pomace.

4.2. DPPH Method for Antioxidant Activity Determination

The reaction mixture involved adding 100 µL of stock solution/sample to 100 µL of 0.3 mM DPPH radical solution in ethanol. Absorbance was measured at λ=517 nm after a 30 min. reaction using a UV-Vis spectrophotometer. Trolox was used as a standard substance, and the calibration curve was established in the range of 0.15 mM to 0.0125 mM, starting from a 1 mM stock solution.[40] Results were expressed in µmol TE/g dry sample of apple pomace.

4.3. FRAP Method for Antioxidant Activity Determination

Determination of the antioxidant power to reduce Fe^{3+} was carried out using a described method with some modifications. A 0.3 M acetate buffer solution at pH 3.6, a 10 mM TPTZ stock solution in 40 mM HCl, and a 20 mM $FeCl_3$ solution in a 10:1:1 ratio were used for the assay. The FRAP reagent was prepared on the day of the analysis and kept at a temperature of 37°C. To 15 µL of sample

or standard solution, 285 μL of FRAP reagent was added, and the mixture was incubated at 37°C for 30 min. After incubation, absorbance was measured at a wavelength of λ = 593 nm. Trolox was used as a standard substance, and the calibration curve was established for concentrations between 0–550 μM.[37] Results were expressed in μmol TE/g dry sample of apple pomace.

5. Pearson correlation and statistics

All extracts and analysis were performed in triplicate. Data statistical analysis was performed by one-way analyses of variance (ANOVA), with Tukey honestly significant difference (HSD) post-hoc test using SPSS 26 statistical software developed by IBM SPSS Corp, USA. The level of statistical significance was set at $p < 0.05$, representing the minimum accepted threshold for statistical significance. In the case of cumulative values, the statistics were analyzed by both one-way ANOVA and Independent Student T-test and the two-sided p-values were determined. The results were the same between the two tests. Levene's test was used to establish the homogeneity of variance. Pearson correlations between TPC, TFC, THA, DPPH, FRAP, CUPRAC, and each AOA normalized to TPC, TFC and THA (n = 12) were determined with SPSS Bivariate Correlations, the heatmap was generated by Conditional Formatting in Microsoft Excel, and the Matrix Scatter Plot was obtained with SPSS Chart Builder.

CONCLUSIONS

We investigated, for the first time to the best of our knowledge, a sequential hydroalcoholic – enzymatic two-step process for eco-friendly extraction of polyphenols from apple pomace. Variant 1, which consists of a hydroalcoholic (ethanolic) extraction as the first step and an enzymatic extraction as the second step gives higher polyphenolic content, but not higher AOA activity than the reversed order of the two extraction steps under the conditions tested.. High positive correlations were obtained between the polyphenol contents and total antioxidant activities, but negative correlations with the relative (specific) AOA were obtained in some cases (THA, CUPRAC), indicating structure-dependent and assay specificity. The influence of apple cultivar and individual step optimization, as well as the molecular composition of each extract needs to be established in future studies. Techno-economic analysis should establish the practical benefit of the 2nd step (S2 EE) in the case of variant 1, as the values obtained were significantly lower than S1 HE. The need to extract only polyphenols or both polyphenols and polysaccharides (pectin) with enhanced AOA, as well as the techno-economic analysis will determine the choice of the best extraction approach.

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