

Article

Optimization of Ultrasonic-Assisted Extraction of Antioxidants in Apple Pomace (var. *Belorusskoje malinovoje*) Using Response Surface Methodology: Scope and Opportunity to Develop as a Potential Feed Supplement or Feed Ingredient

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Abstract: Apple pomace represents an underexploited source of bioactive compounds. This study examines the optimization of total phenolic content (TPC) and antioxidant extraction yield of apple pomace (variety: *Belorusskoje malinovoje*) using response surface methodology. The green extraction technique used was ultrasound-assisted extraction, and it was compared with conventional solvent extraction. The impact of extraction time and amplitude of ultrasound-assisted extraction on the yield of polyphenols and antioxidants has been evaluated. Total phenolic content was determined using an established TPC assay. The antioxidant activity of the apple pomace was determined using established assays 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS^{•+})). Furthermore, the potential of apple pomace as a feed material was explored by assessing its nutritional composition, vitamins, minerals, fatty acids, and amino acid content. The extraction of antioxidants and phenolic compounds was efficiently optimized using RSM. The optimal conditions for TPC and DPPH[•] analyses were achieved with an extraction time of 17.5 min and an ultrasound-assisted extraction amplitude of 20%. Optimal conditions for ABTS^{•+} were 5 min extraction time and 20% amplitude. Conventional and ultrasound-assisted extraction methods yielded comparable results. Moreover, apple pomace exhibits potential as a feed ingredient despite its modest protein content. This study contributes to the utilization of apple pomace by providing additional information on its antioxidant content and nutritional composition, thus contributing to its sustainable utilization in various industries, especially the livestock feed sector.

Keywords: apple pomace; green extraction; optimization; antioxidants; RSM; animal feed



Citation: Malenica, D.; Maciel, L.S.; Herodes, K.; Kass, M.; Bhat, R. Optimization of Ultrasonic-Assisted Extraction of Antioxidants in Apple Pomace (var. *Belorusskoje malinovoje*) Using Response Surface Methodology: Scope and Opportunity to Develop as a Potential Feed Supplement or Feed Ingredient. *Sustainability* **2024**, *16*, 2765. <https://doi.org/10.3390/su16072765>

Academic Editor: Michael S. Carolan

Received: 19 February 2024

Revised: 16 March 2024

Accepted: 16 March 2024

Published: 27 March 2024



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1. Introduction

Utilization of fruit and vegetable waste as livestock feed offers a potential solution for global challenges such as climate change, urbanization, resource scarcity, and increase in current feed prices [1–6]. One of the common by-products of the fruit industry—apple pomace—exhibits promise as an alternative feed ingredient [5–12]. Apple pomace represents solid residue created during the extraction of juice from the apple. It consists of pulp, skin, seeds, and stalk [7,13]. It has been estimated that the annual production of apple pomace can lead to up to 4 million tonnes [12]. This extensive production of apple pomace is especially alarming considering the high moisture content of apples, which makes them more prone to microbiological contamination and, consequently, environmental pollution [1,3,14–16]. Utilization of apple pomace as animal feed material

could help alleviate the negative environmental impact caused by its unsustainable discarding while simultaneously benefiting the animal feed sector, which has been facing numerous challenges [4–6].

Research findings on the inclusion of apple pomace in animal diets are mixed; however, the health properties of apple pomace, like prebiotic and antioxidant effects, are encouraging [6,7,11,12,17–21]. Antioxidant compounds protect animal health by detoxifying harmful free radicals [22,23]. In high-stress situations or during pathological or physiological changes, animals may not be able to produce sufficient amounts of antioxidants to neutralize excess free radicals [22–25]. Adding antioxidant-rich ingredients to diets can prevent the accumulation of free radicals and, therefore, benefit animal health [26–29]. While synthetic antioxidants have proven to be effective, they are also believed to be carcinogenic, toxic, and cause lipid alteration [23,30,31]. Hence, there is a growing interest in utilizing natural antioxidants, which are commonly present in agro-industrial waste [23,32,33]. Choosing the appropriate extraction method is essential for obtaining bioactive compounds. Green extraction techniques aim to reduce environmental impact but are costly compared to conventional methods. Ultrasound-assisted extraction, a novel and efficient technique, has been utilized in this research because of its ability to maximize the yield of specific compounds while saving both time and energy [34–39]. Moreover, the conventional solvent extraction method was employed, and an equivalent volume of solvent was used, thereby demonstrating a comparable environmental impact to ultrasound-assisted extraction. However, it is important to emphasize one of the limitations of solvent extraction, which is that it is time-consuming, especially compared to ultrasound-assisted extraction [40–42].

Extraction optimization of antioxidants has been carried out on apple pomace before [43–52]. While previous studies have investigated antioxidant extraction from apple pomace using different technologies, solvents, and apple cultivars, numerous additional parameters and their corresponding values remain that could potentially impact the extraction process and warrant further exploration. Specifically, for ultrasound extraction of phenolic compounds and antioxidants from apple pomace, parameters such as temperature, concentrations of ethanol, power, and extraction time have been explored [53–55]. Amplitude and time were explored along with different factors involved, including different varieties of apples, temperatures, solvents, and concentrations of solvents used [50,56]. Based on the literature, the antioxidant content depends largely on the variety of apples [44,51,52,57].

To the best of our knowledge, comprehensive research on the nutritional and bioactive properties of the variety *Beloruskoja malinovoje* has yet to be undertaken. Even though certain studies have touched upon the extraction of antioxidants from this variety, a comprehensive investigation into its nutritional properties remains unexplored [52]. Furthermore, the vitamin content within apple pomace is an understudied area since previous studies mainly focused on vitamins C and E [58–61]. A thorough study of the vitamin content in apple pomace is necessary, as it could add to its nutritional value and hold implications for potential applications of this by-product in various industries.

Further study on the valorization of apple pomace in livestock feed production presents an opportunity to reduce fruit waste and benefit animal health. A comprehensive study with a wide range of analyses involving chemical composition and bioactive compounds for apple pomace felt necessary as the information on this has been scattered among several articles [7,43–49,51,57–61]. The main objective of the present research was to optimize the extraction yield of antioxidant compounds from a local variety of apple by-products, which previously exhibited promising antioxidant activity, using response surface methodology (RSM) to assess the impact of different extraction parameters.

The sub-objective of this study was to compare relationships between extraction yield achieved by green extraction and conventional extraction. This comparison aimed to determine whether costly extraction procedures are truly superior and essential and to provide valuable perspectives on perhaps more cost-effective but equally sustainable alternative methods. Proximate analyses have been conducted, followed by analyses of the

mineral, vitamin, fatty acid, and amino acid content, aiming to evaluate its potential as a feed ingredient and to compare it with existing feed ingredients.

2. Materials and Methods

2.1. Sample Selection and Collection

Apple pomace was collected from the Polli Horticulture Research Centre located in Viljandi County in Estonia. The pomace was air-dried using condensing dehydrator CFD 1400, Alpfrigo, at the temperature of +50 °C for 78 h and then ground and packaged in vacuumed polythene bags. The ground material was stored at refrigeration temperature (+4–7 °C) until further analysis.

The studied variety “*Belorusskoje malinovoje*” is cultivated in Estonia but is initially bred in Belarus as a winter variety. The variety was chosen based on a literature review where among 11 cultivars of 3 seasonal groups (autumn, autumn–winter, and late winter) in Latvia, the cultivar *B. malinovoje* exhibited the highest antioxidant results [52]. Given Estonia’s close geographical proximity to Latvia, we found this study particularly important when considering the cultivar selection for further research. Several other Estonian cultivars have been considered; however, based on their antioxidant content and also seasonality, the choice remained with *B. malinovoje* [44]. The apple tree variety of *B. malinovoje* is a winter variety with high yield and early fruiting [62,63]. It has good disease and good winter resistance potential [62]. The identification of this particular variety is based on the fruit: The ripe fruit of this variety is medium to large sized, round, and almost entirely crimson red, while the flesh is white [62,63]. It is juicy with a sweet and sour taste [62,63].

2.2. Proximate Analyses and Fatty Acid Content

Standard methods of the Association of Official Analytical Chemists were used to determine the moisture, crude protein, crude fat, crude fiber, and crude ash of apple pomace [64]. Dry matter (DM) content was determined by heating a feed sample for 2 h at +130 °C to constant weight. Crude ash was determined after ignition at 550 °C for 18 h. Analysis for ether extract content was performed by petroleum ether extraction with the Soxtec System 2043 Extraction Unit (FOSS, Hillerød, Denmark). Crude protein content was analyzed by the Kjeldahl method with a Kjeltac 2300 analyzer (FOSS, Hillerød, Denmark). For determination of crude fiber, ISO 6865:2000 was used [65]. The following calculation was applied for the determination of nitrogen-free extractives (NFE):

$$\text{NFE (\%)} = \text{dry matter} - (\text{crude ash} + \text{crude protein} + \text{crude fiber} + \text{crude fat}).$$
 Feed metabolizable energy was calculated according to Oll and Tölp (1995) and metabolizable protein (MP), as described by Kärt et al. [66,67].

The fatty acid profile was determined using a method described by Sukhija and Palmquist [68].

2.3. Mineral and Vitamin Content

To determine the mineral content of apple pomace, established methods were used. Calcium content was determined flame-photometrically using the EVS-EN ISO 6869:2001 method [69]. Phosphorus content was determined spectrophotometrically using AOAC Official Method 965.17 [62]. The following mineral contents were determined with flame atomic absorption spectroscopy (EVS-EN ISO 6869:2001): calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), iron (Fe), copper (Cu), manganese (Mn), and zinc (Zn).

Vitamin content analysis was performed at the Veterinary and Food Laboratory under the Estonian University of Life Science (Tartu, Estonia) using Agilent HPLC 1200 equipped with degasser, quaternary pump, autosampler, column thermostat, DAD, and FLD detectors for vitamins A, D, E, B1, B2, and B6. B5 was detected by liquid chromatography with tandem mass spectrometry (LC-MS-MS). The column used for all the vitamins was C18. For B vitamins, the mobile phase consisted of acidic water and methanol. For fat-soluble vitamins, the mobile phase contained water–methanol–acetonitrile. Water-soluble vitamins and B1–4 were detected with diode-array detection (DAD) and B6 by using a fluorescence

detector (FLD). The flow rate was 0.5 mL/min. The LC-MS-MS instrument operated in positive ionization mode: capillary voltage 3500 V. The methods used were as follows: EVS-EN 12821:2009 for vitamin D [70], EVS-EN 12822:2014 for vitamin E [71], EVS-EN 12823-1:2014 for vitamin A [72], EVS-EN 14152:2014 for vitamin B2 [73], EVS-EN 14122:2014 for vitamin B1 [74], and EVS-EN 14663:2006 for vitamin B6 [75]. The concentrations were calculated based on the peak area detected in the sample using external calibration.

2.4. Amino Acid Content

For the analysis of amino acid content, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system was used featuring an Agilent 1290 Infinity II quaternary pump, a column thermostat, an autosampler, and an Agilent 6460 Triple Quadrupole (QqQ) mass spectrometer (MS) with Agilent Jet Stream Technology electrospray ionization source (ESI). The chromatographic column was a Zorbax Eclipse Plus C18 (3.0 × 100 mm, 1.8 µm) equipped with a guard column (3.0 × 5 mm, 1.8 µm). The mobile phase consisted of 0.1% aqueous formic acid (A) and acetonitrile (B) at a flow rate of 0.4 mL/min with the following gradient: 0–2 min, 10%; 2–27 min, 10–98%; 27–29 min, 98%; 29–31 min; 98–10% (B). Analysis was carried out in positive ionization mode with a capillary voltage of 3000 V in dynamic multiple reaction monitoring (dMRM) mode. More details regarding the MS parameters, dMRM transitions, and hydrolysis conditions have been published [76]. However, the mass of the sample was modified, and approximately 100 mg of apple pomace was utilized.

2.5. Extraction Procedure

Antioxidants from apple pomace were extracted using both ultrasound-assisted and conventional extraction procedures. The conventional procedure was solvent extraction, during which 96% ethanol, 70% ethanol, and distilled water were used to determine which of the mentioned solvents gives the highest extraction yield. While we initially considered using smaller concentrations of ethanol as well, we chose to use 70% ethanol due to a literature review, which, in general, proved that 70% ethanol is favored over smaller concentrations for the extraction of polyphenols and antioxidants in various plant materials [77,78]. The goal of this study was to compare the highest yield achieved using ethanol to yields achieved with water. We chose 70% ethanol as we anticipated it would yield the highest results over lower ethanol concentrations. Grounded pomace 5 g was mixed with 100 mL of solvent in a glass beaker. After this, the extracts were left for 24 h in a shaker (Thermo Scientific MaxQ 6000 Shaker, Waltham, MA, USA).

The sample powder (5 g) was once again mixed with 100 mL of solvent. For comparison, the sample-to-solvent ratio was the same for both conventional and ultrasound extraction. For ultrasound extraction, 70% ethanol was used based on our results of conventional extraction. The choice was also made based on a literature review, which showed that a similar concentration of ethanol was the most optimal for ultrasound-assisted extraction of antioxidants and phenolic compounds from apple pomace [53]. Before ultrasound extraction, apple pomace samples with solvent were mixed using a magnetic stirrer with no heating applied to samples for 1 h. The ultrasound-assisted extraction of antioxidants was carried out using a UP 400St ultrasonic processor (Ø7 mm titanium horn; Hielscher GmbH, Chamerau, Germany). The conditions for the ultrasound extraction were chosen based on the literature review with some modifications [79]. The number of runs was determined by RSM.

The beaker with the sample was put in a larger beaker with ice to avoid overheating and degradation of the phenolic compounds during the extraction process. In general, the temperature was kept below +30 °C degrees.

After both conventional and ultrasound extraction, the extracts were separated from the residue by filtration using Whatman filter paper. Paper filtration was performed several times until the extract and the residual plant material were properly separated. Recovered extracts were placed in the refrigerator until antioxidant analysis was performed.

Extracts were analyzed for total phenolic content, DPPH• free radical scavenging activity, and ABTS•⁺.

2.6. Response Surface Methodology

For this particular study, response surface methodology coupled with the central composite design was used to evaluate the effect of two independent variables of the UAE process on the extraction yield of total phenolic content (TPC), DPPH• radical scavenging activity, and ABTS•⁺. The significance of the prediction model and the impact of variables were assessed using *p*-values and *R*². Model terms with *p*-values less than 0.0500 are considered statistically significant. The parameters for optimization were extraction time (A, min) and sonicator amplitude (B, %). The ranges of factors were 5–30 min for extraction time and 20–50% for amplitude. In Table 1, the observed responses (TPC, DPPH• and ABTS•⁺) are presented. In Table 2 and Table S1, observed levels of independent variables, determined by RSM, are shown. Table 3 presents an overview of the experimental design for ultrasound-assisted extraction.

Table 1. Responses observed for the central composite design.

Response	Name	Units
R1	TPC	mg GAE eq./g DW
R2	DPPH•	μM TE eq./g DW
R3	ABTS• ⁺	μM TE eq./g DW

mg GA eq./g DW-mg of gallic acid equivalents per g of dry weight; μM TE eq./g DW-μM TE Trolox equivalent (TE) per g of dry weight sample.

Table 2. Observed levels of independent variables.

Name	Units	Type	Min	Max	Coded Low	Coded High	Mean
Time	Min	Numeric Continuous	5.00	30.00	−1↔5.00	+1↔30.00	17.50
Amplitude	%	Numeric Continuous	20.00	50.00	−1↔20.00	+1↔50.00	35.00

Table 3. The experimental design matrix for ultrasound-assisted extraction.

Run	Time (min)	Amplitude (%)
1	30 (+1)	35 (0)
2	5 (−1)	35 (0)
3	5 (−1)	20 (−1)
4	30 (+1)	20 (−1)
5	5 (−1)	50 (+1)
6	30 (+1)	50 (+1)
7	17.5 (0)	20 (−1)
8	17.5 (0)	35 (0)
9	17.5 (0)	50 (+1)

The coded levels are designated as follows: +1 represents the highest observed level, 0 corresponds to the medium observed level, and −1 indicates the lowest observed level of the independent variable.

2.7. Total Phenolic Content

Total phenolic content was measured using the Folin–Ciocalteu (FC) method described by Song et al. (2010) with some modifications [80]. The gallic acid solutions used for the calibration curve were prepared at the following concentrations: 25, 50, 75, 100, 125, 150, 175, 200, 250, and 300 μg/mL. For the calibration curve, 40 μL of each standard was pipetted along with 200 μL of FC (0.2 N) reagent and 160 μL Na₂CO₃ (75 g/L) into a 96-well microplate with 400 μL volume. The microplate was incubated for 30 min in the dark. After that, the microplate was inserted into a microplate spectrophotometer (Biotek, Epoch 2 microplate reader, Winooski, VT, USA), and the absorbance was read at 765 nm wavelength.

Once the calibration curve was made, TPC analyses of apple pomace extracts were performed. Before analyses, all the samples were diluted 40 times (12.5 μL of apple pomace extract + 487.5 μL of solvent used for extraction). The samples were then added the same way as it was carried out for the standard calibration: 40 μL of the diluted sample, 200 μL of FC (0.2 N) reagent, and 160 μL Na_2CO_3 (75 g/L) in microplate, which was then inserted into microplate spectrophotometer. The absorbance was read at 765 nm wavelength. Acquired TPC results were expressed as mg of gallic acid equivalents per g of apple pomace dry weight (mg GA eq./g DW) using regression equation ($R^2 = 0.9992$) acquired from the calibration curve. All chemicals were of analytical grade and were purchased from Stigma (Steinheim, Germany).

2.8. DPPH• Radical Scavenging Assay

DPPH• radical scavenging activity was determined using a spectrophotometric method of Brand-Williams et al. with some modifications [81]. For the calibration curve, 20 μL of Trolox standard, previously prepared in various concentrations, was pipetted, and then 360 μL of 0.1 mM DPPH• assay was added into a 96-well microplate. The microplate was left to incubate in darkness at room temperature for 30 min. After that, the reading was carried out at 515 nm wavelength using a microplate reader. Before the analyses, apple pomace extracts were diluted 10 times (50 μL of apple pomace extract + 450 μL of solvent used for extraction). The DPPH• procedure of apple pomace extracts was carried out in the same way as for the calibration curve described previously: 20 μL of sample and 360 μL of DPPH• assay were pipetted into the microplate, and the absorbance was read at 515 nm wavelength. The final DPPH• values were calculated using the regression equation ($R^2 = 0.9932$) obtained from the calibration curve. The antioxidant capacity of each sample is expressed as μM of Trolox equivalent (TE) per g of dry weight sample. All chemicals used for this experiment were of analytical grade and were purchased from Stigma (Steinheim, Germany).

2.9. ABTS•+ Radical Scavenging Assay

ABTS•+ analyses were performed using a method previously described by Re et al. (1999) with some modifications [82]. ABTS•+ radical cation was made by mixing 7 mM ABTS•+ with 2.45 mM potassium persulfate (1:0.5). After that, it was incubated for 12 h in the dark at room temperature. Prior to the analyses, the ABTS•+ assay was diluted 32 times with 70% ethanol. For the calibration curve, 3 μL of Trolox solutions at various concentrations were pipetted into 96-well microplates, and then 300 μL of diluted reagent was added. The microplate was incubated for 30 min in the dark. Reading was performed at 734 nm wavelength using a microplate spectrophotometer (Biotek, Epoch 2 microplate reader, USA). The ABTS•+ measurement procedure of apple pomace extracts was carried out in the same way as for the calibration curve described previously: 3 μL of sample and 300 μL of assay were pipetted into the microplate, and the absorbance was read at 734 nm wavelength. The final ABTS•+ values were calculated by using a regression equation ($R^2 = 0.9961$) obtained from the calibration curve. ABTS•+ results for each sample are expressed as μM TE Trolox equivalent (TE) per g of dry weight sample. All chemicals utilized for ABTS•+ analyses were of analytical grade and were purchased from Stigma (Steinheim, Germany).

2.10. Statistical Analyses

For statistical analyses, Design Expert 12 (Stat-Ease Inc., Minneapolis, MN, USA) software was used to determine whether factors were statistically significant for the optimization of antioxidant analyses. R Statistical Software (v4.1.2; R Core Team 2021) was used for the generation of plots. Correlations and *t* tests were performed in Microsoft Excel 2017 Data Analysis Add-in. In addition, to evaluate the significance of solvent impact on extraction yield, SAS OnDemand for Academics was used (SAS OnDemand for Academics,

Cary, NC, USA). All results are mean values of three replicate analyses calculated, apart from vitamin content analyses.

3. Results and Discussion

3.1. Proximate Analyses and Fatty Acid Content

Apple pomace showed a relatively low content of crude protein and a moderately low content of crude fiber compared to conventional feed material. The proximate content of this variety of apple pomace is presented in Table 3. Previous studies have reported similar results of proximate analyses of apple pomace, which include low crude protein and crude fat in apple pomace [57,83–91]. However, this variety of apple showed scanty crude fiber content, only up to 14.6%, which is significantly lower than previously reported crude fiber content of dehydrated apple pomace [92–94]. While apple pomace may have low protein and fat content, enhancing its nutritional profile by ensiling it with urea or ammonia or fermenting transforms it into a considerable alternative feed ingredient for ruminants. This process elevates its feeding value to a level comparable to that of grass silage for beef cattle [83]. Based on the literature review that has been carried out for this article, the content of protein and fat of apple pomace from variety *B. malinovje* is comparable to that of citrus pulp [95–97]. The crude fat and crude fiber content of apple pomace has shown to be more similar to pumpkin and citrus pulp; however, pumpkin peel has much greater protein content [95–100]. The results of metabolizable energy and metabolizable protein align with previously reported results and are presented in Table 4 [83,84,89–91].

Table 4. Proximate analysis, vitamin, fatty acid, and mineral content of apple pomace.

Traits		Vitamin Content		Fatty Acid Profile		Mineral Content	
Dry matter, %	89.4 ± 0.28	Vitamin A, Retinol µg/100 g	<2	C14:0	0.32 ± 0.03	Ca, g/kg	1.1 ± 0.08
Crude protein, %	3.0 ± 0.15	Vitamin A, Beta carotene µg/100 g	<20	C16:0	14.6 ± 0.95	P, g/kg	1.5 ± 0.2
Crude fiber, %	14.6 ± 0.25	Vitamin E mg/100 g	2.42	C16:1	0.2 ± 0.04	Na, g/kg	0.04 ± 0
Crude fat, %	3.1 ± 0.2	Vitamin D µg/100 g	<1	C18:0	4.15 ± 0.25	K, g/kg	10.6 ± 0.2
Content of NFE in feed, %	76.2 ± 1.3	B1 vitamin mg/100 g	<0.01	C18:1	17.1 ± 0.95	Mg, g/kg	0.6 ± 0.07
ME, MJ/kg	9.8 ± 0.55	B2 vitamin mg/100 g	0.29	C18:2	55.1 ± 1.4	Zn, mg/kg	10.1 ± 0.5
MP, g/kg	73 ± 0.8	B3 vitamin mg/100 g	0.6	C18:3	3.94 ± 0.3	Copper, mg/kg	6.7 ± 0.2
		B5 Vitamin mg/100 g	0	C18:4	0.09 ± 0	Manganese, mg/kg	5.6 ± 0.1
		B6 Vitamin mg/100 g	0.23	C20:0	2.12 ± 0.2	Fe, mg/kg	11.2 ± 0.28
				C20:1	0.34 ± 0.03		
				C20:2	0.35 ± 0.08		
				C22:0	0.61 ± 0.02		
				C22:1	0 ± 0		
				C24:0	0 ± 0		

ME = metabolizable energy, MP = metabolizable protein; fatty acid composition results are expressed as g/100 g of fatty acids. C14:0—myristic acid; C16:0—palmitic acid; C16:1—palmitoleic acid; C18:0—stearic acid; C18:1—oleic acid; C18:2—linoleic acid; C18:3—alpha-linolenic acid; C18:4—stearidonic acid; C20:0—arachidic acid; C20:1—eicosenoic acid; C20:2—eicosadienoic acid, C22:0—docosanoic acid, C22:1—erucic acid, C24:0—lignoceric acid. Vitamins are expressed in different units.

Regarding fatty acid content, linoleic acid and oleic acid are proven to be two major unsaturated fatty acids of apple pomace. These results align with previous studies on the fatty acid composition of apple pomace [7,101,102]. While oleic and linoleic fatty acids are dominant fatty acids in apple pomace, apple pomace's low-fat content means that this by-product is not considered a rich source of these fatty acids compared to other waste materials, which may yield higher amounts [7].

3.1.1. Minerals and Vitamin Content

Apple pomace measured a relatively high content of vitamin E when compared to conventional feed material [103]. To our knowledge, only a few studies on apple pomace included the vitamin content of apple pomace; thus, the vitamin results obtained in this study were difficult to compare with previous results. Previous studies of apple pomace mostly included vitamins C and E only and often with antioxidants, making it difficult to

understand the value obtained for individual vitamins [58–61,104]. However, based on the results, it can be concluded that pomace derived from this particular variety is a rich source of vitamin E, which is consistent with previous findings in apple pomace [7,102]. Vitamin E significantly influences animal health, particularly in dairy cows, by positively affecting reproductive function, bolstering the immune system, aiding in mastitis prevention, and enhancing milk quality [105]. Even though the concentration of vitamin E for this variety is still quite high, it is lower compared to previously reported vitamin E concentration for apple pomace [7,104]. The results of the vitamin content obtained for apple pomace are shown in Table 3. Moreover, the results of the mineral content of apple pomace are consistent with prior studies [7,11,55]. Based on our results, this variety of apple pomace exhibited high concentrations of potassium, while other minerals are present in lower or trace amounts [7,106].

3.1.2. Amino Acid Content

Based on our results, the amino acid concentration of this variety of apple pomace is low when compared to conventional feed material [103]. Our results showed that two major amino acids in apple pomace are glutamic acid and aspartic acid. The high concentration of glutamic acid in apple pomace is especially interesting, considering its importance for dairy cows. Glutamic acid plays a significant role in protein metabolism, and it is involved in various psychological processes in dairy cows, with a special emphasis on the synthesis of milk protein [107]. Providing an adequate amount of glutamic acid is essential for ensuring animal health and milk production. Amino acid content is presented in Figure 1 and in the chromatogram of amino acids after hydrolysis in dMRM mode, presented in Figure S5. Previous studies involving the amino acid content of apple pomace reported similar results, with general low protein content and glutamic acid and aspartic acid being major amino acids [108,109].

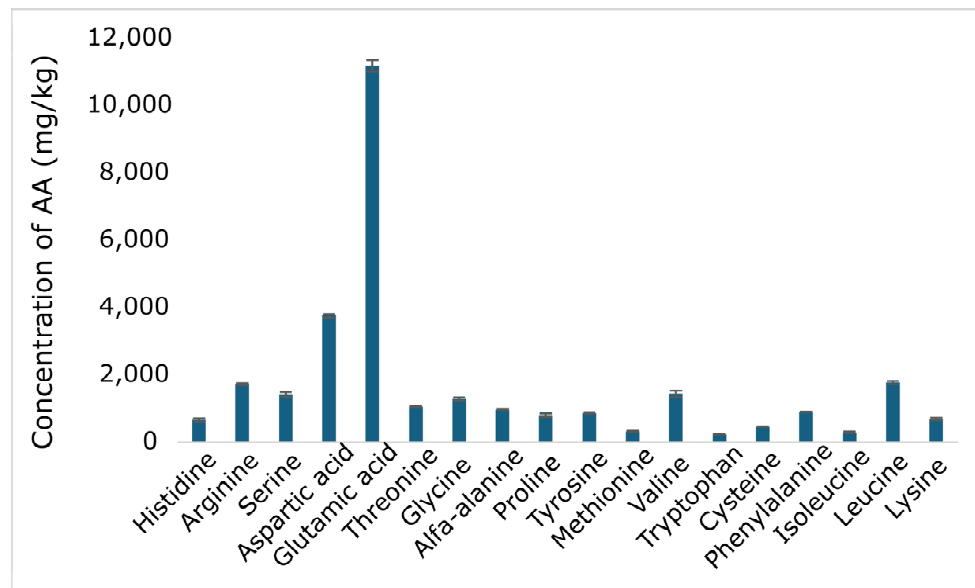


Figure 1. Total amino acid content (mg/kg) of apple pomace. Amino acid composition results are expressed as mg/kg of sample; all values are means $\pm$ standard deviation, $n = 2$.

3.2. Optimization of Conventional Extraction of TPC, DPPH $\bullet$, and ABTS $\bullet^+$

The solvent extraction efficiency was similar across three analyses: TPC, DPPH $\bullet$, and ABTS $\bullet^+$; 70% of ethanol in all three analyses showed the best results and highest yield of antioxidants. Before the analyses, distilled water was expected to show the lowest results; and it was chosen for this experiment to see whether distilled water extraction results could be comparable to the ethanol extraction results. The aim of including distilled water was to evaluate the possibility of eliminating the use of solvents that negatively affect the

environment, even though compared to other solvents (hexane, chloroform, methanol, etc.), ethanol is considered a green solvent and is environmentally preferable [110]. Besides looking from an environmental aspect, economically, it would also make more sense to use distilled water rather than ethanol. Distilled water, however, did not show the lowest results, as the smallest extraction yield for all three analyses was obtained when 96% ethanol was used. In Table 5, mean values of TPC, DPPH•, and ABTS•+ results are presented, respectively. Based on the obtained results and the highest yield for TPC, DPPH•, and ABTS•+ achieved by using 70% ethanol during conventional extraction, ultrasound-assisted extraction was carried out with this solvent. The effect of solvent on the extraction yield of all three analyses—TPC, DPPH•, and ABTS•+—was statistically significant: $p = 0.0006$, $p = 0.0010$, and $p = 0.0017$ respectively (Table S6, Table S7 and Table S8, accompanied by visual representation: Figure S2, Figure S3 and Figure S4 respectively).

Table 5. Mean values of TPC (mg GAE/g DW), DPPH• (μM TE/g DW), and ABTS•+ (μM TE/g DW).

	TPC	DPPH•	ABTS•+
APC96	2.9 ± 0.2	31.34 ± 1.7	84.86 ± 1.6
APC70	4.36 ± 0.1	43 ± 1.8	95.81 ± 1.18
APCW	3.15 ± 0.3	36 ± 2.2	90.7 ± 2.83

APC96 represents apple pomace extracted in 96% ethanol, APC70—apple pomace extracted in 70% ethanol, and APCW—apple pomace extracted in distilled water.

3.3. Optimization of Ultrasound-Assisted Extraction of TPC, DPPH•, and ABTS•+

For optimizing the yield of TPC, DPPH•, and ABTS•+, the chosen extraction method was ultrasound-assisted extraction as this technique is quite efficient, energy and time-saving, and it is suitable for extraction of heat-sensitive compounds. For this study, two independent variables were selected for the optimization of TPC, DPPH•, and ABTS•+ yield: extraction time and amplitude. RSM was employed to ascertain the optimal condition of independent variables, to create a prediction model, and to evaluate the impact of these two factors on the TPC and antioxidant yield. The independent variables are presented in Table 3. In Table 6, actual and predicted values of TPC, DPPH•, and ABTS•+ are displayed, with visual representation of the yields shown in Figure S1. Based on the data acquired and summary statistics involving p -value, F -value, and R^2 , the quadratic model was the best fit for maximizing all three yields (Tables S9–S11).

Table 6. Actual and predicted values of TPC (mg GAE/g DW), DPPH• (μM TE/g DW), and ABTS•+ (μM TE/g DW) of apple pomace extracted by ultrasound-assisted extraction.

Run Order	Time (min)	Amplitude (%)	TPC		DPPH•		ABTS•+	
			Actual Value	Predicted Value	Actual Value	Predicted Value	Actual Value	Predicted Value
1	30	35	3.65	3.66	26.3	24.7	96.0	96.2
2	5	35	2.55	2.68	17.5	17.9	95.7	94.2
3	5	20	3.65	3.50	35.2	34.4	100.7	101.8
4	30	20	4.24	4.15	43.6	43.9	98.4	98.7
5	5	50	1.50	1.52	20.0	20.3	88.0	88.4
6	30	50	2.75	2.83	23.2	24.5	95.9	95.4
7	17.5	20	4.26	4.45	44.4	45.0	98.0	96.6
8	17.5	35	3.95	3.80	26.0	27.1	90.2	91.5
9	17.5	50	2.90	2.80	30.0	28.2	88.0	88.2

All results are mean values of three replicate analyses calculated.

For the optimization of TPC extraction yield, the model p -value implies that the model is significant, as presented in Table 7 and Table S3. In this case, A, B, and A^2 are significant model terms (factors) for the optimization of TPC yield, with A being time and B being the amplitude, as shown in Table 7. p -values indicate that both factors (time and amplitude)

impact the yield of TPC. The predicted R^2 of 0.7614 is in reasonable agreement with the adjusted R^2 of 0.9401; i.e., the difference is less than 0.2 (Table S2). The high R^2 value of 0.9775 implies that approximately 97.75% of the variability in TPC results can be explained by independent variables in the model (Table S2). The adjusted R^2 is also very high, which suggests that even with multiple factors included, the model can explain about 94% of the variability of response (Table S2).

Table 7. p -values of the models for optimization of TPC, DPPH $^{\bullet}$, and ABTS $^{\bullet+}$ yield obtained by Analysis of variance (ANOVA) for quadratic model.

Model and Model Terms	Response 1: TPC	Response 2: DPPH $^{\bullet}$	Response 3: ABTS $^{\bullet+}$
	p -Value	p -Value	p -Value
Model	0.011 *	0.005 *	0.031 *
A-Time	0.013 *	0.019 *	0.236
B-Amplitude	0.003 *	0.002 *	0.008 *
AB	0.235	0.247	0.051
A 2	0.028 *	0.020 *	0.048 *
B 2	0.345	0.005 *	0.505

* Represent values which are statistically significant at $p < 0.05$.

Regarding the optimization of DPPH $^{\bullet}$ yields, the model's F-value of 45.05 and p -value ($p = 0.0051$) indicate that the model is significant, as can be seen in Tables 7 and S4. A, B, A 2 , and B 2 are significant model terms, which tells us that both time and amplitude influence DPPH $^{\bullet}$ yield (Table 7). Just like in the results for TPC, the predicted R^2 is in reasonable agreement with the adjusted R^2 , with the difference being less than 0.2 (Table S2). In addition, high R^2 and adjusted R^2 values suggest that the model is a good fit and that it can explain a large percentage of variability of DPPH $^{\bullet}$ extraction yield (Table S2).

For maximizing ABTS $^{\bullet+}$ yield, the p -value ($p = 0.03$) suggests the model created is significant, as presented in Table 7 and Table S5. In this quadratic model, B (amplitude) and A 2 are significant model terms. In addition, R^2 of the ABTS $^{\bullet+}$ optimization model is high: 0.9552, suggesting that 95% variability in ABTS $^{\bullet+}$ extraction yield can be explained by independent variables used in the model (Table S2). However, the predicted R^2 of 0.56 is not as close to the adjusted R^2 of 0.88 as one might normally expect (Table S2). For this model, reducing the number of terms could be helpful.

Final equations describing the extraction yield of TPC (mg GAE/g DW) (1), DPPH $^{\bullet}$ (2), and ABTS $^{\bullet+}$ (3) were the following:

$$Y1 = 3.38 + (0.149 \times A) - (0.015 \times B) + (0.00088 \times A \times B) - (0.004019 \times A^2) - (0.000782 \times B^2) \quad (1)$$

$$Y2 = 77.84924 + (1.81633 \times A) - (3.38551 \times B) - (0.006917 \times A \times B) - (0.037173 \times A^2) + (0.042141 \times B^2) \quad (2)$$

$$Y3 = 120.5616 - (1.22422 \times A) - (0.784751 \times B) + (0.013556 \times A \times B) + (0.023644 \times A^2) + (0.003827 \times B^2) \quad (3)$$

where Y1 represents the yield of TPC (mg GAE/g DW), Y2 represents the yield of DPPH $^{\bullet}$ (μ M TE/g DW), Y3—ABTS $^{\bullet+}$ (μ M TE/g DW), A—time, and B—amplitude.

In Figure 2, a three-dimensional (3D) response surface plot is represented to show the visual effect of factors on each response, TPC (a), DPPH $^{\bullet}$ (b), and ABTS $^{\bullet+}$ (c) extraction yield, as well as the relationship between the two factors. From the plot, it can be concluded that increasing the amplitude negatively affected the yield of TPC (Figure 2a). This can also be concluded by Equation (1). At the same time, with the increase in the time of extraction, TPC extraction increased as well. The lowest amplitude (20%) and middle set time (17.5 min) provided the highest yield of TPC, while the lowest amplitude and highest set time were a close second.

Based on the given Equation (2), which helps us understand the impact of the independent variable on the response, in this case, DPPH $^{\bullet}$, we can conclude that increasing amplitude has a negative effect on DPPH $^{\bullet}$ extraction yield. This has been further proved by a 3D response surface plot, which provides the visual of the factor's individual and

combined influence on the response (DPPH[•]), which is shown in Figure 2b. Much like with the results of TPC, the lowest set amplitude (20%) accompanied by the middle set time 17.5 provided the highest DPPH[•] results. This also implies there is a positive correlation between TPC and DPPH[•] results.

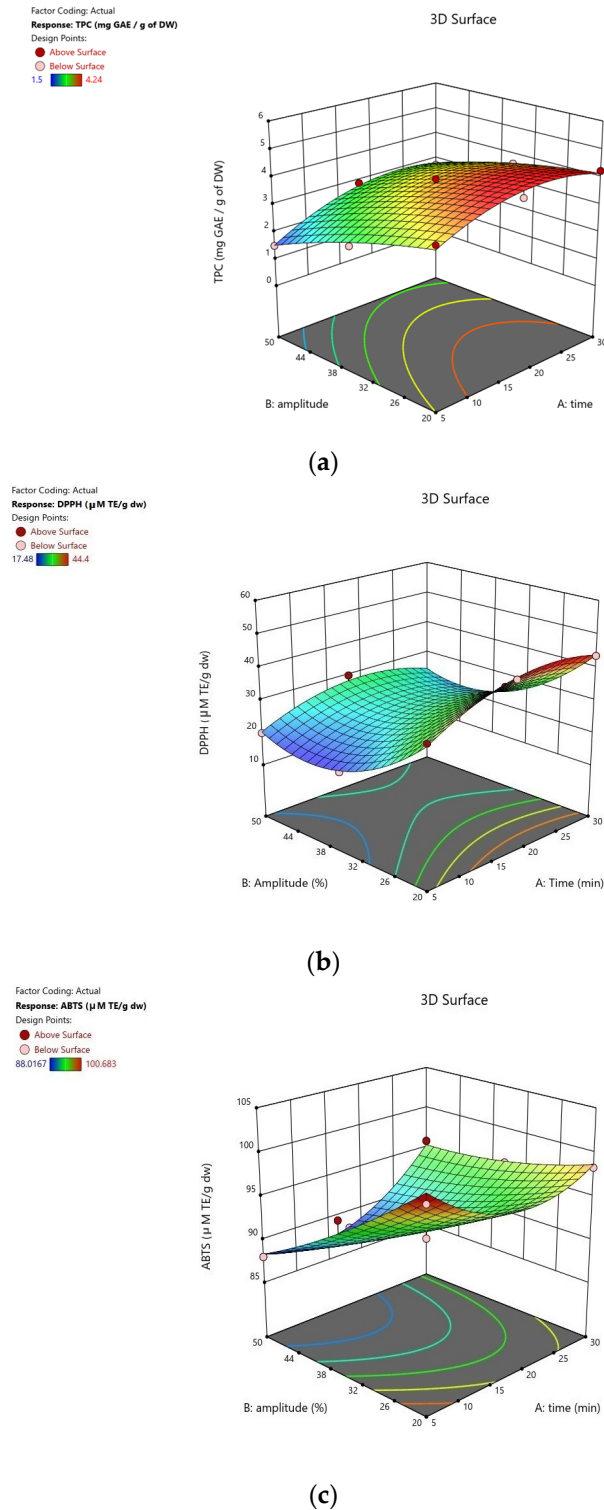


Figure 2. Three-dimensional response surface plot showing the effects of ultrasonic time (A) and amplitude (B) on TPC extraction yield (a), DPPH[•] extraction yield (b), and ABTS^{•+} extraction yield (c). Blue color presents the lowest results, and red color shows the highest results for TPC yields (mg GAE/g DW), DPPH[•], and ABTS^{•+} (μM TE/g DW).

Looking at Equation (3), we can see that increasing both time and amplitude has a negative effect on $\text{ABTS}^{\bullet+}$ yield. This is visually shown in the 3D response surface plot, which is presented in Figure 2c. Based on this, we can also include that the correlation between $\text{ABTS}^{\bullet+}$ extraction yield and TPC and $\text{DPPH}^{\bullet}$ is slightly less. The highest $\text{ABTS}^{\bullet+}$ yield was obtained with the lowest amplitude (20%) and lowest extraction time (5 min). The $\text{ABTS}^{\bullet+}$ assay yielded the highest results with the shortest extraction time, unlike TPC and $\text{DPPH}^{\bullet}$, where the highest results were achieved with medium extraction time. The decline in $\text{ABTS}^{\bullet+}$ results associated with prolonged extraction time could be attributed to the decomposition of antioxidative compounds within the sample [111,112]. Furthermore, the highest $\text{ABTS}^{\bullet+}$ results obtained with the shortest extraction time could be attributed to the rapid response of certain antioxidants, leading to increased activity during shorter extraction time, while other antioxidants may require a longer period of time to reach their optimal antioxidant activity [113]. It is important to emphasize that while both $\text{DPPH}^{\bullet}$ and $\text{ABTS}^{\bullet+}$ provide valuable information about the antioxidant capacity of the material, they might not extract the same antioxidants [113]. In addition, the antioxidants they extract could exhibit different response times influenced by their distinct chemical properties and interactions with other components in the matrix [113]. Moreover, some antioxidants could be more sensitive to certain extraction conditions such as ultrasound intensity, temperature, extraction duration, etc. [114–116]. Differences in assay conditions, especially the concentration of radicals and reaction kinetics, might contribute to the highest antioxidant yield being achieved with different extraction times [113].

3.4. Comparison between Conventional and Ultrasound-Assisted Extraction and Antioxidant Content of Apple Pomace

The results for TPC, $\text{DPPH}^{\bullet}$, and $\text{ABTS}^{\bullet+}$ from apple pomace correspond with results reported in previous studies carried out on different varieties of apple pomace [47,50,53,56]. In this study, TPC values for the variety *B. malinovoje* were lower than the results previously presented for this variety; however, $\text{DPPH}^{\bullet}$ results are in agreement [52]. While this study reports lower total phenolic content (TPC) results compared to previous findings, several factors might explain this variance. Differences in extraction techniques, methodologies, variations in the treatment of apple pomace, and seasonal variations could contribute to the observed differences in antioxidant content [117–120]. Understanding and recognizing the factors that influence is very important for the optimization of antioxidants of apple pomace.

In addition, the positive values of linear correlation coefficients indicate that analyses are positively correlated one with the other ($\text{TPC} \times \text{DPPH}^{\bullet} = 0.78$, p value: 0.01388; $\text{DPPH}^{\bullet} \times \text{ABTS}^{\bullet+} = 0.52$, p value: 0.1497 and $\text{TPC} \times \text{ABTS}^{\bullet+} = 0.57$ p value: 0.109); however, the correlation is not as high as expected. Previously reported correlation between results of TPC, $\text{DPPH}^{\bullet}$, and $\text{ABTS}^{\bullet+}$ of apple pomace has been higher [47]. The correlation between TPC and $\text{DPPH}^{\bullet}$ results is statistically significant. In addition, $\text{ABTS}^{\bullet+}$ results are higher than $\text{DPPH}^{\bullet}$ results. This can be attributed to the fact that $\text{ABTS}^{\bullet+}$ assay applies to hydrophilic and hydrophobic antioxidant systems, while the $\text{DPPH}^{\bullet}$ applies to hydrophobic systems only [121–123]. This can result in $\text{ABTS}^{\bullet+}$ capturing more antioxidant capacity, leading to higher readings [121–123].

In addition, the difference in results between $\text{ABTS}^{\bullet+}$ and $\text{DPPH}^{\bullet}$ could be explained by higher radical reactivity and reaction kinetics in $\text{ABTS}^{\bullet+}$ assay than in $\text{DPPH}^{\bullet}$ where the reduction in radicals occurs more slowly [124–127]. Due to higher radical reaction, absorbance decreases faster too, which may lead to higher antioxidant activity values observed in the $\text{ABTS}^{\bullet+}$ assay compared to the $\text{DPPH}^{\bullet}$ assay [111–113,122]. Antioxidant compound solubility can also lead to differences in results [128,129]. In addition, $\text{ABTS}^{\bullet+}$ is more sensitive than $\text{DPPH}^{\bullet}$ assay, and it can detect antioxidants with low concentrations, which can further explain the higher results obtained [122]. The choice of solvent can play a significant role and influence the results as well. $\text{ABTS}^{\bullet+}$ radicals are soluble in both organic and aqueous solutions, while $\text{DPPH}^{\bullet}$ radicals are soluble in organic mediums

only [122,124,127]. Therefore, the choice of solvent can affect the solubility and reactivity of antioxidants and affect the results. Also, because the chemical structure of ABTS^{•+} and DPPH[•] radicals is different, it impacts their reactivity with antioxidants, so some antioxidants may be more efficient at scavenging ABTS^{•+} radicals compared to DPPH[•] radicals, which can result in higher readings in ABTS^{•+} assay [113].

The difference between TPC, DPPH[•], and ABTS^{•+} of apple pomace obtained by conventional extraction using 70% ethanol, and TPC, DPPH[•], and ABTS^{•+} results obtained by ultrasound extraction using optimal conditions is not statistically significant ($p = 0.368$, $p = 0.128$, and $p = 0.122$, respectively). Interestingly, TPC results from conventionally extracted apple pomace using 70% ethanol are higher than results acquired by using optimal conditions for ultrasound extraction. However, as previously mentioned, the difference between the two is not statistically significant. For both DPPH[•] and ABTS^{•+}, extraction yield was higher when optimal conditions for ultrasound-assisted extraction were used, compared to yield obtained by conventionally extracted apple pomace with 70% ethanol, but as previously stated, the difference was not statistically significant. Based on the results obtained, further research on extraction conditions and different extraction techniques is advised.

4. Conclusions

Based on our comprehensive investigation, it can be concluded that the apple pomace variety *B. malinovoje* has potential as a feed ingredient despite its low protein and fiber content. It is a rich source of vitamin E and glutamic acid, which could especially be beneficial to dairy cows. Moreover, the extraction yield of TPC, DPPH[•], and ABTS^{•+} has been optimized using RSM, and the impact of independent variables—time and amplitude—was established. The time and amplitude proved to be of high significance. The optimal conditions for TPC and DPPH[•] analyses were found to be an extraction time of 17.5 min and an ultrasound-assisted extraction amplitude of 20%. For ABTS^{•+}, optimal conditions were achieved with a 5 min extraction time and 20% amplitude. Further research is warranted to improve the nutritional content of apple pomace, especially in enhancing its protein and fiber content. Enhancing protein content could potentially be achieved through ensiling apple pomace with urea or by fermentation and cultivation of filamentous fungi with pomace. Fiber content could be enhanced by adding material rich in fiber with apple pomace. Besides enhancing fiber and protein content, we propose further research involving optimizing vitamin E yield, studying the effect of apple pomace on animal health, and determining the optimal percentage of incorporating apple pomace in the diets of different animals. By addressing these areas, apple pomace of var. *B. malinovoje* could be utilized as a sustainable and nutritious ingredient in animal feed production, contributing to both agricultural sustainability and animal wellbeing.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su16072765/s1>, Figure S1: Presentation of actual and predicted yield of TPC (a), DPPH (b) and ABTS^{•+} (c); Table S1: Independent variables and their levels; Table S2: R² values of the model predicting TPC, DPPH[•] and ABTS^{•+} extraction yield; Table S3: Analysis of variance (ANOVA) of model for the yield of TPC from apple pomace; Table S4: Analysis of variance (ANOVA) of model for the yield of DPPH[•] from apple pomace; Table S5: Analysis of variance (ANOVA) of model for the yield of ABTS^{•+} from apple pomace; Figure S2: Distribution of TPC results for apple pomace conventionally extracted using different solvents; Figure S3: Distribution of DPPH[•] results for apple pomace conventionally extracted using different solvents; Figure S4: Distribution of ABTS^{•+} results for apple pomace conventionally extracted using different solvents; Table S6: Results of ANOVA procedure for TPC results of apple pomace conventionally extracted using different solvents (96% ethanol, 70% ethanol, distilled water); Table S7: Results of ANOVA procedure for DPPH[•] results of apple pomace conventionally extracted using different solvents (96% ethanol, 70% ethanol, distilled water); Table S8: Results of ANOVA procedure for ABTS^{•+} results of apple pomace conventionally extracted using different solvents (96% ethanol, 70% ethanol, distilled water); Table S9: Model Summary Statistics for TPC results from apple pomace obtained

with ultrasound-assisted extraction; Table S10: Model Summary Statistics for DPPH• results from apple pomace obtained with ultrasound-assisted extraction; Table S11: Model Summary Statistics for ABTS•+ results from apple pomace obtained with ultrasound-assisted extraction; Figure S5: Chromatogram in MRM mode of amino acids after hydrolysis.

Author Contributions: D.M., experimental works, designing of the experiments and methodology, interpretation of results, and writing—original draft and editing; L.S.M., experimental work, methodology, investigation, and editing of the draft; M.K., supervision and editing of the draft; K.H., supervision; R.B., supervision, editing and funding. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the European Union’s Horizon 2020 research and innovation program under grant agreement No. 810630 (VALORTECH). In addition, funding was received from Mobilitas Pluss ERA-Chair support [Grant No. MOBEC006 ERA Chair for Food (By-) Products Valorisation Technologies of the Estonian University of Life Sciences]. The author (R.B.) acknowledges the base funding of the Estonian University of Life Sciences (No. P210162VLTQ) provided to support research and development activities. The authors L.S.M and K.H. were supported by the European Regional Development Fund (TK141 “Advanced materials and high-technology devices for energy recuperation systems”). The work was performed using the instrumentation at the Estonian Centre of Analytical Chemistry.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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