Ultrasound-Assisted Extraction of Pectin from *Malus domestica* ‘Fălticeni’ Apple Pomace

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**Abstract:** The use of an ultrasonic treatment for the extraction of pectin from *Malus domestica* ‘Fălticeni’ apple pomace, its effects on extraction yield and galacturonic acid content, and degree of esterification of the extracted pectin were investigated. The optimization of the extraction process showed that the highest yield of 9.183% pectin, with a 98.127 g/100 g galacturonic acid content and 83.202% degree of esterification, was obtained at 100% amplitude, pH of 1.8, SLR of 1:10 g/mL, and 30 min. The pectin obtained in optimal extraction conditions was compared to commercial citrus and apple pectin in terms of chemical composition (determined by FT-IR), thermal behaviour (analyzed by differential scanning calorimetry), rheological properties, and morphological structure (analyzed by scanning electron microscopy). By comparison to commercial citrus and apple pectin samples, the FT-IR analysis of pectin extracted by ultrasound treatment confirmed the high degree of esterification and showed similarity to that of apple pectin (88.526%). It was found that the thermal behaviour of the pectin obtained by ultrasound-assisted extraction was influenced by the narrower distribution of molecular weights and the orderly molecular arrangement, while the rheological properties (high viscosity, G₀, and G₁) of this sample were influenced by the morphological structure and the galacturonic acid content. The correlation coefficient showed a strong positive relationship between viscosity and galacturonic acid content ($r = 0.992^{**}$).

**Keywords:** apple pomace; pectin; *Malus domestica* ‘Fălticeni’; ultrasound; optimization

1. Introduction

Pectins have numerous applications in the food industry as technological adjuvants—the commercially available pectins are almost exclusively produced from citrus peel and apple pomace, two by-products of fruit juice manufacturing [1,2]. Apart from these main sources, other raw materials such as banana peel [3,4], watermelon rind [5], mango peel [6,7], sisal waste [8], sunflower heads [9], pistachio green hull [10,11], and tomato waste [2] were used for extraction, and the resulting yield and the composition and properties of the obtained pectin were studied and reported. This interest for finding other sustainable pectin sources arises from a growing demand in the market and from the attempt to obtain pectins with diverse functional properties by valourizing various side streams [1,11].

Defined as a group of cell wall polysaccharides that are heterogeneous in structure, pectins are composed of at least 17 different monosaccharides that are interconnected through more than 20 different chemical bonds [12,13]. Galacturonic acid, in the form of1,4 linked α-D-galacturonic acid (GalA) residues, was identified as the most prevailing building block in pectin structure and is a predominant component of the homogalacturonan (HG) and rhamnogalacturonan I (RG-I) structural regions [14]. In the European Union, according to EU regulation No. 231/2012, food-grade pectin should have a content of galacturonic acid not less than 65%.
The extraction of pectin from a raw material is a multiple stage physicochemical process that includes a pretreatment, an extraction operation, and a post-extraction stage [11]. In the commercial manufacturing process, the extraction operation involves the use of hot water (60–100 °C) acidified to a pH in a range from 1.5 to 3.0 and is completed in 0.5–6 h [15]. Because the overall process is time-consuming and the resulting yield can be limited, other non-conventional extraction techniques that include the use of microwave, ultrasound, subcritical water, and enzymes have been applied for the isolation of pectin [16].

The ultrasound-assisted extraction (UAE) is a process that combines the use of acoustic energy with that of an extraction solvent to isolate target compounds from various plant matrices [17]. Some of the major advantages of UAE when compared to the conventional extraction methods are low energy consumption, reduced treatment time, less solvent usage, increased safety of the operators, and improved extraction yield [18,19]. The increase of extraction efficiency, alongside the reduction of the extraction time, is determined by two mechanisms—ultrasonic cavitation and mechanical mixing effect. Ultrasonic waves generate a mechanical effect in the solvent, resulting in faster movement of molecules and a disruption of the plant cell walls when the cavitation bubbles collapse on the surface of the extraction material, which leads to higher penetration of the solvent into the solid matrix [20,21]. The extraction process is also enhanced by a localized heating (thermal effect) that stimulates the diffusion of the targeted compound into the solvent.

For pectin, which is a soluble fiber, the cavitation and cell disruption caused by ultrasonic waves may improve mass transfer and therefore enhance the extraction process [17]. UAE was applied as a singular extraction technique to extract pectin from grapefruit peel [22], sour orange peel [23], mango peel [6], sisal waste [24], and sunflower heads [25] among other by-products and wastes. Ultrasounds were also applied in sequential extractions, together with techniques such as microwave- [26,27] and enzyme-assisted extraction [8].

*Malus domestica* ‘Fălticeni’ is an apple hybrid developed at the Fruit Research and Production Station Fălticeni (Suceava, Romania) in 1962 through free pollination of the Jonathan variety. This autumn apple hybrid was developed with the intent to be resistant against disease, frost, drought, and other stress factors. The fruits have the best quality from September to December and are red-skinned, of medium size and spherical shape, flattened, with a weight of around 150 g. As a means to increase the economic viability of the apples from this hybrid variety, we previously reported on the first extraction of pectin from *Malus domestica* ‘Fălticeni’ apple pomace [28].

In this study, pectin was extracted from *Malus domestica* ‘Fălticeni’ apple pomace by applying an ultrasonic treatment. This is the first report on the application of a non-conventional extraction technique, namely ultrasound-assisted extraction, to isolate pectin from this plant source. The pomace used in the extraction process was obtained by processing the apples into juice in the geographical region of Fălticeni, Suceava, Romania. The extracted pectin was characterized in terms of yield, galacturonic acid content, and degree of esterification (DE). The purpose of this research was to optimize the extraction and to understand the influence of the working conditions on the yield and physicochemical properties of the extracted pectin and compare them with those of commercial citrus pectin and commercial apple pectin. It should be mentioned here that determination of DE was included to ensure that, under optimal conditions, high-esterified pectin was obtained, which provided the means for an accurate comparison between this sample and the commercial apple and citrus pectin samples, which have high DE.

2. Materials and Methods

2.1. Material and Chemicals

The pomace used for pectin extraction was obtained by extracting the juice from *Malus domestica* ‘Fălticeni’ apples in a small-scale plant, in the Fălticeni area of Suceava, Romania. After juice extraction, the apple pomace was dried at 60 °C in an oven with air circulation until constant weight. The dried
pomace was powdered and then sieved with a Retsch AS 200 shaker (Retsch GmbH, Hann, Germany) to obtain pomace with particlesizes of 125–200 µm.

All chemical reagents, including citric acid, ethyl alcohol, D-galacturonic acid, m-hydroxydiphenyl, and commercial apple (Sigma-Aldrich, Hamburg, Germany, product number 93854) and citrus pectin (Sigma-Aldrich, Hamburg, Germany, product number P9135) were of analytical grade and were purchased from Merck KGaA (Darmstadt, Germany).

2.2. UAE Experimental Set-Up and Extraction Procedure

Pectin extraction from *Malus domestica* 'Fälticeni' apple pomace was carried out using an ultrasonic device (Sonopuls HD 2070, Bandelin, Berlin, Germany) with a flat tip probe (KE 76, Bandelin, Berlin, Germany) operated at 20 kHz and maximum power of 70 W. The ultrasonic device probe was submerged at 15 mm depth into the extraction mixture prepared with apple pomace and distilled water acidified with citric acid to different pH values (1.5, 2, 2.5) in appropriate quantities to obtain solid-to-liquid ratios (SLR) of 1:10, 1:15, and 1:20 (w/v), respectively. Sonication was made at different amplitudes (20%, 60%, and 100%) for 10, 20, and 30 min, respectively.

After the ultrasonic treatment was completed, the mixture was centrifuged (4000 rpm, 40 min) to separate the extract from the solid mass. The extract was then filtered and precipitated in equal parts with cold ethyl alcohol (>96%, v/v) for 12 h at 4–6°C. The precipitated pectin was separated by centrifugation (4000 rpm, 40 min), washed three times by 96% ethyl alcohol, and dried at 50°C to a constant weight in a hot air oven.

The extraction efficiency, expressed as pectin yield, was calculated using the formula in Equation (1).

\[
Pectin \text{ yield} \,(\%) = \frac{m_0}{m} \times 100 \tag{1}
\]

where \(m_0\) is the weight of dried pectin (g) and \(m\) is the weight of dried apple pomace powder (g) [24].

2.3. Physicochemical Properties of Pectin

2.3.1. Determination of Galacturonic Acid Content

The galacturonic acid content of pectin was analyzed according to the method developed by Filisetti-Cozzi and Carpita [29]. Sample preparation for the analysis was made as described previously [30]. In brief, 20 mg of dry pectin were dissolved in 50 mL of warm (40 °C) distilled water, and then the volume was brought to 100 mL with distilled water. From the resulting solution, 400 µL were taken and transferred to glass tubes, then 40 µL of 4 M sulfamic acid solution were added, and the content of the tubes was vigorously mixed, followed by the addition of 2.4 mL of sulfuric acid containing 75 mM of sodium tetraborate, after which the content was mixed again. Hydrolysis of pectin was carried out by placing the tubes in a water bath (100 °C) for 20 min. After that, the samples were cooled down in an ice bath (10 min). To each sample, 80 µL of m-hydroxydiphenyl solution in sodium hydroxide (0.05%, w/v) were added, and the content was mixed. After 10–30 min, the absorbance was read at 525 nm against the reagent control (containing 0.05% sodium hydroxide solution instead of the m-hydroxydiphenyl solution) with a UV-3600 Plus UV-Vis-NIR spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

2.3.2. Determination of Degree of Esterification

The degree of esterification of pectin was determined using the method described previously by Franchi et al. [31]. Briefly, sample preparation was made by dissolving dry pectin (50 mg) in boiled distilled water (10 mL). The solution that was obtained was titrated with 0.1 N solution of NaOH using phenolphthalein as indicator. The volume of sodium hydroxide solution that was used for titration was recorded as \(V_1\). Then, 20 mL of 0.5 M solution of NaOH were added, and the solution was kept for 30 min under continuous stirring (400 rpm) for saponification. After this, 20 mL of 0.5 M solution of
HCl were added for neutralization, and a final titration with NaOH solution was made, recording the used volume as \( V_2 \). The degree of esterification of pectin was calculated using Equation (2).

\[
\text{Degree of esterification (\%)} = \frac{V_2}{V_1 + V_2} \times 100
\] (2)

2.4. FT-IR Analysis of Pectin

The sample extracted by UAE in optimal conditions and the commercial pectin samples were subjected to FT-IR analysis using a Spectrum Two infrared spectrophotometer (PerkinElmer, Waltham, MA, USA). The characteristic spectra were recorded (three scans for each sample) in the frequency range of 4000–400 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\) [22]. Spekwin32–optical spectroscopy software (Version 1.72.2) was used to display the spectra.

2.5. Thermal Analysis

Thermal analysis of the pectin samples (pectin extracted by UAE in optimal conditions, commercial apple pectin, and citrus pectin) was carried out in triplicate with the differential scanning calorimetry technique (DSC). DSC measurements of dried pectin (5 mg), weighted in aluminum pan and placed in the instrument (DSC 8500, PerkinElmer, Waltham, MA, USA) together with an empty pan used as reference, were performed over a temperature range of 0–300 ℃ at a heating rate of 10 ℃/min in dynamic inert nitrogen atmosphere (flow rate of 20 mL/min).

2.6. Pectin Solutions Rheology

2.6.1. Preparation of Pectin Solutions

Samples of pectin extracted by UAE in optimal conditions, commercial apple pectin, and commercial citrus pectin, respectively, at 3% (w/w) were obtained using deionized water adjusted to pH = 4 under continuous stirring for 12 h at 40 ℃. The samples were cooled to room temperature and stored under refrigeration prior to the rheological analysis.

2.6.2. Dynamic Viscosity Measurement

The pectin dynamic viscosity was determined with Mars 40 rheometer (Thermo Haake, Germany) using a cone (Ø 35 mm, 2°)–plate system. Each sample was left to rest for 10 min prior to the analysis to allow the recovering of the structure and achieve the desired temperature. All the rheological measurements were performed at 20 ℃. The shear rate (\( \gamma \), s\(^{-1}\)) was ranged between 0–100 s\(^{-1}\), while the shear stress (\( \tau \), Pa) and dynamic viscosity (\( \eta \), Pa·s) were measured. Each measurement was done in triplicate.

2.6.3. Dynamic State

The dynamic rheological properties of pectin solutions were determined using Mars 40 rheometer (Thermo Haake, Dieselstraße, Germany) coupled with a cone (Ø 35 mm, 2°)–plate system. Each sample was left to rest for 10 min prior to the analysis to allow the recovering of the structure and achieve the desired temperature. The stress sweeps were done at 1 Hz for determining the viscoelastic region. The stress was chosen in the linear viscoelastic region, and the frequency ranged between 0.1 to 10 Hz. The rheological parameters used were: loss modulus \( G'' \) (Pa) and elastic modulus \( G' \) (Pa). The measurements were made at 20 ℃. Each measurement was made in duplicate.

2.6.4. Creep and Recovery Analysis

The creep and recovery analysis was performed at a constant stress of 1Pa. The stress was applied and maintained for 180 s and then released to allow sample recovery for another 180 s. Creep
parameters were determined by computing a constant stress (σ) over time (t) and expressed using the creep compliance (J) function, as shown in Equation (3) in terms of shear deformation (γ).

\[ J(t) = \frac{\gamma(t)}{\sigma} \quad (3) \]

The creep and recovery data were subjected to Burger model, which consists of a Maxwell model and a Kelvin-Voigt model, associated with series. The system deformation per unit stress, namely the compliance (J), is a function of time and calculated using Equation (4) [32].

\[ J(t) = \frac{1}{G_0} + \frac{1}{G_1} \left[ \frac{t}{\eta_1} \right] + \frac{t}{\eta_0} \quad (4) \]

where \( J(t) \) = compliance at any time (1/Pa), \( t \)-time (s), \( G_0 \) = elastic modulus of the Maxwell unit, \( \eta_0 \) = viscosity of the liquid filling the dashpot of the Maxwell element (Pa·s), \( G_1 \) = shear modulus of the Kelvin-Voigt unit, and \( \eta_1 \) = viscosity of the liquid filling the dashpot of the Kelvin-Voigt element (Pa·s).

2.7. Surface Morphology Analysis

With the purpose of studying the structural features of pectin, scanning electron microscopy (SEM; SU-70, Hitachi, Tokyo, Japan) was conducted. Dried pectin powder of the sample extracted by UAE in optimal conditions and the commercial apple and citrus pectin samples were fixed to the sample table with double-sided adhesive carbon tape. The images were taken at an accelerating potential of 5 kV and a magnification of 300x.

2.8. Experimental Design and Statistical Analysis

A four factors, each having three levels, Box-Behnken design was adapted in this study to investigate and optimize the effect of the independent variables—amplitude (X₁), pH (X₂), SLR (X₃), and time (X₄)—on the extraction yield, galacturonic acid content, and degree of esterification of pectin. The coded levels of the variables are shown in Table 1. All calculations and graphics were carried out using the statistical software Design Expert 11 (trial version, Minneapolis, MN, USA). Triplicate experiments were made in order to validate the optimal extraction conditions.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude (%)</td>
<td>20 60  100</td>
</tr>
<tr>
<td>pH</td>
<td>1.5 2 2.5</td>
</tr>
<tr>
<td>Solid-to-liquid ratio (g/mL)</td>
<td>1:10 1:15 1:20</td>
</tr>
<tr>
<td>Time (min)</td>
<td>10 20 30</td>
</tr>
</tbody>
</table>

The Pearson correlation was carried out using Unscrambler X 10.1 (Camo, Norway).

3. Results and Discussion

3.1. Model Fitting and Statistical Analysis

The extraction of pectin from Malus domestica ‘Fälticeni’ apple pomace was designed using the Box-Behnken method with four parameters according to the information presented in Table 2. A total of 29 experiments were carried out with various combination of independent variables—amplitude (20–100%), pH (1.5–2.5), SLR (1:10–1:20 g/mL), and time (10–30 min)—and the experimental and predicted data that was recorded for extraction yield, galacturonic acid content, and degree of esterification of pectin is presented in Table 2.
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The model used to predict the evolution of pectin yield and its galacturonic acid content and degree of esterification was a second-order (quadratic) polynomial response surface model that was applied to fit the experimental data obtained by Box-Behnken design. The quadratic model was selected because of the higher $R^2$, adjusted $R^2$, and predicted $R^2$ than the linear and 2FI models and the low probability value ($p < 0.0001$). The results of the analysis of variance, presented in Table 3, showed that the quadratic model can explain and predict most of the variation in pectin yield ($R^2 = 0.9829$), galacturonic acid content ($R^2 = 0.9472$), and degree of esterification ($R^2 = 0.9606$) of extracted pectin. The square polynomial equations that describe the combined effect of amplitude ($X_1$), pH ($X_2$), SLR ($X_3$), and time ($X_4$) on the extraction yield (Equation (5)), galacturonic acid content (Equation (6)), and degree of esterification (Equation (7)) of pectin are shown below.

\[
Y(\%) = 2.371 + 1.322 \cdot X_1 - 2.402 \cdot X_2 - 0.062 \cdot X_3 + 0.839 \cdot X_4 - 0.966 \cdot X_1 \cdot X_2 + 0.132 \cdot X_1 \cdot X_3 + 0.982 \cdot X_1 \cdot X_4 - 0.074 \cdot X_2 \cdot X_3 - 0.984 \cdot X_2 \cdot X_4 - 0.267 \cdot X_3 \cdot X_4 + 0.676 \cdot X_1^2 + 1.736 \cdot X_2^2 + 0.035 \cdot X_3^2 + 0.589 \cdot X_4^2 \]

(5)

GalA (g/100 g) = 99.085 + 2.014 \cdot X_1 + 3.572 \cdot X_2 - 1.374 \cdot X_3 - 0.577 \cdot X_4 - 12.134 \cdot X_1 \cdot X_2 + 6.011 \cdot X_1 \cdot X_3 + 10.499 \cdot X_1 \cdot X_4 + 0.834 \cdot X_2 \cdot X_3 - 0.725 \cdot X_2 \cdot X_4 - 2.120 \cdot X_3 \cdot X_4 - 8.098 \cdot X_1^2 - 4.495 \cdot X_2^2 + 0.341 \cdot X_3^2 - 6.150 \cdot X_4^2

(6)
Table 3. Analysis of variance (ANOVA) of constructed quadratic model.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degree of Freedom</th>
<th>Mean Square</th>
<th>F-Value</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Pectin Yield %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>132.28</td>
<td>14</td>
<td>9.45</td>
<td>57.40</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude</td>
<td>20.99</td>
<td>1</td>
<td>20.99</td>
<td>127.52</td>
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</tr>
<tr>
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<td>1</td>
<td>69.28</td>
<td>420.90</td>
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<tr>
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<td>0.04</td>
<td>1</td>
<td>0.04</td>
<td>0.28</td>
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<tr>
<td>Time</td>
<td>8.45</td>
<td>1</td>
<td>8.45</td>
<td>51.36</td>
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</tr>
<tr>
<td>Amplitude × pH</td>
<td>3.73</td>
<td>1</td>
<td>3.73</td>
<td>22.68</td>
<td>0.0003</td>
</tr>
<tr>
<td>Amplitude × SLR</td>
<td>0.06</td>
<td>1</td>
<td>0.06</td>
<td>0.42</td>
<td>0.5251</td>
</tr>
<tr>
<td>Amplitude × time</td>
<td>3.86</td>
<td>1</td>
<td>3.86</td>
<td>23.44</td>
<td>0.0003</td>
</tr>
<tr>
<td>pH × SLR</td>
<td>0.02</td>
<td>1</td>
<td>0.02</td>
<td>0.13</td>
<td>0.7204</td>
</tr>
<tr>
<td>pH × time</td>
<td>3.88</td>
<td>1</td>
<td>3.88</td>
<td>23.54</td>
<td>0.0003</td>
</tr>
<tr>
<td>SLR × time</td>
<td>0.28</td>
<td>1</td>
<td>0.28</td>
<td>1.74</td>
<td>0.2081</td>
</tr>
<tr>
<td>R²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9829</td>
</tr>
<tr>
<td>Adjusted R²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9658</td>
</tr>
<tr>
<td>(B) Galacturonic Acid Content g/100 g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>2088.87</td>
<td>14</td>
<td>149.20</td>
<td>17.93</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude</td>
<td>48.68</td>
<td>1</td>
<td>48.68</td>
<td>5.85</td>
<td>0.0298</td>
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<tr>
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<td>153.16</td>
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<td>153.16</td>
<td>18.40</td>
<td>0.0007</td>
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<tr>
<td>SLR</td>
<td>22.68</td>
<td>1</td>
<td>22.68</td>
<td>2.73</td>
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<tr>
<td>Time</td>
<td>4.01</td>
<td>1</td>
<td>4.01</td>
<td>0.48</td>
<td>0.4991</td>
</tr>
<tr>
<td>Amplitude × pH</td>
<td>589.02</td>
<td>1</td>
<td>589.02</td>
<td>70.78</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amplitude × SLR</td>
<td>144.56</td>
<td>1</td>
<td>144.56</td>
<td>17.37</td>
<td>0.0009</td>
</tr>
<tr>
<td>Amplitude × time</td>
<td>440.97</td>
<td>1</td>
<td>440.97</td>
<td>52.99</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pH × SLR</td>
<td>2.78</td>
<td>1</td>
<td>2.78</td>
<td>0.33</td>
<td>0.5723</td>
</tr>
<tr>
<td>pH × time</td>
<td>2.11</td>
<td>1</td>
<td>2.11</td>
<td>0.25</td>
<td>0.6227</td>
</tr>
<tr>
<td>SLR × time</td>
<td>17.99</td>
<td>1</td>
<td>17.99</td>
<td>2.16</td>
<td>0.1636</td>
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<tr>
<td>R²</td>
<td></td>
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<td></td>
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<tr>
<td>Adjusted R²</td>
<td></td>
<td></td>
<td></td>
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<td>0.8943</td>
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<tr>
<td>(C) Degree of Esterification %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
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<td>14</td>
<td>163.12</td>
<td>24.40</td>
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</tr>
<tr>
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<tr>
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<td>1523.70</td>
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<tr>
<td>SLR</td>
<td>12.83</td>
<td>1</td>
<td>12.83</td>
<td>1.92</td>
<td>0.1876</td>
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<tr>
<td>Time</td>
<td>17.45</td>
<td>1</td>
<td>17.45</td>
<td>2.61</td>
<td>0.1285</td>
</tr>
<tr>
<td>Amplitude × pH</td>
<td>84.09</td>
<td>1</td>
<td>84.09</td>
<td>12.58</td>
<td>0.0032</td>
</tr>
<tr>
<td>Amplitude × SLR</td>
<td>0.76</td>
<td>1</td>
<td>0.76</td>
<td>0.11</td>
<td>0.7401</td>
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<tr>
<td>Amplitude × time</td>
<td>119.36</td>
<td>1</td>
<td>119.36</td>
<td>17.85</td>
<td>0.0008</td>
</tr>
<tr>
<td>pH × SLR</td>
<td>30.09</td>
<td>1</td>
<td>30.09</td>
<td>4.50</td>
<td>0.0522</td>
</tr>
<tr>
<td>pH × time</td>
<td>0.26</td>
<td>1</td>
<td>0.26</td>
<td>0.03</td>
<td>0.8450</td>
</tr>
<tr>
<td>SLR × time</td>
<td>30.31</td>
<td>1</td>
<td>30.31</td>
<td>4.53</td>
<td>0.0515</td>
</tr>
<tr>
<td>R²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9606</td>
</tr>
<tr>
<td>Adjusted R²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9213</td>
</tr>
</tbody>
</table>

3.2. Effect of Process Variables

3.2.1. Effect on Pectin Yield

The RSM plots (Figure 1) were used for the study of the influence of the parameters on the response variables (yield, GalA, DE). For pectin yield, the response surface plots are presented in Figure 1A–F. As these three-dimensional graphics and the results of the analysis of variance in Table 3 show, with the
exception of SLR, all the applied factors highly influenced the extraction yield of pectin. Pectin yield varied between a minimum of 1.68% (60% amplitude, pH of 2.5, 1:20 g/mL SLR, and 20 min) and a maximum of 9.18%, resulted from an extraction at 60% amplitude, pH of 1.5, 1:15 g/mL SLR, and 30 min extraction time. Pectin yield might be attributed in this case to the following two factors: (a) the lower maximum working power (70 W) of the ultrasonic device, considering that other researchers stated the use of ultrasound generating probes operated at higher output power, e.g., 450 W [8], 800 W [33,34], and (b) the absence of heating in the UAE process, which allowed for an accurate comparison between the non-conventional extraction technique and citric acid extraction of pectin [7].

The effects of sonication amplitude on pectin yield are shown in Figure 1A–C. As can be observed, the increase in amplitude had a positive influence on the recovery of pectin from apple pomace. Overall, the best values for extraction yield were obtained at the maximum amplitude (100%). However, a combination between an amplitude of 60% and a prolonged extraction time (30 min), in the same conditions of SLR and pH (1.5 and 1:15 g/mL), resulted in a maximum pectin yield of 9.18% that was slightly higher than the 9.14% yield obtained when the extraction was carried out at a 100% amplitude for 20 min. This decrease in extraction yield may be attributed to a reduction in the efficiency of ultrasonic energy transmitted into the extraction mixture, as also observed by other authors [9,35]. Beside the combined effect of amplitude and time, the interaction of amplitude and pH was also significant for the extraction efficiency.

To evaluate the influence of the pH value of the water-citric acid mixture on pectin yield, the experiments were carried out in a pH range of 1.5–2.5 (Figure 1A,D,E). As expected, pectin yield was significantly higher at a pH of 1.5, corroborating the conclusion that in high acidic pH conditions the insoluble pectin constituents are hydrolyzed into soluble pectin, thus increasing the transfer of the polysaccharide from the plant material into the extraction solvent [36,37]. Apart from the amplitude-pH interaction mentioned before, the interaction between pH and extraction time had significantly influenced the pectin yield.

The effect of SLR on pectin yield was studied between 1:10 g/mL and 1:20 g/mL. As the three dimensional graphics in Figure 1B,D,F show, the extraction yield did not change significantly with the variation of this process parameter, an observation that was confirmed by the results of the analysis of variance ($p > 0.05$). Regarding the influence of this parameter on pectin yield reported in other studies on the UAE of this polysaccharide from various sources, it was found that high solid-to-liquid ratios enhance the surface area between plant material and extraction solvent, therefore leading to increased pectin yields [24,34]. It can be assumed that varying the range of study above a SLR of 1:20 g/mL might lead to a different conclusion regarding the involvement of this working parameter in the cavitation effect occurring in an extraction assisted by ultrasound treatment and consequently its impact on pectin yield.

The influence of sonication time on pectin yield was also studied and the results are presented in Figure 1C,E,F in the form of combined effect with amplitude, pH, and SLR, respectively. Pectin recovery through a UAE is based on the cavitation effect of ultrasound waves that induces enlargement of the pores within the plant cell walls (swelling), accelerating the penetration of solvent into the plant material by diffusion, and finally resulting in a disruption of the solid matrix and liberation of the polysaccharide to the outer medium [16,20,33,38]. In the case of this study, pectin yield increased with the duration of the ultrasound treatment and the maximum yield was obtained after 20 min (60% amplitude, pH of 2.5, 1:20 g/mL SLR) of exposure of apple pomace to ultrasonic waves. The slight decrease of yield observed at the maximum sonication amplitude and extraction time can be attributed to a decomposition of pectin by excessive localized heating and overexposure to ultrasound treatment.
Figure 1. Response surface plots showing the effect of extraction parameters on pectin yield (A–F), the galacturonic acid content (G–L), and degree of esterification (M–R).

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recovery through a UAE is based on the cavitation effect of ultrasound waves that induces enlargement of the pores within the plant cell walls (swelling), accelerating the penetration of solvent into the plant material by diffusion, and finally resulting in a disruption of the solid matrix and liberation of the polysaccharide to the outer medium [16,20,33,38]. In the case of this study, pectin yield increased with the duration of the ultrasound treatment and the maximum yield was obtained after 20 min (60% amplitude, pH of 2.5, 1:20 g/mL SLR) of exposure of apple pomace to ultrasonic waves. The slight decrease of yield observed at the maximum sonication amplitude and extraction time can be attributed to a decomposition of pectin by excessive localized heating and overexposure to ultrasound treatment.

3.2.2. Effect of Extraction Parameters on GalA Content

The evolution of pectin content in galacturonic acid with the conditions of UAE are presented in Table 2 and Figure 1G–L, while the results of the ANOVA regarding the influence of sonication, pH, SLR, and time on this chemical parameter are given in Table 3. According to the values in Table 2, all pectin samples had higher content of GalA than that reported for apple pectin by other authors [39–41]. Considering the GalA content obtained with citric acid for a conventional heating extraction method of pectin from the same plant source, that ranged from 84.25 to 93.90 g/100 g [28], it can be stated that the high GalA content was strongly determined in this case by the structural composition of pectin in *Malus domestica* ‘Fălticeni’ apple pomace and was to a lesser extent dependent on the application of an ultrasound treatment in the extraction process. Sonication amplitude has shown a statistically significant influence on the GalA content of the extracted pectin. The highest GalA content of 99.86 g/100 g was obtained for an extraction at 100% amplitude, pH of 1.5, SLR of 1:15, and 20 min extraction time. Figure 1G,H illustrate that GalA content increased with the sonication amplitude when the pH of the extraction solution decreased and the SLR was above 1:15 g/mL. A major impact on pectin’s GalA content was also displayed by the combined increase in amplitude of the UAE and extraction time (Figure 1I).

With respect to the pH of the citric acid solution used for pectin extraction, the previous indication that low pH increases the GalA content of the extracted pectin [42] was not applicable in the case of this study (Table 3). In the studied pH range of 1.5–2.5, higher GalA contents were determined when the pH was above 1.5, as can be deduced from Figure 1G (amplitude-pH interaction) and 1K (time-pH interaction). These two interactions manifested a statistically significant influence on the GalA content of pectin.

The proportion of solid material to citric acid solution had no effect on the GalA content of the extracted pectin, as seen in Table 3 and Figure 1H,J,L. In a similar way, the duration of the exposure to ultrasound did not significantly influence the content of pectin in GalA (Table 3, Figure 1L,K,L). The only combined effect that was statistically significant for the changes in GalA content was that of pH and time; as observed for run no. 5 and 6, as well as 14 and 27, at a 60% sonication amplitude and the lowest (1.5) and highest (2.5) pH values, the GalA content of pectin slightly decreased with the extraction time. At high pH values, this decrease in the GalA content occurs simultaneously with the reduction of pectin yield, highlighting a degradation of apple pectin molecule when the ultrasound treatment was applied for a longer time.

3.2.3. Effect of Process Variables on DE

The degree of esterification of pectin, which is an important parameter for its applications in the industry, is related to the technological characteristics of the solubility of pectin, the emulsification capabilities, and the ability of the polysaccharide to form gels [43,44]. Table 2 shows the experimental DE, alongside the predicted values, and Table 3 the ANOVA results for this parameter. The interactions between sonication amplitude, pH, SLR and time that determine the evolution of the DE of pectin are shown in Table 3 and Figure 1M–R. According to the data presented in Table 2, all samples had a DE above 50%, ranging from 60.34% (run no. 10, 20% amplitude, pH of 1.5, 1:15 g/mL SLR and 20 min
extraction time) to 94.75% (run no. 26, 60% amplitude, pH of 2.5, 1:10 SLR g/mL and 20 min) and were classified as pectin with high esterification degree.

Similar to pectin yield, the sonication amplitude of 60% resulted in the extraction of a pectin with the highest DE (94.75%), however, the extraction pH was at the highest level (2.5) and the SLR and extraction time were at lower levels. In the same conditions that gave a pectin with the highest DE, pectin yield was very low (2.31%), indicating that there is no similar tendency in the evolution of the two parameters. Figure 1M–O show that DE reduced as the sonication amplitude increased from 60% and 100%. While no simultaneous major modification of GalA content occurred, these results show that the de-esterification of pectin chains taking place in some conditions of the ultrasound treatment was not accompanied by changes in the main structural polygalacturonic chain of pectin, corroborating the conclusions regarding the effects of ultrasounds on the structure of apple pectin reported previously [45]. Determinant factors for the changes of the DE of pectin were in this study the interactions between acoustic amplitude and pH and time, respectively (Table 3).

The pH was an extraction parameter with a major influence of the evolution of DE, as seen in Table 3 and Figure 1M,P,Q. The influence of pH on DE was more pronounced when the pH of the citric acid solution was between 1.5 and 2, suggesting that harsh extraction conditions may lead to a de-esterification of pectin [23,46,47]. The combined effect of pH and sonication amplitude had a significant statistical influence of the evolution of DE, while the interactions between pH and SLR and pH and time manifested no influence on this parameter.

Figure 1N,P,R and Table 3 show no major effect of SLR on the DE of pectin. This is also applicable to explain the influence of extraction time (Figure 1O,Q,R) on DE. A similar conclusion was made in the case of these two process parameters for GalA content, which leads to the conclusion that the variation of SLR and time does not determine significant modifications in the chain structure and the esterification of the GalA residues in the extracted pectin. To conclude, the only two extraction parameters that determine important variations in the physicochemical properties of the pectin recovered through a UAE were the sonication amplitude and the pH of the citric acid-water mixture used for extraction. It is to be expected that, when operating over the optimum sonication amplitude and at very low pH, breakage in the structure of pectin will occur.

3.3. Optimization and Validation of Extraction Conditions

The desirability function approach was used to optimize the multiple characteristics (pectin yield, GalA content, and DE) concurrently [48]. In the desirability function approach, first each characteristic, $y_i$, was converted into an individual desirability function, $d_i$, which varied over the range shown in Equation (7) [21].

$$0 \leq d_i \leq 1$$

For the purpose of achieving the maximum extraction yield of pectin from Malus domestica ‘Fälticeni’ apple pomace and a maximum GalA content and DE of the extracted pectin, sonication amplitude, pH, SLR, and extraction time were optimized. Using the response surface methodology for optimization, the optimal conditions to obtain a 9.183% yield, 98.127 g/100 g GalA content, and 83.202% DE were the following: 100% amplitude, pH of 1.8, SLR of 1:10 g/mL, and 30 min extraction time ($d = 0.857$).

Considering that the average values of triplicate experiments performed in optimal conditions (pectin yield of 9.074%, GalA content of 94.491 g/100 g, and DE of 76.715%) were well correlated with the predicted values, it was concluded that the optimal conditions for UAE of pectin were valid.
3.4. FT-IR Analysis of Apple Pectin

To study the different structural particularities of pectin extracted from *Malus domestica* ‘Fălticeni’ apple pomace and compare them to two commercial pectins (apple and citrus), FT-IR analysis was used. The FT-IR spectra of pectin obtained by UAE in the optimal conditions and commercial pectin samples are shown in Figure 2. Through a general comparison between the spectra, in the region 4000–2400 cm\(^{-1}\) the extracted pectin sample was similar to the commercial citrus pectin, while from 2400 to 400 cm\(^{-1}\) the spectrum resembled that of the commercial apple pectin. The major absorption peaks reported around 3434–3360 cm\(^{-1}\) [23,49] were absent from the three spectra. The absorption peak characteristic for polysaccharides that was found at 2919 cm\(^{-1}\) in both the commercial citrus pectin and UAE pectin spectra was due to C–H stretching of CH\(_2\) groups [22]. All samples had an absorption peak at 1743–1732 cm\(^{-1}\), which was attributed to ester carbonyl (C=O) stretching, and a peak at 1636–1606 cm\(^{-1}\) that indicated (C=O) stretching vibration of carboxylate ion. The ratio of the peak area at 1743 cm\(^{-1}\)(COO–R) over the sum of the peak areas of 1743 cm\(^{-1}\) and 1636 cm\(^{-1}\) (COO\(^-\)) was quantified as DE [50]. In accordance to the analysis of DE, pectin obtained by UAE was high-esterified (DE of 76.715%, Table 4) and seemed more similar to the commercial apple pectin sample than to the commercial citrus pectin sample in terms of DE. The weak band at 1440 cm\(^{-1}\) was also attributed to stretching of carbohydrate groups. Very important for the structure analysis of pectin by FT-IR were the intense absorption peaks between 1300 and 800 cm\(^{-1}\), that were collectively referred to as the ‘fingerprint’ region of carbohydrates because these are unique to a compound and allow the identification of the major chemical groups [51]. The peak at 1225 cm\(^{-1}\) corresponded to C–C bond in the ring structure of pectin, while the absorption peaks between 1120–990 cm\(^{-1}\) were considered the range for the spectral identification of GalA in pectin molecules [52,53]. The major peak at 1015 cm\(^{-1}\) was attributed to the presence of pyranose and 830 cm\(^{-1}\) was referred to the absorption of α-D-mannopyranose, similar to previous studies [22,49].

![Figure 2. FT-IR spectra of pectin extracted from apple (*Malus domestica* 'Fălticeni') pomace by UAE under optimal conditions and commercial apple and citrus pectin.](image)

**Table 4.** Galacturonic acid content and degree of esterification (DE) of ultrasound-assisted extraction (UAE) pectin and commercial citrus and apple pectin samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Galacturonic Acid Content g/100 g (^a)</th>
<th>DE % (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAE pectin</td>
<td>94.491</td>
<td>76.715</td>
</tr>
<tr>
<td>Commercial citrus pectin</td>
<td>87.601</td>
<td>50.584</td>
</tr>
<tr>
<td>Commercial apple pectin</td>
<td>88.835</td>
<td>88.526</td>
</tr>
</tbody>
</table>

\(^a\) determined by the method described in Section 2.3.1; \(^b\) determined by FT-IR.
3.5. Thermal Analysis

Thermal analysis of the pectin sample extracted by UAE under optimal conditions, alongside the analysis of the commercial apple and citrus pectin samples for comparison, provided useful data regarding the thermal behaviour of pectin and thus information about its properties and possible target applications. The thermograms obtained by DSC analysis are presented in Figure 3. As shown in the thermograms, for all samples, no endothermic peaks were recorded. On the other side, exothermic transition was observed at 243 °C for commercial citrus pectin, 246 °C for pectin extracted from Malus domestica ‘Fălticeni’ apple pomace by UAE under optimal conditions, and 255 °C for commercial apple pectin. An interesting observation was that the commercial sample of pectin from apple presented some small exothermic peaks around 180 °C and 235 °C. The temperature of degradation of apple pectin extracted by UAE was close to the value of 243.86 °C reported for apple pectin extracted at 170 °C by subcritical water [39]. The exothermic peaks correspond to the degradation of pectin in the heat processing, which is generally dependent on its chemical constituents. Sharper exothermic peaks were correlated with shorter degradation range, a concentrated distribution of molecular weight, and more ordered molecular arrangement [54]. From this point of view, pectin extracted by UAE had a slightly sharper exothermic peak, which suggested a narrower distribution range of molecular weight and an orderly molecular arrangement. The lower degradation temperature of the pectin sample extracted in this study by comparison to the commercial apple pectin was explained by the differences in the chemical structure of the two samples that were confirmed by FT-IR analysis.

![Figure 3](image_url)  
**Figure 3.** Differential scanning calorimetry (DSC) thermograms of pectin extracted by UAE under optimal conditions and commercial apple and citrus pectin.

3.6. Rheological Properties

3.6.1. Flow Behaviour of Pectin Solutions

Figure 4 presents the flow curves of the three pectin samples; all curves present a shear thinning behaviour of a non-Newtonian fluid due to the weakness of the pectin intermolecular forces with increasing shear rate [55,56]. It can be observed that the origin of pectin had no influence on the thinning behaviour of the solutions, as all of them had the same behaviour, showing viscosity decrease with the increasing of shear rate. Regarding the dynamic viscosity, it was observed that the UAE sample had a higher viscosity than the commercial pectin solutions, which implies that different extraction conditions and raw materials influence the flow behaviour of pectin solutions [56]. The viscosity of UAE pectin at a shear rate of 1 s⁻¹ was 21.07 Pa·s and was higher than in the case of commercial citrus
pectin (1.35 Pa-s) and commercial apple pectin (0.39 Pa-s); this value was also higher than the viscosity measured in the case of gabiroba pectin in the same conditions (approx. 0.2 Pa-s) [57] and viscosity obtained for 5% pectin solutions of cacao pod husks [58] and apple pomace [59], which was lower than 0.2 Pa-s.

![Figure 4. Flowcurves of pectin solutions: commercial citrus pectin (△), commercial apple pectin (□), and ultrasound extracted apple pectin (△); η—dynamic viscosity, γ—shear rate.](image)

Hua et al. [60] observed that the source of pectin and extraction procedure influences the viscosity of the solutions obtained because high methoxyl content means a small number of molecules and a greater distance between molecules, resulting in low viscosity of pectin extracted from the conventional heating. According to the Pearson correlation, a negative correlation was observed between the viscosity and 1634 cm\(^{-1}\) wavenumber transmittance values \((r = -0.9991^{**})\), which was responsible for (C=O) stretching vibration of the carboxylate ion and a highly positive correlation between viscosity and galacturonic acid content \((r = 0.992^{**})\).

### 3.6.2. Viscoelastic Properties of Pectin Solutions

The viscoelastic properties of pectin solutions are presented in Figure 5. For this analysis it was determined the elastic modulus \((G')\) and loss modulus \((G'\prime\prime)\) in the linear viscoelastic region. The elastic modulus \((G')\) represents the elastic component of the stress, while the loss modulus \((G'\prime\prime)\) measures the energy lost through viscous flow [61]. From Figure 5, it can be observed that all the samples had a higher \(G'\prime\prime\) than \(G'\) in the 0.1–10 frequency region. However, in the case of pectin extracted from apple pomace by UAE, at higher frequency, the \(G'\) had similar values to \(G'\prime\prime\). The behaviour change of the viscoelastic properties can be due to the formation of a three-dimensional network structure because of the high frequencies applied to the pectin solution. Rodsamran & Sothornvi [56] observed that the pectin solutions from lime peel at 1% and 2% had \(G'\prime\prime > G'\), while at concentrations higher than 3%, the \(G'\) was higher than \(G'\prime\prime\). The viscoelastic parameters for commercial apple and citrus pectin were in agreement with those reported by Barbieri et al. [57] in the case of pectin from gabiroba.
3.6.3. Creep and Recovery

Creep

The creep test (Figure 6) represents a graphical representation of the compliance in function of time. The creep analysis is from 0 to 180 s, while the recovery analysis is from 180 s to 360 s.

![Figure 6. Creep and recovery test of pectin solutions: commercial citrus pectin (CP, ♦), commercial apple pectin (AP, □), and ultrasound extracted apple pectin (UAE, △), f—frequency.](image)

The coefficients of the Burger model are presented in Table 5. For all the samples analysed (commercial pectins and UAE pectin) the regression coefficients were higher than 0.956. The Burger model coefficients reflect the structure of a system as: $G_0$—indicates the rigidity of the structure while the $G_1$—reflects the cohesive force and the resistance to deformation of the system [62,63]. The values of $G_0$ and $G_1$ were similar for commercial apple pectin and commercial citrus pectin, while the UAE pectin had values much higher, indicating that the structure of the pectin solution was influenced by the source and the extraction technique applied. The $f(t)$ values were higher for commercial citrus and apple pectin than UAE pectin solution.
Table 5. Burger model parameters for UAE apple pectin, commercial citrus pectin, and commercial apple pectin.

<table>
<thead>
<tr>
<th>Sample</th>
<th>G₀</th>
<th>G₁</th>
<th>η₁</th>
<th>η₀</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAE pectin</td>
<td>10.200</td>
<td>0.001</td>
<td>1.328</td>
<td>1.294</td>
<td>0.956</td>
</tr>
<tr>
<td>Commercial citrus pectin</td>
<td>0.197</td>
<td>4.700·10⁻⁶</td>
<td>0.040</td>
<td>0.039</td>
<td>0.979</td>
</tr>
<tr>
<td>Commercial apple pectin</td>
<td>0.043</td>
<td>9.600·10⁻⁷</td>
<td>0.090</td>
<td>0.090</td>
<td>0.980</td>
</tr>
</tbody>
</table>

Recovery

According to the recovery phase, all the samples reached at 180 s the maximum deformation (Figure 6). All pectin samples exhibited a non-Newtonian behaviour, with a decreasing of strain response during the stress application, and the recovery was much better in the case of UAE pectin solution (Figure 6).

3.7. Morphological Characteristics of Pectin Samples

SEM analysis was applied to observe the morphological features of the pectin obtained by UAE from Malus domestica 'Fălticeni' apple pomace and compare them to those of commercial citrus and apple pectin. As Figure 7 shows, the CP sample appears to be very different from the other two samples, having a more compact and wrinkled surface that seems to be very hard; this sample is very similar in terms of morphology with the native high-esterified commercial citrus pectin sample analyzed previously by Fracasso et al. [64], who stated that this type of material triggers the formation of groats that are difficult to solubilize. On the other side, the AP sample seemed to be softer, more fragmented and suggested a tendency to curl easily. The pectin sample that was obtained by UAE was similar to AP in terms of fragmentation and had a slight tendency to curl; however, it was closely packed and less smooth. It was also noted a more homogenous distribution of the fragments sizes and an increased number and size of inter-particle voids in the structure of the UAE sample, which were correlated with an enhanced water uptake by immobilization and inclusion [65]. This, together with the high GalA content (Table 4) that directly influences the formation of the pectin network [64], might explain why the solutions obtained with this pectin sample were stronger.
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**Figure 7.** Scanning electron microscopy (SEM) images of commercial citrus pectin (CP), commercial apple pectin (AP), and pectin from *Malus domestica* ‘Fălticeni’ pomace extracted by UAE (UAE); 5 kV, 300× magnification.

### 4. Conclusions

Pectin was extracted from *Malus domestica* ‘Fălticeni’ apple pomace by UAE using four factors, each at three levels—sonication amplitude (20%, 60%, and 100%), pH (1.5, 2, and 2.5), SLR (1:10, 1:15, and 1:20), and time (10, 20, 30 min) Box-Behnken response surface design. The amplitude of the ultrasound treatment applied for the extraction of pectin was found to strongly influence the yield and the DE of the extracted pectin, while the extraction pH had a great impact on all three responses (yield, GalA content, and DE). The optimal conditions for extraction were 100% amplitude, pH of 1.8, SLR of 1:10 g/mL, and 30 min. Under these conditions, pectin yield was 9.183% and had a 98.127 g/100 g GalA content and 83.202% DE.

The pectin sample obtained by UAE under optimal conditions were compared to commercial citrus and apple pectin samples by FT-IR, DSC, rheological analysis, and SEM. The first two techniques highlighted some particularities of the pectin sample extracted by UAE such as the narrower distribution range of molecular weight, the orderly molecular arrangement, and the high DE that was similar
to that of commercial apple pectin. The analysis of the morphological characteristics of this sample indicated a determination pattern between the distribution of the fragment sizes of this sample and its GalA content on one side, and the water uptake capacity on the other side. The viscosity of UAE pectin solution was much higher than that of the solutions made using commercial pectin, which maybe because of the high concentration of galacturonic acid. When also considering the high DE, this might explain why the viscosity, \( G_0 \) and \( G_1 \), were higher for the UAE pectin sample. The purity, structure and rheological behaviour of pectin extracted by UAE from *Malus domestica* ‘Fălticeni’ apple pomace indicated promising applications of this soluble fiber.

Considering the results of this study, future research will be focused on the application of other non-conventional extraction techniques (such as microwave-assisted extraction) to extract pectin from this plant source and also on a comparison between the yield, composition, and properties of pectin obtained by different extraction methods.

**Author Contributions:** Conceptualization, F.D. and M.O.; Methodology, F.D. and M.O.; Investigation, F.D.; Resources, M.O.; Writing—Original Draft Preparation, F.D.; Writing—Review and Editing, F.D. and M.O.; Project Administration, M.O.; Funding Acquisition, M.O.

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

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