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COMPARATIVE EVALUATION OF DIFFERENT EXTRACTION METHODS FOR ANTHOCYANIN RECOVERY FROM RED ONION PEEL: ENZYME-ASSISTED, ULTRASOUND-ASSISTED, AND ULTRASOUND-ASSISTED ENZYMATIC METHODS

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Damar, I. *(2025).* Kırmızı soğan kabuğundan antosiyanin geri kazanımı için farklı ekstraksiyon yöntemlerinin karşılaştırmalı değerlendirmesi: Enzim destekli, ultrason destekli ve ultrason destekli enzimatik yöntemler. GIDA (2025) 50 (3) 389-405 doi: 10.15237/ gida.GD25040

ABSTRACT
In this study, enzyme-assisted extraction (EAE), bath and probe type ultrasound-assisted extraction (UAE-B, UAE-P), and ultrasound-assisted enzymatic extraction using bath and probe-type ultrasound (UAEE-B, UAEE-P) were compared to conventional extraction (CE) for recovering anthocyanins from red onion peel. Extracts were analyzed for total phenolic (TPC), total flavonoid (TFC), total monomeric anthocyanin (TMA), antioxidant activities (AA-ABTS, AA-DPPH), individual anthocyanins, and colour parameters. UAEE-B achieved 33.12% higher extraction yield than CE. UAEE-B showed more TPC compared to other methods, while UAEE-B and UAEE-P exhibited statistically significant TFC and TMA content. The extraction methods influenced the concentration of individual anthocyanins in distinct ways. EAE resulted in the highest AA-ABTS, while combined ultrasound- and enzyme-assisted methods showed the greatest efficacy in the AA-DPPH. The colour variation observed was 2.17±0.91 for UAEE-B and 3.48±0.24 for UAEE-P. In conclusion, combining ultrasound- and enzyme-assisted extraction techniques detected to be beneficial for recovering anthocyanins from red onion peel.
Keywords: Red onion peel, anthocyanin, ultrasound-assisted extraction, enzyme-assisted extraction, ultrasound-assisted enzymatic extraction

KIRMIZI SOĞAN KABUĞUNDAN ANTOSİYANİN GERİ KAZANIMI İÇİN FARKLI EKSTRAKSİYON YÖNTEMLERİNİN KARŞILAŞTIRMALI DEĞERLENDİRMESİ: ENZİM DESTEKLİ, ULTRASON DESTEKLİ VE ULTRASON DESTEKLİ ENZİMATİK YÖNTEMLER

ÖZ
Bu çalışmada, kırmızı soğan kabuklarından antosiyaninleri geri kazanmak için enzim destekli ekstraksiyon (EAE), banyo ve prop tipi ultrason destekli ekstraksiyon (UAE-B, UAE-P) ve banyo ve prob tipi ultrason destekli enzimatik ekstraksiyon (UAEE-B, UAEE-P) geleneksel ekstraksiyona (CE) ile karşılaştırılmıştır. Ekstraktlar toplam fenolik (TPC), toplam flavonoid (TFC), toplam monomerik

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antosiyanin (TMA), antioksidan aktivite (AA-ABTS, AA-DPPH), bireysel antosiyaninler ve renk parametreleri açısından analiz edilmiştir. UAEE-B ile CE'den %33.12 daha yüksek ekstraksiyon verimi elde edilmiştir. UAEE-B diğer yöntemlerle karşılaştırıldığında daha fazla TPC gösterirken, UAEE-B ve UAEE-P istatistiksel olarak daha yüksek TFC ve TMA içeriği sergilemiştir. Ekstraksiyon yöntemleri, bireysel antosiyaninlerin konsantrasyonunu farklı şekillerde etkilemiştir. EAE en yüksek AA-ABTS ile sonuçlanırken, kombine ultrason ve enzim destekli yöntemler AA-DPPH'de en büyük etkinliği göstermiştir. Gözlemlenen renk değişimi UAEE-B için 2.17±0.91 ve UAEE-P için 3.48±0.24'dir. Sonuç olarak, ultrason ve enzim destekli ekstraksiyon tekniklerinin birlikte kullanılmasının kırmızı soğan kabuğundan antosiyaninleri geri kazanmak için faydalı olduğu tespit edildi.
Anahtar kelimeler: Kırmızı soğan kabuğu, antosiyanin, ultrason destekli ekstraksiyon, enzim destekli ekstraksiyon, ultrason destekli enzimatik ekstraksiyon

INTRODUCTION

Onion (*Allium cepa* L.) is one of the most critical vegetables cultivated throughout all over the world since ancient times. The production of onion has increased by 25% on a global scale in the last decade and its production has reached nearly 98 million tons (Bains et al., 2023; Lipsa et al., 2024). Onion wastes contain onion peels, roots, the outer two-layered fleshy part, upper and lower parts of onions, and damaged onions (Ersoy et al., 2020). It has been reported that approximately 0.6 million tons of onion waste is produced annually in the European Union as a result of domestic and industrial processing (Kumar et al., 2022). The characteristic composition of onions (caused by sulfur-containing components) limits their use as fertilizer and causes environmental problems. Additionally, the rapid proliferation of phytopathogenic agents in environments where these wastes are present makes onion waste unsuitable for use as animal feed or for landfill management (Chadorshabi et al., 2022).

The chemical composition of various onions (white, yellow and red) contains high amounts of bioactive compounds such as phenolic, flavonoids, anthocyanins, triterpenoids and organosulfurs (Samota et al., 2022). Studies have proven that flavonoid synthesis increases in the peel to protect onions from soil microorganisms and UV light. Therefore, flavonoids are found 20 times more in the peel than in the edible part (Chadorshabi et al., 2022, Bains et al., 2023). Additionally, the concentration of bioactive compounds in red onions has been found to be significantly higher than in other types of onions (Gorrepati et al., 2024). The characteristic peel colour, ranging from yellow to red/purple, are related to the type of flavonoid compounds. The yellow colour of the peel is due to quercetin, and the red and purple colour is due to the presence of anthocyanins (Celano et al., 2021; Gorrepati et al., 2024). Chadorshabi et al. (2022) reported that the main anthocyanin of red onions was cyanidin 3-glucoside (10.04-233 mg/100 g) and various anthocyanins were determined in some red onion varieties, namely, cyanidin 3-laminaribioside and less amounts of cyanidin, peonidin, and pelargonidin glucosides. Anthocyanins act as a functional agent for health. Therefore, onion peel extracts have been stated to have anticancer, antiobesity, antibacterial, neuroprotective, cardioprotective, and antidiabetic activities (Lipsa et al., 2024). However, the stability of anthocyanins is easily impressed by different factors such as pH, temperature, light, and sugars (acylated and unacylated) (Mirzazadeh et al., 2024).

Therefore, the extraction of anthocyanins from red onion peel without degradation is of critical importance for producing high-value bioactive preparations that could serve as natural alternatives to synthetic colorants and antioxidants in the food industry (Santos et al., 2022). Conventional extraction methods (CE) have some disadvantages such as high solvent usage, long duration, degradation of target components and low yield. Therefore, nowadays, new and green techniques such as ultrasound, microwave, supercritical fluid and enzyme-assisted extraction, which include using non-toxic alternative solvents, safe and sustainable natural

resources, are increasingly preferred in order to enhance the extraction efficiency and process efficiency of anthocyanins (Mirzazadeh et al., 2024). Ultrasound-assisted extraction (UAE) among these methods is based on the effect of mechanical sound waves at frequencies above the human ear's audibility (>20 kHz) on cell walls via cavitation. Ultrasound waves create compression and relaxation cycles in liquid media, creating "cavitation bubbles". These bubbles grow over several cycles, reach a critical size and eventually burst violently, producing high temperature (~5000 K) and pressure (~2000 atm). This process increases the permeability of cell walls, accelerates mass transfer and provides improved penetration (Chemat and Khan 2011; Vinatoru et al., 2017). In enzyme-assisted extraction (EAE), various enzymes such as cellulases, pectinases and hemicellulases are used to break down cell walls of natural matrix. These enzymes cause disruption of the cell wall structure and releases components sugars, proteins, essential oils, and phenolic compounds (Kitryte et al., 2017). The use of enzymes offers advantages such as shorter processing temperatures and times. However, enzyme use has serious limitations such as loss of enzyme activity over time and high cost. Recent studies have reported that ultrasound-assisted enzymatic extraction (UAEE) (ultrasound and enzyme-combined extraction) enhances extraction yield of phenolic compounds, anthocyanins, and carotenoids from fruit and vegetable waste (Tan et al., 2020; Ma et al., 2024; Meral and Demirdoven, 2024; Patil et al., 2024).

In the literature, different extraction methods have been used to recovery anthocyanins and phenolic compounds from dry onion peel. Benito-Román et al. (2021) reported that UAE increased the productivity seven times higher than CE. Jin et al. (2011) emphasized that MAE is more advantageous than CE and UAE for obtaining quercetin from onion peel. Hammad et al. (2024) optimized UAE and EAE for the extraction of phenolic compounds from dried red onion peel. Antioxidant activity results revealed that EAE was more effective than UAE under optimum conditions. Mirzazadeh et al. (2024) examined various modern techniques for anthocyanin extraction from red onion peel, including solvent extraction, UAE, subcritical water extraction, MAE, pulsed electric field extraction, supercritical fluid extraction, and high hydrostatic pressure-assisted extraction. Their study reported that high hydrostatic pressure-assisted extraction was the most effective method in terms of extraction efficiency and total anthocyanin content.

The aim of this study was to evaluate the influence of the different techniques on the extraction of anthocyanins from red onion peel. Therefore, innovative green extraction methods such as enzyme-assisted extraction (EAE), bath and probe type ultrasound-assisted extraction (UAE), and both bath and probe-type ultrasound-assisted enzymatic extraction (UAEE-B, UAEE-P) were compared with conventional extraction (CE). The extraction efficiency, total phenolic content, flavonoid content, monomeric anthocyanin content, antioxidant activities, concentration of individual anthocyanins and colour parameters of the extracts obtained by these methods were determined. These results will provide the way for the sustainable transformation of these extracts into high-value- added products for food, cosmetics, and pharmaceutical industries.

MATERIAL AND METHODS

Materials

Chemicals and reagents

Folin-Ciocalteu reagent, potassium chloride, gallic acid, sodium acetate, sodium nitrite, sodium carbonate, aluminum chloride hexahydrate, sodium hydroxide, (+)-catechin, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), methanol, citric acid, and the cellulolytic enzyme mixture Viscozyme® L (*Aspergillus aculeatus*, V2010, fungal beta-glucanase units (FBU)/g ≥100), cyaniding 3-glucoside (C3G), cyaniding 3-rutinoside (C3R), and peonidin 3-glucoside (P3G) were purchased from Sigma-Aldrich (Darmstadt, Germany). Delphinidin 3-rutinoside (D3R) was purchased from Extrasynthese (Geney, France). Ethanol was obtained from Isolab (Istanbul, Turkey), and sodium citrate dihydrate was purchased from Tekkim (Bursa, Turkey).

Red Onion Peel

The outer dry protective layers of red onions were obtained from a local market in Edirne in October 2024. The peels were dried at 25±1°C for 24 hours, then ground using a Waring blender (Waring 8011 Eb, Vernon Hills, Illinois, USA) and sieved through a 1 mm mesh. The dry matter content of the resulting red onion peel powder (ROPP) was determined to be 92.75±0.03% using the oven-drying method, and the value was calculated gravimetrically. The ROPP samples were stored at +4°C until used for extraction studies.

Extraction Methods

The parameters of all extraction methods were determined based on the enzyme's optimum pH value, solid-to-solvent ratio, and temperature conditions, to allow for comparison with the enzyme-assisted methods (Kaur et al., 2016; Nguyen and Nguyen, 2018). Specifically, the extraction durations in bath-type and probe-type ultrasound-assisted extraction, as well as their enzyme-assisted versions, were determined to maintain the enzyme's maximum activity under its optimum temperature and pH conditions, ensure the device's temperature stability, and minimize the risk of enzyme deactivation caused by thermal fluctuations.

Conventional Extraction (CE)

For the extraction process, ROPP samples were prepared at a 1:20 (g/mL) ratio using 0.1 M citric acid-sodium citrate buffer (pH 5.5), adjusting the final solution pH to 4.5. The samples were incubated in a shaking water bath (Memmert, Schwabach, Germany) at 50°C for 2 hours. After incubation, the samples were cooled to room temperature and subsequently filtered through filter paper. The supernatants were collected after centrifugation at 5000 rpm for 10 minutes at 25°C (Sigma 3K 30, Osterode am Harz, Germany). The extracts were kept into amber-colored bottles and stored at -18°C until analysis.

Enzyme-Assisted Extraction (EAE)

The enzymatic extraction of ROPP was performed using the commercially available Viscozyme® L enzyme. The extraction process involved preparing ROPP samples at a 1:20 (g/mL) ratio using 0.1 M citric acid-sodium citrate buffer, adjusting the final solution pH to 4.5 (Kaur et al., 2016; Kitryte et al., 2017). Subsequently, 1.0% (w/v) Viscozyme® L enzyme was added to the mixture (Nguyen and Nguyen 2018). To ensure optimal enzyme activity, the extraction temperature was set to 50°C (Kaur et al., 2016; Nguyen and Nguyen 2018). The samples were incubated at 50°C in a magnetic stirrer at 200 rpm for 2 hours. At the end of the extraction period, enzyme inactivation was carried out by placing all samples in a 90°C water bath for 5 minutes (Memmert, Schwabach, Germany), followed by cooling to approximately 25°C (Hefzalrahman et al., 2022). The filtration and centrifugation steps following extraction were performed identically to the conventional extraction method.

Ultrasound-Assisted Extraction (UAE)

Bath-Type Ultrasound-Assisted Extraction (UAE-B)

Bath-type ultrasound-assisted extraction was performed using an ultrasonic bath (Isolab, Turkey) with 60 W ultrasonic power and a frequency of 40 kHz (dimensions: 150 × 138 × 65 mm, W × D × H). ROPP samples were prepared at a 1:20 (g/mL) solid-to-solvent ratio using 0.1 M citric acid-sodium citrate buffer. The extraction was carried out at 50°C for 30 minutes in the ultrasonic bath. The bath temperature was regularly monitored using a calibrated thermometer, and ice was added when necessary to maintain a stable temperature. The preparation of ROPP and UAE-B extraction is presented in Figure 1. Filtration and centrifugation steps were performed identically to the conventional extraction method.

Probe-Type Ultrasound-Assisted Extraction (UAE-P)

Probe-type ultrasound-assisted extraction was carried out using a Bandelin ultrasonic homogenizer (Sonopuls HD 4200, Berlin, Germany), which consisted of a generator (GM 4200), an ultrasonic transducer (UW 200), an amplifier (SH 200 G), and a titanium probe (TS 109, diameter: 9 mm). ROPP samples were

prepared at a 1:20 (g/mL) solid-to-solvent ratio using 0.1 M citric acid-sodium citrate buffer. The extraction was conducted at a constant 20 kHz ultrasound frequency, with an amplitude of 50% at 50°C for 5 minutes. To maintain a stable extraction temperature, an ice jacket was placed around the double-walled extraction vessel. A calibrated thermometer was used to monitor the temperature throughout the process, ensuring that it remained within the 50±5°C range. Filtration and centrifugation steps were carried out identically to the conventional extraction method.

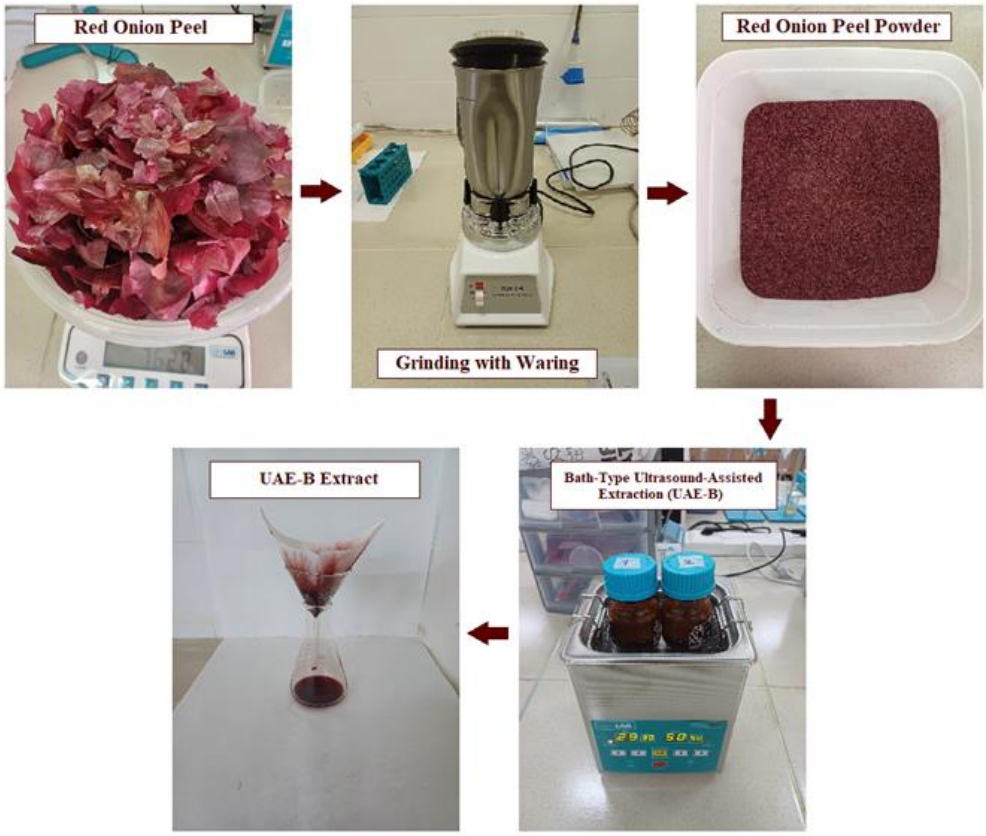

Fig. 1. Preparation of ROPP and UAE-B extraction

Ultrasound-Assisted Enzymatic Extraction (UAEE)

Bath-Type Ultrasound-Assisted Enzymatic Extraction (UAEE-B)

Bath-type ultrasound-assisted enzymatic extraction was performed using the same ultrasonic bath (Sonopuls HD 4200, Berlin, Germany) as described in the bath-type ultrasound-assisted extraction (UAE-B) procedure. ROPP samples were prepared according to the conditions specified for enzyme-assisted extraction (EAE). A 1.0% (w/v) Viscozyme® L enzyme was then added to the mixture. The extraction was conducted at 50±2°C for 30 minutes in the ultrasonic bath. The bath temperature was regularly monitored using a calibrated thermometer, and ice was added when necessary to maintain a stable extraction temperature. At the end of the extraction process, all samples were placed in a 90°C water bath for 5 minutes to inactivate the enzyme (Memmert, Schwabach, Germany), followed by cooling to approximately 25°C (Hefzalrahman et al., 2022). Filtration and centrifugation steps were performed identically to the conventional extraction method.

Probe-Type Ultrasound-Assisted Enzymatic Extraction (UAEE-P)

For probe-type ultrasound-assisted enzymatic extraction, ROPP samples were prepared following the conditions specified for enzyme-assisted extraction (EAE). A 1.0% (w/v) Viscozyme® L enzyme was then added to the mixture. The extraction was performed using the same ultrasonic homogenizer and operational settings described for probe-type ultrasound-assisted extraction (UAE-P). The ultrasound application time using the probe-type system was set to 5 minutes, taking into consideration the need to maintain a constant temperature during treatment and to prevent enzyme inactivation. The extraction was conducted at 50% amplitude and 50±5°C for 5 minutes under continuous ultrasonic treatment. After extraction, samples were subjected to enzyme inactivation by placing them in a 90°C water bath for 5 minutes (Memmert, Schwabach, Germany), followed by cooling to approximately 25°C (Hefzalrahman et al., 2022). Filtration and centrifugation steps were carried out identically to the conventional extraction method.

Parameters evaluated on the extraction

Extraction yield

To calculate the extraction efficiency, the water-soluble part of the extracts was dried in an oven at 50°C until the weight remained constant. The extraction yield was calculated based on the initial amount of dry weight (DW) of the ROPP as follows:

$$Extraction\ yield\ (g/100\ g\ DW) = \frac{Water\ soluble\ fraction\ weight\ (g)}{Initial\ dry\ weight\ of\ ROPP\ (g)} x100 \quad (1)$$

Extracts Characterization

Total phenolic content was determined using the Folin-Ciocalteu method, following the procedure proposed by Shahidi et al. (2001). The results were expressed as mg gallic acid equivalent (GAE) per g of extract. Total flavonoid content of ROPP was evaluated using the method recommended by Wannes et al., (2010). The results were given as milligrams of catechin equivalent per g of extract. Total monomeric anthocyanin content (TMA) was determined using the pH differential method, as described by Wrolstad et al. (2005). The results were expressed as mg cyanidin 3-glucoside (C3G) per g of extract. Antioxidant activity was assessed using two different methods. DPPH radical scavenging assay conducted according to the method proposed by Zhang and Hamauzu (2004). ABTS radical scavenging assay performed based on the procedure described by Re et al. (1999). The antioxidant activity results were expressed as mmol Trolox equivalent (TE) per g of extract. To account for extraction efficiency, the results were also expressed per unit of dry matter (Syrpas et al., 2021). Total phenolic content (TPC), total flavonoid content (TFC), and total monomeric anthocyanin content (TMA) were expressed as mg/g dry weight (DW). Antioxidant activity was expressed as μmol TE/g DW.

Colour measurement

The colour parameters with CIELAB indices (International Commission on Illumination, Vienna) of ROPP extracts were measured with Minolta CM 3600d spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan). L* (lightness/brightness, 0 = black, 100 = white), a* (redness/greenness, a+ = red, a- = green) and b* (yellowness/blueness, b+ = yellow, b- = blue) colour intensity values were read. The total colour difference (ΔE*) for anthocyanin extracts compared to the anthocyanin extract from CE was calculated using L*, a* and b* values by means of the following equation:

$$\Delta E^* = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \quad (2)$$

Where, subscript '0' indicates the values of extracts obtained CE methods. L*, a*, and b* are values of extracts after the other extraction methods.

Identification of anthocyanin by HPLC-DAD

Anthocyanin profile of ROPP was determined by modifying the method suggested by Nour et al. (2013) using reverse phase high performance liquid chromatography (RP-HPLC) instrument (Agilent 1200 system, Agilent Technologies, Santa Clara, CA, USA). The ROPP extracts obtained by CE, EAE, UAE, UAEE-B, and UAEE-P

methods were filtered through a 0.45 μm polyvinylidene fluoride (PVDF) filter and placed in amber vials. Chromatographic separation of anthocyanins was carried out using a C18 RP-column (EC Nucleosil, 150 × 4.6 mm, 3 μm) column at 40°C and 530 nm with 20 μL sample. Solvent A (acetonitril:water:formic acid, 10:89:1, v/v) and solvent B (acetonitril:water:formic acid, 89:10:1, v/v) used as the mobile phase at 0.5 mL min^{-1} in gradient mode: 0–15 min linear from 3% to 10% solvent B, 15–20 min linear from 10% to 15% solvent B, 20–25 min linear from 15% to 20% solvent B, 25–28 min linear from 20% to 25% solvent B, 28–30 min linear from 25% to 35% solvent B, 30–35 min linear from 35% to 50% solvent B, 35–38 min linear from 50% to 3% solvent B, and 38–40 min isocratic 3% solvent B. Anthocyanins were identified by comparing the retention times of the extracts with those of external standards, and their concentrations were calculated based on calibration curves constructed using these standards, allowing quantification in terms of their own specific equivalents.

Statistical Analysis

The experiments were conducted in independent duplicate, with two parallel analyses for each replicate. The results were expressed as the mean±standard error (SE) of the analyses. Comparisons between means were performed using one-way analysis of variance (ANOVA) with SPSS 27.0 software (SPSS, IBM Corp, USA) Inc., Chicago, IL). Differences between means were analyzed using Duncan's multiple comparison test at a 95% confidence level ($P< 0.05$).

RESULTS AND DISCUSSION

Extraction yields

Anthocyanin recovery from ROPP was investigated using conventional extraction (CE), enzyme-assisted extraction (EAE), ultrasound-assisted extraction (UAE), and their combination, ultrasound-assisted enzymatic extraction (UAEE), to evaluate the efficiency of different techniques in maximizing extraction yield. A comparative view of the effects of six different extraction methods on extraction yields is given in Figure 2.

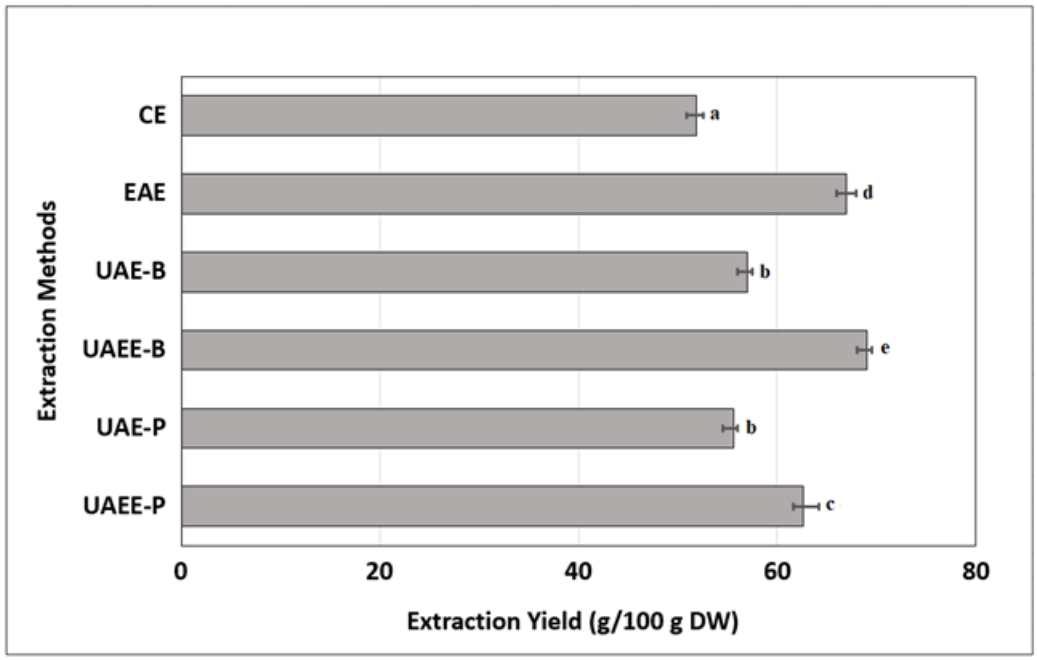

Fig. 2. Comparison of extraction yields obtained from ROPP with different extraction methods. Different superscript letters indicate significant differences ($P< 0.05$).

Extraction efficiency is influenced by multiple factors, including the chemical composition of the plant matrix, particle size, solvent type and concentration, temperature, and extraction time. Among these, the extraction technique plays a crucial role, as it directly impacts both the yield and quality of the target bioactive compounds (Pagano et al., 2021; Jha and Sit, 2022). The results obtained in this study showed that there were significant differences among the methods used for extraction ($P< 0.05$). It was determined that CE produced the lowest yield (51.87±0.68 g/100 g DW) compared to ultrasound and enzyme-assisted techniques. UAE-B (56.99±0.47 g/100 g DW) and UAE-P (55.57±0.42 g/100 g DW) showed no statistically significant difference in yield ($P> 0.05$), although they demonstrated improved extraction efficiency (approximately 8.50%) compared to CE, but were outperformed by their enzyme-assisted counterparts. The effect of ultrasound is primarily attributed to cavitation phenomena, where the rapid formation and collapse of microscopic bubbles lead to localized shear forces that facilitate the breakdown of cell structures. As a result, this phenomenon enhances the interaction between the solvent and the target compounds, enabling better penetration of the plant matrix by the solvent. During the extraction process, it promotes a more efficient transfer of bioactive compounds from the cellular structure into the solvent, facilitating their release from the matrix into the extraction medium (Chemat and Khan 2011, Vinatoru et al., 2017). UAE-B required 30 minutes to achieve comparable yields, whereas UAE-P reached the same yield in just 5 minutes, which is associated with increased efficiency and reduced energy consumption. This shows a clear advantage of the probe technique in terms of time efficiency and makes it a more practical option for large-scale or time-sensitive extractions.

Among the tested extraction methods, UAEE-B achieved the highest yield (69.05±0.46 g/100 g DW), followed by EAE (66.96±0.97 g/100 g DW) and UAEE-P (62.60±1.54 g/100 g DW), highlighting the effectiveness of enzymatic and ultrasound-assisted techniques in improving extraction efficiency. Notably, under the applied conditions, the use of Viscozyme L alone resulted in 29.1%, 17.5%, and 20.0% higher extraction yields compared to CE, UAE-B, and UAE-P, respectively. Several studies have confirmed the extraction-enhancing effects of Viscozyme L, a commercially available cellulolytic multi-complex (cocktail) enzyme containing carbohydrases such as arabanase, cellulase, hemicellulase, β-glucanase, xylanase, and pectinase (Kaur et al., 2016). Islam et al. (2023) demonstrated that Viscozyme L hydrolyzes various cell wall and membrane polysaccharides, leading to the effective release of bound and unbound bioactives from plant matrices. Kumar et al. (2019) further supported this, emphasizing that Viscozyme breaks down cell wall polysaccharides, overcomes the physical barrier of phenol-protein-polysaccharide linkages, and facilitates the release of anthocyanins and phenolics. Additionally, Mushtaq et al. (2015) used SEM micrographs to visually confirm that Viscozyme L and other cocktail enzymes degrade the pomegranate peel cell wall, increasing surface area and enhancing the extraction of bound phenolics.

Recent studies have increasingly focused on optimizing extraction techniques by modifying existing methods or integrating multiple approaches, such as enzyme-assisted ultrasound extraction (UAEE), to enhance both efficiency and bioactive compound recovery (Tan et al., 2020; Ma et al., 2024; Meral and Demirdöven, 2024; Patil et al., 2024). In this study, the highest extraction efficiency was achieved using the UAEE-B, which exhibited 9.3% higher efficiency than UAEE-P. The enhanced performance of UAEE-B can be attributed to the synergistic effect of ultrasound and enzymatic treatment, which induces favorable conformational modifications while preserving structural integrity, thereby improving biomolecule extraction, solvent infiltration, and mass transfer (Han et al., 2024). In contrast, the lower efficiency of UAEE-P may be stated by unstable mixing conditions during the process, which can cause localized temperature spikes that negatively impact enzyme activity. Kaur et al. (2016) emphasized that temperature plays a crucial role in disrupting the cell wall and enhancing enzyme

penetration for phenolic release; however, excessive heat can reduce enzymatic activity, leading to suboptimal extraction yields. Moreover, ultrasonic probe extraction involves multiple interacting parameters, such as amplitude, duration, and temperature, while enzymatic extraction is influenced by factors like enzyme concentration, pH, temperature, and solid-liquid ratio. When both techniques were combined, the interaction of numerous variables may have created challenging conditions that adversely affected enzyme stability and activity. In contrast, in this study, UAEE-B is considered to create a more homogeneous and thermally stable environment, which may enhance enzyme efficiency by maintaining controlled temperature conditions and ensuring uniform cavitation distribution.

Phenolic compounds of extracts

The TPC, TFC, TMA, and individual anthocyanin contents of ROPP extracts obtained using different extraction methods are presented in Table 1. According to Table 1, TPC and TFC amounts of 34.08 mg GAE/g DW and 13.44 mg CE/g DW, respectively, were detected in EAE, while 30.56 mg GAE/g DW of TPC and 11.73 mg CE/g DW of TFC were found in UAE-B. Hammad et al. (2024) optimized the extraction of TPC and TFC components from red onion peel using EAE with the commercial enzyme Viscozyme L and UAE employing an ultrasonic bath system. The TPC and TFC values obtained under these EAE conditions ranged from 23.57 to 41.33 mg GAE/g DW and 13.18 to 21.70 mg quercetin equivalent (QE)/g DW, respectively, which are consistent with the results of our study. However, in UAE higher TPC and TFC values, ranged between 58.93 and 84.02 mg GAE/g DW and 15.94 to 36.24 mg QE/g DW, respectively were obtained. This discrepancy could be attributed to several factors, including using of 80% ethanol as the extraction solvent, the application of higher ultrasonic power, and longer extraction durations.

Table 1. Comparison of the TPC, TFC, TMA and individual anthocyanins of the ROPP extracts obtained different extraction methods

	TPC (mg GAE/g DW)	TFC (mg CE/g DW)	TMA (mg C3G/g DW)	D3R (mg/kg DW)	C3G (mg/kg DW)	C3R (mg/kg DW)	P3G (mg/kg DW)
CE	25.78±0.77^{a}	10.85±0.21^{b}	0.97±0.05^{a}	2.84±0.28^{ab}	268.06±4.59^{a}	94.94±1.11^{a}	7.95±0.53^{a}
EAE	34.08±0.77^{c}	13.44±0.14^{d}	1.20±0.02^{b}	4.23±0.03^{c}	345.06±7.73^{c}	116.80±2.55^{bc}	10.37±0.42^{b}
UAE-B	30.56±0.52^{b}	11.73±0.01^{c}	1.25±0.02^{c}	3.36±0.05^{b}	316.14±2.93^{b}	123.98±1.05^{c}	10.40±0.24^{b}
UAEE-B	36.26±0.04^{d}	14.39±0.16^{e}	1.36±0.02^{d}	3.30±0.08^{b}	313.19±6.62^{b}	115.51±1.79^{bc}	9.37±0.37^{b}
UAE-P	25.51±0.64^{a}	8.37±0.15^{a}	1.19±0.01^{b}	2.54±0.06^{a}	303.21±9.44^{b}	116.64±4.76^{bc}	9.15±0.23^{b}
UAEE-P	33.73±0.67^{c}	14.19±0.22^{e}	1.33±0.03^{d}	4.36±0.31^{c}	299.08±10.78^{b}	111.40±3.81^{b}	9.31±0.65^{b}

CE: conventional extraction, EAE: enzyme-assisted extraction, UAE: ultrasound-assisted extraction, and UAEE-B: bath-type ultrasound-assisted enzymatic extraction, UAEE-P: prop-type ultrasound-assisted enzymatic extraction, D3R: delphinidin 3-rutinoside, C3G: cyanidin 3-glucoside, C3R: cyanidin 3-rutinoside, and P3G: peonidin 3-glucoside, different superscript letters in the same column means significant differences among the groups at $P< 0.05$.

The highest TPC was obtained using UAEE-B, which reached 36.26±0.04 mg GAE/g DW and was significantly different from the other methods ($P< 0.05$). Similarly, the highest amounts of TFC and TMA were also detected in UAEE-B and UAEE-P ($P> 0.05$). UAEE-B provided an increase of 40.65% in TPC, 32.63% in TFC, and 40.21% in TMA compared to CE. UAEE-B had 42.14% more TPC and 71.92% more TFC compared to UAE-P, while it also had 13.33% more TMA compared to EAE. Overall, the combination of EAE and UAE methods demonstrated greater efficiency in component yield for all three groups compared to using either EAE or UAE alone, in addition to providing an advantage over CE. These results highlight the critical role of enzymes in enhancing phenolic extraction and emphasize that their combined use

with ultrasound significantly improves the recovery of bioactive compounds. This combined approach not only enhanced extraction efficiency but also proved to be more effective than the use of each individual method on its own. Similarly, Kumar et al. (2020) reported that Viscozyme L and microwave treatment provided a synergistic advantage, showing that enzyme-assisted microwave extraction (EMAE) was more effective than both EAE and MAE alone in enhancing phenolic extraction and antioxidant activity from pomegranate peel. In line with this, Davidson et al. (2023) investigated the extraction of polyphenols and oil from raspberry pomace using various extraction methods, including control extraction, EAE, UAE, two sequential extraction approaches (UAE → EAE, EAE → UAE) and synergistic approach of US and alkaline protease (UEAE). The findings indicated that the UEAE outperformed the individual extraction methods, yielding a higher recovery of polyphenols. In this context, the use of enzymes alone enhances extraction efficiency by breaking down the cell wall structure and facilitating the release of phenolic compounds. Enzymes hydrolyze polysaccharides and the lignocellulosic matrix in the cell wall, making it easier for phenolic compounds to migrate from inside the cell into the extraction medium (Kumar et al., 2022; Islam et al., 2023). This process removes cellular barriers, allowing solvents to penetrate more effectively and improving the recovery of phenolic compounds (Ribeiro, 2024). On the other hand, ultrasound applications enhance mass transfer and increase solvent-sample interactions by physically disrupting cell walls through the mechanical cavitation effect. Ultrasonic waves generate microjets and shockwaves that break down the cell structure, thereby improving the solubility of phenolic compounds and enhancing extraction efficiency (Chemat and Khan, 2011; Vinatoru et al., 2017). Although each method offers distinct advantages, the combined use of hydrolytic effect of the enzyme and cavitation phenomenon of ultrasound (US) facilitates both the chemical and mechanical disruption of cellular structures while also improving solvent penetration, leading to a higher extraction yield of phenolic compounds.

Anthocyanins are a key characteristic of red onion varieties, giving them their distinct red/purple colour. These compounds are predominantly found in the skin and outer fleshy layers, while in the edible portion, they are restricted to a single layer of epidermal cells (Celano et al., 2021). In this study, the main identified anthocyanins using different extraction methods were D3R, C3G, C3R, and P3G. C3G has been identified as the predominant anthocyanin of ROPP (Table 1). Celano et al. (2021) emphasized that cyanidin is the predominant anthocyanin in red onion and identified three cyanidin derivatives (cyanidin 3-laminaribioside, cyanidin 3-malonilglucoside, cyanidin 3-malonillaminaribioside) in two different red onion varieties using UHPLC-HRMS/MS. Similarly, Chadorshabi et al. (2022) reported that cyaniding 3-glucoside is the major anthocyanin in onion peel, with anthocyanin concentrations ranging from 10.04 to 233 mg/100 g. In addition, Gorrepati et al. (2024) identified 34 anthocyanins in the acidified methanol extract of onion skin using LC–MS [UHPLC-Orbitrap MS], including 10 cyanidin, 10 delphinidin, 4 peonidin, 4 petunidin, 3 pelargonidin, and 2 malvidin derivatives and stated that cyanidin-3-(6-malonylglucoside), delphinidin, and delphinidin-3-galactoside were the predominant pigment in dark red variety.

The results in Table 1 demonstrated that the CE method was not effective enough to extract individual anthocyanins compared to other extraction methods, providing statistically significantly lower anthocyanin content of up to 23.6% ($P< 0.05$), indicating its lower efficiency in anthocyanin recovery. The insufficient efficiency of the CE can be attributed to oxidative degradation due to longer extraction times and the thermal and pH instability of anthocyanins, which makes them more susceptible to degradation (Celano et al., 2021). Additionally, the use of citrate buffer alongside solvents such as ethanol and methanol in CE may have also contributed to the lower extraction yields (Kitryte et al., 2017). When examining the concentrations of individual anthocyanins in ROPP extracts, contrary to the results obtained for TMA, the synergistic methods did not exhibit significant

advantages, particularly for C3R and P3G (P> 0.05). Notably, the EAE method was statistically superior for C3G, while EAE and UAEE-P methods demonstrated a statistically significant advantage over other methods for D3R (P< 0.05). Differences in interactions between extraction methods and individual anthocyanins can be attributed to two factors: one is the improved efficiency of Viscozyme-based EAE extraction, in particular, by targeting the structural integrity of the plant cell wall (Kumar et al., 2022). The synergistic effect of enzymes such as cellulase, xylanase, and β-glucanase facilitates the hydrolysis of cellulose and hemicellulose, effectively degrades the cell wall matrix, and increases the accessibility of intracellular anthocyanins. This enzymatic degradation, combined with optimal enzyme concentration and temperature, promotes the release of bound water molecules and hydrophilic anthocyanins, thereby improving extraction efficiency (Kumar et al., 2022). And the other can be the differences in the chemical structure, stability, and extraction sensitivity of anthocyanins and the intracellular localization of these compounds.

Antioxidant activity of extracts

The antioxidant activity values obtained by ABTS and DPPH of ROPP extracts for the extraction methods are presented in Figure 3. The lowest AA-ABTS value (94.85±0.28 μmol TE/g DW) was obtained using UAE-P for ROPP extract. The extracts obtained through the CE and UAE-B showed values of 123.67±3.00 and 122.20±1.05 μmol TE/g DW, respectively, with no statistically significant difference (P> 0.05). The highest AA-ABTS value (199.35±1.13 μmol TE/g DW) was achieved using the EAE method. This was followed by UAEE-B (172.71±9.87 μmol TE/g DW) and UAEE-P (164.30±2.42 μmol TE/g DW), where enzymatic treatment was combined with ultrasonic bath and probe, respectively. The lowest AA-DPPH value (64.14±1.29 μmol TE/g DW) was obtained using the CE for ROPP extract. This was followed by the UAE-B and UAE-P methods, with values of 71.19±0.29 and 71.09±1.30 μmol TE/g DW, respectively. The UAEE-P method yielded a higher value (85.51±0.30 μmol TE/g DW), while the highest AA-DPPH values were obtained using the EAE and UAEE-B methods, with values of 91.16±0.99 and 93.20±0.78 μmol TE/g DW, respectively. There was no statistically significant difference between the EAE and UAEE-B methods (P> 0.05).

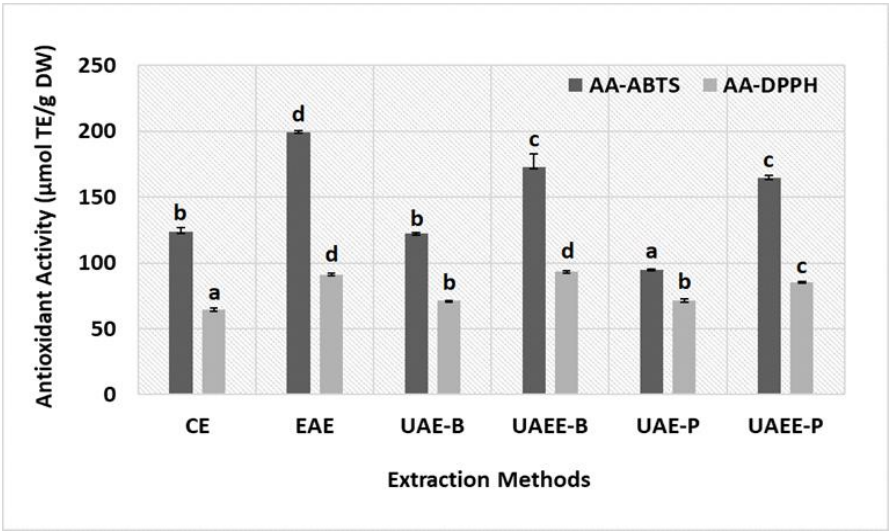

Fig. 3. CE: conventional extraction, EAE: enzyme-assisted extraction, UAE: ultrasound-assisted extraction, and UAEE-B: bath-type ultrasound-assisted enzymatic extraction, UAEE-P: prop-type ultrasound-assisted enzymatic extraction, different superscript letters indicate significant differences among the groups (P< 0.05).

As seen in Figure 3, the antioxidant activity obtained using the ABTS radical is higher than the values obtained with the DPPH radical for all methods. Since using a single method for antioxidant activity assessment is not scientifically recommended due to the complex composition of plant samples, the electron transfer-based DPPH and ABTS decolorization assays are among the most frequently employed methods to determine the antioxidant capacity of plant extracts (Islam et al., 2023). ABTS is effective in measuring the antioxidant activity of both water-soluble (hydrophilic) and fat-soluble (lipophilic) compounds, making it a versatile method. In contrast, DPPH is more specific to lipophilic antioxidants and is predominantly used in organic solvent-based systems (Santos et al., 2022). Similarly, in a study conducted by Santos et al. (2022), the radical scavenging capacity (%) obtained with ABTS was higher than that obtained with DPPH, which was attributed to the lower presence of lipophilic compounds in purple onion peel extracts.

The results of this study state that the CE has a limited effect on antioxidant capacity. The lower yield of TPC, TFC, and TMA obtained with CE compared to other techniques suggests that this method is ineffective in efficiently transferring bioactive compounds into the solvent. This limitation may be attributed to the degradation of heat-sensitive compounds and the fact that the extraction efficiency of CE is restricted by solvent diffusion. Ultrasound-assisted methods, particularly UAE-B and UAE-P, provided up to 11% higher antioxidant activity in the DPPH assay compared to CE. However, ultrasound alone resulted in lower antioxidant activity than enzyme-assisted methods. A comparable trend was observed in the study conducted by Hammad et al. (2024), where EAE of purple onion peel extracts yielded higher antioxidant activity than ultrasound-assisted extracts. This finding suggests that enzymes enhance the release of phenolic compounds by breaking down polysaccharide and protein structures in the cell wall, thereby improving their extraction efficiency. One of the most striking findings is that the highest ABTS value was obtained using the EAE method, whereas the highest DPPH values were observed for both UAEE-B and EAE. This result suggests that commercially available enzyme applications may play a selective role in extracting different antioxidant compounds including water and methanol-soluble antioxidative phenolics and exhibit varying effects depending on the radical type being assessed (Kaur et al., 2016). Additionally, antioxidant activity values determined by DPPH were found to be higher in enzyme-assisted extraction methods compared to those without enzymatic treatment. This may be attributed to the fact that DPPH is a sterically hindered radical, and its reactivity is influenced by molecular accessibility. Browning products or intermediate reaction compounds may react more rapidly with DPPH than larger, bulkier antioxidant molecules, potentially leading to higher measured antioxidant activity (Buyuktuncel, 2013).

Colour measurements of extracts

The different extraction methods have varying effects on the L*, a*, and b* colour parameters of the extracts, as shown in Table 2. Similarly, the values of L*, a*, b* were significantly affected by different extraction methods, which was following the previous studies on carotenoids from lemon peels (Meral and Demirdöven, 2024) and anthocyanins from blue pea flower (Gamage and Choo, 2023). In terms of colour parameters, the extracts obtained using EAE, UAEE-B, and UAEE-P exhibited similar behavior. These extracts were observed to have a lighter colour, a more intense red colour, and higher yellow values. There is no statistically significant difference among all colour parameters between the CE and UAE-B processes ($P > 0.05$). However, the UAEE-P extract was found to have a more yellowish colour compared to these methods. This situation can be associated with the negative impact on enzyme activity due to greater temperature variation during extraction. Additionally, Tiwari et al. (2010) show that higher amplitude levels and treatment times have adverse effects on the anthocyanin content of grape juice. In this study, even though there is no increase in amplitude levels, the localized high temperature and pressure, depending on the sample-to-solvent

ratio, are thought to cause anthocyanin degradation.

Table 2. The colour coordinates and change of anthocyanin extracts of ROPP obtained from various extraction methods

	L*	a*	b*	ΔE*
CE	9.34±0.10^{a}	31.00±0.26^{a}	11.20±0.18^{a}	0.00 ±0.00^{a}
EAE	10.20±0.11^{b}	33.03±0.26^{b}	12.82±0.18^{bc}	2.73±0.67^{bc}
UAE-B	8.90±0.05^{a}	30.06±0.10^{a}	10.46±0.08^{a}	1.28±0.19^{ab}
UAEE-B	10.03±0.19^{b}	32.62±0.44^{b}	12.48±0.33^{b}	2.17±0.91^{bc}
UAE-P	10.58±0.42^{b}	33.60±0.98^{b}	13.59±0.66^{cd}	3.74±0.91^{c}
UAEE-P	8.69±0.18^{a}	30.26±0.38^{a}	14.30±0.22^{d}	3.48±0.24^{c}

Different superscript letters in the same column means significant differences among the groups at $P< 0.05$.

Regarding the parameter ΔE*, while greater changes were observed in extracts obtained from UAE-P, smaller changes of ΔE* were obtained for UAE-B. Santos et al. (2022) associated the ΔE* in anthocyanin-rich extracts with the thermosensitive behavior of anthocyanins, noting that high temperatures modulate these compounds, leading to yellowish or brown colour indicative of pigment degradation. Moreover, Patras (2019) attributed higher ΔE* values primarily to variations in the red component (Δa*), with a lesser influence from lightness (ΔL*) and an even smaller impact from changes in the blue component (Δb*). Additionally, the observed colour shifts in the presence of different compounds were associated with factors such as pH influence, tautomeric form interconversion, copigmentation effects, anthocyanin polymerization, and browning. Colour differences are considered very distinct when ΔE* is more than 3, distinct when ΔE* is between 3 and 1.5, and a small difference when ΔE* is less than 1.5 (Gamage and Choo, 2023). Accordingly, UAE-P and UAEE-P extracts had very distinct colour difference compared to the CE. This situation is consistent with the decrease in L*, a*, and b* values and the possible anthocyanin degradation. On the other hand, the UAEE-B extract, having the highest bioactive compound content, shows a ΔE* of 2.17, which falls within the moderate range of colour difference. Given that anthocyanins are responsible for the characteristic red/purple colour of red onion varieties (Celano et al., 2021), this moderate ΔE* value can be considered a favorable outcome, as it indicates that colour alterations due to polymerization and degradation are relatively limited, thereby preserving the extract's chemical stability, visual characteristics and potentially maintaining its functional properties. This situation indicates that the extract's anthocyanin content, colour, and stability index support its potential application in various bio-industries, such as the pharmaceutical, cosmetic, and food sectors (Santos et al., 2022).

CONCLUSION

This study highlights the potential of green extraction techniques for the efficient recovery of anthocyanins from red onion peel, a significant food industry waste. For this purpose, traditional extraction methods were compared with enzyme-assisted, ultrasound-assisted and combined techniques (ultrasound and enzyme), evaluating extraction yield and the characteristic properties of the obtained extract. The results indicate that these environmentally friendly approaches can enhance extraction efficiency and extract characteristics while preserving the structural integrity and bioactivity of anthocyanins compared to conventional extraction. The use of a complex enzyme mixture, such as Viscozyme, in the extraction process facilitated the disruption of red onion peel cell wall integrity. As a result, enzyme-assisted and combined methods provided a significant advantage over ultrasound-only techniques in terms of both extraction yield and bioactive compound concentration. Similarly,

in terms of antioxidant activity, the use of a single enzyme and the combined application of enzymes with ultrasound demonstrated a significant advantage. Although the extraction methods had different effects on individual anthocyanins, the total monomeric anthocyanin content in the combined methods, including both UAEE-B and UAEE-P, was found to be statistically different from the other methods. When the colour change values calculated according to the traditional method, which are an indicator of the stability of anthocyanins in the extract, were compared, it was found that UAEE-B was more advantageous than UAEE-P. Finally, when comparing the application time, it should not be overlooked that the procedure carried out with the UAEE-P takes 5 minutes and the procedure carried out with the UAEE-B takes 30 minutes. In conclusion, this study has demonstrated that the combined application of enzymes and ultrasound offers significant advantages in anthocyanin recovery from red onion skins, particularly in preserving production quality. Additionally, future research should focus on optimizing the factors influencing these combined methods to develop more specific and sustainable extraction techniques specifically suited for ROPP while also assessing their economic feasibility. This approach would enable the environmentally friendly and cost-effective production of anthocyanin extracts from ROPP, facilitating their use in the food, pharmaceutical, and other industries both as functional ingredients and natural colorants.

In addition, it was observed that integrated extraction processes involving ultrasound application and enzyme addition yielded higher results in only 30 and 5 minutes, respectively, compared to the conventional method, which required 2 hours at 50°C. This highlights the time-saving advantage of these "green" extraction techniques. However, in order to determine whether these methods are truly more sustainable and economically viable, analyses on carbon footprint and cost are needed. For these methods to present a viable alternative at an industrial scale, comprehensive evaluations must be conducted, taking into account equipment requirements, energy consumption, and enzyme costs.

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