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Ultrasound-assisted extraction of antioxidant bioactive compounds from wastes of rapeseed industry and their application in delaying rapeseed oil oxidation

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Competing interests

The authors declare no conflict of interest.

Availability of data and material

Not applicable

Code availability

Not applicable

Abstract

Rapeseed meal ethanol-wash solutes (EWS) are wastes generated from rapeseed meal as a pretreatment step to reduce the presence of antinutritional compounds. This study focused on employing and optimizing an ultrasound assisted extraction (UAE) of antioxidant bioactive compounds from EWS. The second aim was to fortify rapeseed oil with the best extract obtained, and to optimize this process. Both optimizations were carried out with the Response Surface Methodology (RSM), considering the combinations amplitude-time and concentration-time of homogenizing the sample with the oil as the assessed variables in the first and second process, respectively. The UAE achieved a higher content of total phenolic compounds (TPC) and antioxidant activity (AOA) than a conventional extraction. The parameters used for obtaining the optimum value of TPC (99.30 mg GAE/g) and AOA (148.99 mg TE/g) were the combinations of amplitude and time of 10% - 9.24 min and 15% - 0.76 min, respectively. The optimum extracts were mainly composed of phenylacetic acid and ferulic acid. The fortification of rapeseed oil with the optimum extracts delayed the oxidation up to 45% while the second optimization process allowed to increase this value to almost 61%. Therefore, EWS could be an alternative to synthetic antioxidants.

Keywords: Rapeseed meal, by-products valorization, antioxidants, oil stability, optimization

1. Introduction

Rapeseed (*Brassica napus*) seeds are rich in oil, containing about 40-50% (Chmielewska et al., 2021). The composition of rapeseed oil depends on many factors such as the seed variety, growing conditions, drying temperature, and the method of extraction (Ghazani et al., 2014). It is known for its high concentration of oleic (50-66%), linoleic (21%), and α-linolenic acid (6-14%) (Lin et al., 2013). Nevertheless, to counteract its vulnerability to oxidation, rapeseed oil contains some antioxidant compounds such as phytosterols, tocopherols and polyphenols (Ghazani and Marangoni, 2013). It also contains sulfur compounds; however, they are highly reduced during refining steps of oil production (Ghazani and Marangoni, 2013; Wongsirichot et al., 2022; Zeb, 2021).

The extraction of oil from rapeseed grains generates 39 million meters of tons of meal every year, which have to be disposed (Hussain et al., 2022; Wongsirichot et al., 2022). Due to its high amount of proteins, rapeseed meal is included in animal feed. However, it contains antinutritional compounds such as glucosinolates, phytates, and lignocellulosic fiber that limit its use. Moreover, rapeseed meal could be employed as a fertilizer or as a source for the production of fuel, antimicrobials, surfactants, biopolymers, and proteins for human consumption (Di Lena et al., 2021; Wongsirichot et al., 2022).

Polyphenols present in rapeseed meal have been reported to interfere negatively with the digestibility, functional, and organoleptic properties of the protein isolates generated from this source (Kalaydzhiev et al., 2020a; Von Der Haar et al., 2013). To achieve a better quality of the proteins extracted, a previous research washed the rapeseed meal with an ethanol solution (Kalaydzhiev et al., 2020b). The ethanol-wash liquids, collected and vacuum concentrated, turned into a new product, ethanol-wash solutes (EWS). The characterization of the EWS demonstrated significant presence of phenolic compounds, responsible for antimicrobial and antioxidant properties of the product (Georgiev et al., 2021). In addition, they enhanced the oxidative stability of several types of oil (Cisneros-Yupanqui et al., 2021). Although direct application of the EWS into food systems is an easy and inexpensive approach, an additional treatment enhancing the concentration of bioactive compounds in the product might increase its antioxidant capacity and applicability in food processing.

The extraction of several bioactive compounds from vegetables can be carried out with the use of the conventional techniques. However, many of them have some critical points like the requirement of high temperatures, the long extraction time, high solvents use, these factors could destroy interesting bioactive compounds (Aryanti et al., 2021; Muñiz-Márquez et al., 2013; Wen et al., 2019). On the other hand, the employment of green technologies, such as the ultrasound-assisted

extraction (UAE) is an attractive approach used within the food industry to avoid the negative impact of the conventional extraction techniques (Wen et al., 2019). It has been applied to a wide range of materials (Aryanti et al., 2021; Q. Li et al., 2022; Y. Li et al., 2022). UAE reduces significantly the extraction time, solvents use and energy while preserving the natural properties of biomolecules (Carreira-Casais et al., 2021). There are two different ways to apply UAE: ultrasonic bath and ultrasonic probe, the latter being preferred because the energy is delivered to the sample more uniform with less loss and the effect is concentrated in a specific zone with more efficiency (Kumar et al., 2021). Besides UAE, other potential emerging technologies for extraction of phenolic compounds from different food sources also exist. Their mechanisms, advantages, and disadvantages are outlined in Table 1.

To the best of the author's knowledge, limited number of studies have been focused on the EWS. Considering this, the present work provides novelty by using ultrasound to extract the antioxidant bioactive compounds from EWS. Then, two extraction-operating parameters (amplitude and time) were optimized, considering the response surface methodology (RSM) to improve the concentration of total phenolic compounds (TPC) and antioxidant activity (AOA). In addition, a commercial rapeseed oil was fortified with the optimum EWS extracts for the first time to assess its oxidative stability with a further second optimization of the concentration and the time of homogenizing the sample with the oil. The employment of the EWS underlies the concept of circular economy, allowing a complete valorization of the rapeseed meal by a waste-free technology.

2. Material and Methods

2.1. Chemical and reagents

Rapeseed oil was bought in a local market (Padova, Italy) and the rapeseed meal was supplied by a Bulgarian company. It was generated at an industrial scale with thermal operation at 110-115 °C followed by oil extraction with hexane at 60-65 °C for 1 hour. Folin Ciocalteau's phenols reagent, sodium carbonate, 2,3,5-triphenyltetrazolium chloride (TPTZ), hydrochloric acid, iron chloride, and sodium acetate were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Ethanol-wash solutes (EWS) from rapeseed meals

Rapeseed meal was ground and sifted to produce 0.315 mm particles. Then, 200 g of the meal was mixed with aqueous ethanol solution (75%) to a final volume of 800 mL at room temperature and constant agitation for 30 minutes and then vacuum-filtered. This process was repeated four times. The final residue of the process (after the ethanol washing) was dried and further used for protein extraction. The ethanol-wash aliquots were collected, mixed, vacuum concentrated (RV 3 V Rotary Evaporator, IKA®, Germany) at 50 °C to the 0.3-fold reduction of the volume and freeze-dried

(Lyovac GT2, Leybold-Heraeus, Germany) to obtain EWS powder (Cisneros-Yupanqui et al., 2021).

2.3. Ultrasound-assisted extraction (UAE) of antioxidant bioactive compounds

The extraction of phenolic compounds from the EWS was carried out following the methodology previously reported (Cisneros-Yupanqui et al., 2021) with slight modifications. Water was used as the extraction solvent at a ratio of 1/50 (w/v) for 20 minutes under constant shaking (220 rpm). Then, ultrasound-assisted extraction (UAE) was performed with SONOPULS ultrasonic homogenizer at 20 kHz ± 500 Hz frequency. The KE76 tip was used for the sonication. The independent variables studied were amplitude (x_1, 10-35%) and time (x_2, 2-8 min). After the UAE, the samples were centrifuged at 9500 g at 4 °C for 5 minutes and a second extraction was carried out under the same conditions. In addition, a conventional extraction was prepared following the same procedure, except for the ultrasound application (Figure 1) and compared to the UAE at an amplitude of 25% for 2 minutes.

2.4. Experimental design and statistical analysis

A response surface methodology (RSM) based on a Central Composite Face (CCF); 2^2+2 full factorial was conducted to optimize the UAE method and the fortification of rapeseed oil with the optimum extract obtained. The experimental designs and statistical analyses were performed with Statgraphics® Centurion XIX (StatPoint Technologies, Inc., Warrenton, VA, USA) and ten planned experiments were performed for each optimization process. Models and regression were significant when $p < 0.05$. The experimental factors assessed were amplitude (x_1) from 10-35% and time of extraction (x_2) from 2-8 min in the first optimization. The second one considered time of homogenizing the sample with the oil (x_1) from 3-10 min and concentration of extract in the oil (x_2) from 6-30% as the parameters assessed. In both cases, the results and the experimental factors are linked by a second order polynomial function (***Equation 1***):

$$Y = \beta_0 + \sum_{i=1}^{a} \beta_i X_i + \sum_{i=1}^{a} \beta_{ii} X^2{}_i + \sum_{ij=1\ (i\neq 1)}^{a} \beta_{ij} X_i X_j \qquad \textbf{(Equation 1)}$$

where the estimated response is represented by Y, β_0 is a constant, β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interactive coefficients, respectively. X_i and X_j are the levels of the experimental factors.

Besides the RSM, the data presented the mean ± standard deviation of three independent experiments (n = 3) by using an analysis of variance (ANOVA) one way, followed by the post-hoc

LSD (p < 0.05), using Statgraphics Centurion XVI (StatPoint Inc., Rockville, MD, USA). Correlations between the variables were performed by using the same software.

2.5. Analytical determinations

2.5.1. Total phenolic compounds (TPC)

The TPC was assessed by the Folin-Ciocalteau methodology (Campos et al., 2022). Briefly, 500 µL of a diluted sample was mixed with 250 µL of 1 N Folin-Ciocalteau's reagent and 1250 µL of 7.5% $NaCO_3$. A blank solution was prepared with water instead of a sample. After waiting 30 minutes under darkness, the absorbance value was measured at 755 nm, using a Varian Carry 50 Bio UV/Vis spectrophotometer. The results were expressed as mg of gallic acid equivalent per g of dried matter (mg GAE/g).

2.5.2. Antioxidant activity (AOA)

The AOA was evaluated using a spectrophotometric method assay, the ferric reducing antioxidant potential (FRAP), as previously reported (Cisneros-Yupanqui et al., 2021). The FRAP reagent was prepared by mixing 2.5 mL of 0.01 M of TPTZ in HCl 40 mM, 2.5 mL of an aqueous solution of 0.02 M $FeCl_3$, and 25 mL of 0.2 M of sodium acetate buffer (0.2 M Sodium acetate/ 0.2 M acetic acid). A FRAP volume of 900 µL was mixed with 100 µL of sample and incubated at 37°C for 30 minutes. A blank solution was prepared with the dilution solvent. The absorbance was measured at 593 nm using a Varian Carry 50 Bio UV/Vis spectrophotometer. The results were expressed in mg of Trolox equivalent per g of dried matter.

2.5.3. Phenolic compounds identification

The phenolic profile of the rapeseed EWS was assessed by HPLC analysis, using a Thermo Finnigan SpectraSystem UV6000LP (Thermo Finnigan, San Jose, CA, USA) HPLC system with a diode array detector as previously described (Cisneros-Yupanqui et al., 2021). The phenols present in the sample were identified based on the retention time of the corresponding commercial standards (pyrogallol, benzoic acid, protocatechuic acid, phenylacetic acid, protocatechuic aldehyde, syringaldehyde, and ferulic acid). The separation of the compounds was performed on SupelcosilTM LC-18 column under the following operating conditions: mobile phase, 18 mL n-butanol (solvent A)/1.5 mL 50% v/v acetic acid (solvent B); flow rate, 0.6 mL/min; isocratic flow; wavelength, 214 nm, 275 nm, and 310 nm; temperature, 25 °C; and running time, 60 min. The assay was performed in triplicate.

2.5.4. Oxidative stability

First, oxidative stability was assessed on a mixture of rapeseed oil with rapeseed EWS (30%, w/w) extracted with either the optimum parameters for AOA or TPC, corresponding to 0.89% and 0.71% of total antioxidant compounds in the fortified oil (w/w), respectively. In addition, a mixture of rapeseed oil with the extract conventionally extracted (0.60% total antioxidant compounds, w/w) as well as a control (rapeseed oil + 30% water) were prepared. Then, oxidative stability was also carried out in all the samples submitted to the second optimization process. All these treatments were mixed for 20 minutes in an orbital shaker (220 rpm) and then immersed in a beaker with ice for a further ultrasound treatment at amplitude 25% for 2 minutes (steps of 30 seconds). After sonication, the samples in ice bath were homogenized using Ultra Turrax T-25 (Janke and Kunkel,

$$\text{AAI} = \frac{\text{IT of oil with antioxidants}}{\text{IT of oil without antioxidants (C)}}$$

Germany) operated at 12.000 rpm for a specific time, according to the treatment. Samples (3 g) were tested using the official Rancimat method at 110 °C and insufflated with 20 L/h of air. The oxidative stability was assessed by the time of induction (IT), which is the time (h) before starts increasing water conductivity (μS/min), due to compounds obtained from lipid oxidation. The antioxidant activity index (AAI) was calculated using the following ratio (Cisneros-Yupanqui et al., 2021):

3. Results and Discussion

3.1. Ultrasound-assisted extraction (UAE)

Table 2 shows the comparison of TPC and AOA of extracts obtained by either a conventional method or an UAE at 2 minutes with an amplitude of 25%. While the quantification of TPC by Folin-Ciocalteau reagent is a straightforward and widely accepted approach, the choice of a method for the evaluation of AOA may be difficult, especially when assessing samples with unknown and complex composition as EWS. There are many methods such as Oxygen Radical Absorption Capacity (ORAC), Hydroxyl Radical Antioxidant Capacity (HORAC), Cupric Reducing Antioxidant Power (CUPRAC) test, 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) test, and the [2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl] (DPPH) test (Munteanu and Apetrei, 2021). They differ in their principle, sensitivity, and reproducibility. The β-carotene bleaching assay is also a common method for evaluating antioxidant activity, but it has not been recommended for the evaluation of samples containing metals (Dawidowicz and Olszowy, 2010). Several metals (Cu, Fe, Mn and Zn) have been previously found in EWS (Georgiev et al., 2021), limiting the potential use of the β-carotene bleaching assay. Since there is no single method that universally suits the

evaluation of any systems (Munteanu and Apetrei, 2021), the current study employed the FRAP one to monitor the influence of the UAE on the AOA of the EWS.

Many types of solvents at different concentrations are employed to extract polyphenols or antioxidant compounds from plants, so the final choice depends on the nature of the matrix (Muñiz-Márquez et al., 2013; Rao et al., 2021). In this study, distilled water was chosen because of the plausible high UAE yield achievement (Szydłowska-Czerniak et al., 2010). Besides its good affinity to phenolic compounds, water is cheap, not toxic, and environmentally friendly. The extraction was carried out at room temperature since the yield can be affected by high temperatures, reducing the dielectric constant (Bampouli et al., 2014).

The conventional extraction obtained the lowest values in the two variables ($p < 0.05$). UAE increases the extraction yield and it reduces the time needed compared to the conventional techniques because it combines the acoustic cavitation generated by ultrasounds with the solvent of extraction (Falleh et al., 2012; Rodsamran and Sothornvit, 2019). The bubbles collapse generated by ultrasound damage the cell tissue, producing littler particles, enhancing the contact with the solvent and favoring the release of the compounds (Rao et al., 2021). In addition, UAE reduces both the solvent required and the energy consumption (Silva and Saldaña, 2020).

The use of UAE increased the values of TPC and AOA by 7.93% and 10.41%, respectively (Table 2). These results agreed with studies that employed similar parameters to this one. The AOA of *Limonium sinuatum* flower increased by 9% with the UAE (Xu et al., 2017). However, the time of extraction was 9.8 min against the 2 min used in the present study. The TPC and AOA of citrus peels extract increased by 15% and 12%, respectively, when the extraction was carried out at room temperature (Saini et al., 2019). The nature of the samples, amplitude, time, and the temperature used in the UAE are some of the factors that should be considered in data interpretation (Teh and Birch, 2014; Wang et al., 2020).

On the other hand, the application of ultrasound in this study was performed through a probe, which obtains higher concentrations of TPC than the ultrasonic bath, as reported in walnut shells (Han et al., 2018). The employment of the ultrasonic probe may break the cell structure more efficiently, allowing a better penetration of solvents. Moreover, it favors mass transfer, helping in the disruption of cells with the consequent enhancement of bioactive compound extraction (Rodrigues and Fernandes, 2009). Moreover, UAE has obtained a better extraction yield than conventional methodologies not only at laboratory scale, but also at pilot one. However, the effect was less significant in the latter (Tamminen et al., 2022).

3.2. Optimization of the UAE of antioxidants bioactive compounds

Amplitude and time were the two factors considered in this study to optimize the UAE of TPC and AOA. Table 3 shows the TPC and AOA experimental values, which are within the range of 75.18 - 96.64 mg GAE/g and 92.26-125.81 mg TE/g, respectively. Additionally, one row was added for the conventional extraction to confirm the difference obtained when using the UAE. The polynomial equations, without considering the unsignificant terms, are the following:

$$TPC = 85.2777 - 0.0832305 * \text{Amplitude}(x1) + 2.26873 * \text{Time}(x2) - 0.0661024 * x1x2 \quad \textit{(Equation 2)}$$

$$AOA = 123.172 + 3.49712 * \text{Amplitude}(x1) - 3.21046 * \text{Time(x2)} - 0.108217 * x12 \quad \textit{(Equation 3)}$$

The theoretical optimum TPC and AOA were obtained at the combination amplitude-time of 10% - 9.24 min (99.30 mg GAE/g) and 15% - 0.76 min (148.99 mg TE/g), respectively. These values were 25% and 50% higher than the TPC and AOA values from a conventional extraction, respectively. Similar findings were reported in soursop, where the optimization of UAE increased the TPC by 32-37% (Aguilar-Hernández et al., 2019) and in olive leaves, with an increase of 11% (Hasnia et al., 2019). To verify the validity of the regression model, the equations have been tested experimentally, obtaining 89.10 ± 3.44 mg GAE/g and 122.26 ± 2.62 mg TE/g, for TPC and AOA, respectively. The possible difference with the theoretical values could be due to other factors besides amplitude and time that were not considered in this research. Despite these considerations, the difference was less than 20% compared to the theoretical values and in agreement with the literature (Aourach et al., 2021; Drevelegka and Goula, 2020).

The regression coefficients and F-ratios of both response variables (TPC and AOA) are shown in Table 4 and Table 5, respectively. For TPC, the independent variables and the interaction AB (amplitude-time) had a significant influence ($p < 0.05$) on both responses. Amplitude obtained the highest linear influence while the interaction AB had the lowest one (F-ratio 213.36 and 31.06, respectively). Regarding the AOA, the influence of the independent variables was significant ($p < 0.05$) as well as the quadratic interaction amplitude. The greatest significance was observed for amplitude (F-ratio 43.68) and the lowest for time (F-ratio 20.99). In this case, the influence of the interaction amplitude-time was not significant. F-ratios show that amplitude had a major impact on TPC (F-ratio 213.36) and AOA (F-ratio 43.683) values. When the amplitude increases, there is a major release of phenolic compounds; however, the yield of extraction is reduced after a peak because of polyphenols destruction (Kaur et al., 2021; Osorio-Tobón, 2020). Moreover, high amplitudes generate more bubbles, reducing their impact with the matrix and forming a layer around the probe that reduces the transmission of ultrasounds (Kumar et al., 2021). As shown in Figure 2, the values of TPC were high up to around an amplitude of 25%. At higher values, the

bioactivity decreased steadily until reaching 50% amplitude. The AOA was high until 30% amplitude, then it decreased, and the minimum value was obtained at 50% amplitude. By increasing this factor, the temperature has the same trend while the viscosity and surface tension of the solvent decrease (Han et al., 2018; Oroian et al., 2020).

In this study, the temperature was controlled by using an ice bath; however, there could be a local influence in the extraction of compounds. Similarly to previous research (Rodsamran and Sothornvit, 2019), the lowest TPC was obtained at an amplitude of 40%, which suggests that polyphenols can be damaged at amplitudes over 40%. The accurate amplitude that increases the TPC and AOA yield depends on the matrix. For instance, areca nuts and pomegranate employed 50% and 30%, respectively (Chavan and Singhal, 2013; Foujdar et al., 2020). The best amplitude for obtaining the highest AOA was 80% for *Bauhinha fortificata* (Palsikowski et al., 2020) and 90% for sugar beet molasses powder (Babaoğlu et al., 2022). As seen in Table 3, to obtain the maximum values of TPC and AOA, it was necessary to use lower amplitudes than the ones employed in the matrixes mentioned above because of the sensitive compounds that EWS contain such as polyphenols and other antioxidant compounds. Moreover, the optimal amplitude necessary to extract compounds from the matrix depends on the stability of antioxidant molecules (Kumar et al., 2021; Nguyen and Nguyen, 2018).

As reported in Table 4, the time has a minor but important impact on TPC and AOA. The time of extraction does not have to be too long. This situation could increase the TPC yield; however, no more compounds are released from the matrix when the solvent reaches saturation, causing possible damage to the phenols (Kaur et al., 2021). Prolonging the time of extraction facilitates the oxidation of compounds due to some environmental factors like light or oxygen (Saci et al., 2018). There are many studies about the time influence of UAE on vegetable matrixes, but they used longer times of extraction than the ones assessed in this research (Kaur et al., 2021; Leksawasdi et al., 2022; Mrkonjić et al., 2021; Qiu et al., 2018; Szydłowska-Czerniak and Tułodziecka, 2014; Teh and Birch, 2014; Weremfo et al., 2022; Wu et al., 2021). Probably, the effect of the ethanol wash has concentrated the antioxidant compounds inside the rapeseed EWS, reducing the time of extraction required. Figure 2 shows that time had a positive influence on the TPC value since it started increasing after 6 minutes. In the case of AOA, the highest value was obtained after the first minutes and then it decreased.

The optimal time of extraction depends on the stability of the molecules during sonication. Each study from the literature has its optimal time of sonication for TPC and AOA. In the case *Polygonum aviculare* leaves, 60 minutes were required for obtaining the optimum TPC (Wu et al., 2021) and 17.3 minutes for Turkey berries (Weremfo et al., 2022). On the other hand, the best

values for AOA were reached after 10 minutes for green soybean pods (Leksawasdi et al., 2022), and 70 minutes for wild thyme (Mrkonjić et al., 2021). In the case of rapeseed meal, it was reported that 18 (Szydłowska-Czerniak and Tułodziecka, 2014) or 20 min (Teh and Birch, 2014) of bath sonication were sufficient to obtain the maximum level of antioxidant compounds.

Nevertheless, more than assessing both factors independently, the TPC value was influenced by the combination of time and amplitude, which interaction was significant ($p < 0.05$, Table 4). Oregano leaves required 10 minutes and an amplitude of 80% (Rout et al., 2021) while olive mill leaves, 8.33 minutes and 70% amplitude (Martínez-Patiño et al., 2019). Likewise, the combination of time and amplitude is important for the AOA; however, in this study, the interaction was not significant ($p > 0.05$).

According to Table 5, both models have a significant lack of fit ($p > 0.05$), showing a good predictability of the responses. The coefficient of determination (R^2) for TPC and AOA was 0.82 and 0.55, respectively while the adj-R^2 were 0.77 and 0.43, respectively. TPC value has a good prediction for its R^2 because is over 0.75 (Sousa et al., 2021). Despite the fact AOA presented a low R^2, it is acceptable because of the F-ratio values and the significance of the lack of fit. Previous studies indicated that the models do not fit perfectly with the prediction, but their reliability is not affected (Hossain et al., 2012).

3.3. Determination of the oxidative stability

Table 6 shows the oxidative stability of rapeseed oil fortified with the EWS extracts (30% w/w), presenting the optimum TPC and AOA values. Rapeseed oil samples alone and fortified with a conventional extract were assessed as controls. The oxidative stability is influenced by the oil composition, processing, heat, light, oxygen, free fatty acids, prooxidants, and antioxidants (Madhujith and Sivakanthan, 2019). Previous studies have indicated that it is possible to increase the induction time (IT) of edible oils by adding extracts from pumpkin peel, pomegranate peel, moringa leaves, vine tea, *Fumaria parviflora*, fig pulp, and skin (Jia et al., 2021; More et al., 2022; Razavi and Kenari, 2021; Salami et al., 2020).

As shown in Table 6, the rapeseed oil without any fortification (control) has the lowest oxidation stability with an IT of 9.96 ± 0.20 h. The rapeseed oil, fortified with the conventional extract, achieved a stability increase by 18% ($p < 0.05$). However, better results were obtained with the extracts obtained by UAE. To mix the different extracts with the rapeseed oil, a sonication was carried out, enhancing the transfer of antioxidant components from the extract to the oil (Cisneros-Yupanqui et al., 2021).

The oil fortified with the optimum TPC value extract (0.71% total antioxidant compounds in the oil, w/w) increased the stability by 25% ($p < 0.05$). However, the effect was not significantly different from the one obtained with the conventional extract. These data suggest that the optimum TPC sample prolonged IT, but the phenolic compounds extracted may exert other stronger bioactivities than delaying oil oxidation. Moreover, measuring the TPC with the Folin-Ciocalteau reagent could lead the reaction among polyphenols and other compounds like sugars, proteins, amino acids, carotenoids, and vitamins (Szydłowska-Czerniak et al., 2010), overestimating the TPC values of the extracts employed. The assessment of the samples phenolic profile could be useful to understand the behavior of these compounds. Table 7 shows that the pyrogallol was the only one present in the conventional extract (1.39 ± 0.35 mg/g), which partially explains its low capacity to delay the rapeseed oil oxidation.

Furthermore, this phenolic compound was found in the extract with the optimum TPC value along with phenylacetic acid (11.93 ± 2.09 mg/g), benzoic acid (0.14 ± 0.01 mg/g), protocatechuic aldehyde (0.20 ± 0.04 mg/g) and syringaldehyde (0.07 ± 0.01 mg/g). It is interesting to note that phenylacetic acid is present in a considerable amount in RS-TPC; however, it was not detected either in the conventional extract or in RS-AOA. This could be explained by the stability of this phenolic acid, which has been shown to decrease at an intense agitation (Leuba et al., 1989). Therefore, a higher amplitude applied in RS-AOA could generate the dissociation of the phenylacetic acid into other molecules not detected. In addition, the extract with the optimum AOA contained ferulic acid (0.73 ± 0.01 mg/g) and protocatechuic acid (0.05 ± 0.01 mg/g), unlike the other samples.

On the other hand, the extract with the optimum AOA (0.89% total antioxidant compounds in the oil, w/w) improved the oil stability by 45% ($p < 0.05$), presenting the longest AAI. These results suggest that the AOA is not determined only by polyphenols, but by other compounds. The antioxidant properties of polyphenols depend on the number and position of hydroxyl groups. Molecules with a higher number of hydroxyl groups have a higher antioxidant capacity (Nićiforović and Abramovič, 2014). Although pyrogallol is the molecule with the highest content of hydroxyl groups and it is present in the extract with the optimum TPC, the extract with the optimum AOA contained a richer phenolic profile with more compounds containing hydroxyl groups, so it could have a stronger AOA to delay the oil oxidation. The hydroxyl groups can act in two ways: donating their hydrogen atoms to radicals (primary antioxidants) or chelating free metals (secondary antioxidants) (Gutiérrez-Del-río et al., 2021; Nićiforović and Abramovič, 2014). It is reported that polyphenols have a synergistic effect with other antioxidant compounds, enhancing their

bioactivity. Rapeseed meal has high concentrations of α-tocopherol (Sharaf eldin et al., 2018), which might partially be transferred to the rapeseed EWS due to the high affinity of these compounds to ethanol (Saini and Keum, 2016). In addition, EWS contains 242.05 ± 3.11 μmol/g glucosinolates, which were related to exerting antioxidant properties (Georgiev et al., 2021). The AOA of glucosinolates has preventive effects against diabetes, cardiovascular alterations, bacterial infections, and oxidative stress (Sánchez-Pujante et al., 2017).

3.4.Optimization of the oil fortification

Based on the results obtained in the section 3.3, the optimum AOA extract was chosen to optimize the IT obtained during the oil fortification. The factors considered in this study were the time of homogenizing and the concentration of extract in the oil. Table 8 shows the IT as well as the AAI experimental values, which are within the range of 10.64 – 16.49 h and 1.11-1.67, respectively. Additionally, the corresponding final concentration of total antioxidant compounds in the oil was calculated and included in a different column. Likewise, a row was added for the control, which was obtained when the oil was mixed with water at the maximum concentration (35%, w/w) for 11.4 min.

The polynomial equation obtained is the following (***Equation 4***):

$$IT = 10.4312 + 0.14496 * \text{Time}(x1) + 0.0451163 * \text{Concentration}(x2) - 0.0176605 * x12 + 0.00405072 * x1x2 + 0.0018156 * x22 \quad \textit{(Equation 4)}$$

Table 9 and Table 10 represent the regression coefficients and F-ratios of the IT, showing that only the concentration of extract added in the oil had a positive significant influence ($p < 0.05$) on the variable response (F-ratio 56.46), increasing the oxidative stability. This behavior is confirmed in Figure 3. However, the time of mixing did not present an impact on the IT, as also appreciated in Figure 4. The concentration of antioxidant compounds in the final mixture (extract + oil) could be related to the increase of the IT since a significant Pearson correlation was found between these two variables (r= 0.8924 [p<0.05]). There are other natural antioxidant extracts from sage, savory and borage that were reported to have an AAI of 1.68, 1.06, and 1.12, respectively (Bandoniene et al., 2002). Some studies have been able to confirm that some antioxidant compounds are directly correlated to the induction time, leading to a decrease in the oxidative stability when their concentration is low in the mixture (De Leonardis et al., 2013).

According to Table 10, the model has a significant lack of fit ($p > 0.05$), presenting a good predictability of the responses with a coefficient of determination (R^2) of 0.83 and an adj-R^2 of 0.76.

The theoretical optimum IT (15.39 h) was obtained at 8.12 minutes of mixing when adding 35% of extract, which represents 1.04% of total antioxidant compounds in the oil and reaching a final AAI of 1.61 (taking as a control the IT of 9.56h from Table 8). This value is significantly higher than the 1.45 obtained before the optimization, indicating the success of this process. To verify the validity of the regression model, the equations have been tested experimentally, achieving an IT of 15.27 h with an AAI of 1.60. Since the difference was less than 20% compared to the theoretical values, the prediction was accurate, in agreement with the literature (Aourach et al., 2021; Drevelegka and Goula, 2020).

Moreover, the fortification of rapeseed oil with the synthetic antioxidant BHT (0.02%), which is stable at high temperatures and soluble in oil (Yehye et al., 2015), improved its stability by 6%. This AAI was similar to the one obtained in a previous research, where BHT delayed the rapeseed oil oxidation by 9% (Cisneros-Yupanqui et al., 2021). In both cases, the AAI exerted by this synthetic antioxidant was significantly lower than the one obtained during the optimization and similar to the one reached when rapeseed oil has been fortified with 0.03% of total antioxidant compounds, as shown in the run 7 in Table 8 (AAI, 1.11). Natural antioxidants are usually employed in a higher concentration than the synthetic ones since they are not pure substances, so the active fraction is lower than the addition in the mixture (Pokorný, 2007). Therefore, the rapeseed EWS extract could substitute totally or partially BHT, as previously suggested when employing other natural extracts (Taghvaei and Jafari, 2015). It represents a strategy for employing a novel food waste in the replacement of synthetic additives, which have been related to toxicological issues (Fadda et al., 2022).

4. Conclusions

This study was focused on the recovery of bioactive compounds from rapeseed EWS by using UAE. This emerging technology enhanced the extraction, obtaining higher values of TPC and AOA than the ones with the conventional method. Combinations of amplitude and time of 10% - 9.24 min and 15% - 0.76 min were used for obtaining the optimum value of TPC (99.30 mg GAE/g) and AOA (148.99 mg TE/g), respectively. Phenylacetic and ferulic acid were the most predominant phenolic compounds. When rapeseed oil was fortified with the optimum extracts, a significant delay of the oil oxidation ($p < 0.05$) was detected, especially with the optimum AOA, which was chosen to continue with an optimization of the oxidative stability, in terms of the IT. Mixing rapeseed oil with the optimum AOA extract at a final total antioxidant compounds concentration of 1.04% in oil (w/v) for 8.12 minutes could be a promising way to upcycle EWS while reducing the use of synthetic additives. Further studies regarding the costs of this technique might be conducted for a

better and more complete evaluation of the procedure. The sensory evaluation of the oil fortified with ultrasound-assisted extracts from the EWS should be further considered as well.

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Table 1. Emerging technologies employed to extract phenolic compounds

Emerging extraction technologies	Mechanism	Advantages	Disadvantages	Examples	References
Microwave Assisted Extraction (MAE)	-Based on the formation of high-energy electromagnetic waves that can change the solvent's molecular rotation and ionic mobility without altering the sample -The heat generated facilitates the diffusivity of solvents in the sample to improve the diffusion of target phytochemicals out of the sample	-Phenolic compounds absorb microwave energy -Offers an improved performance, low solvent consumption, and energy-saving combined with high automation	-MAE has not been found useful in the extraction of polymeric polyphenols such as anthocyanins and tannins due to the possibility that this technology destroys polyphenols with different hydroxyl-type and heat-sensitive substituents -The main problems in using this technique for polyphenol extraction include the necessity to carefully select the type of material, solvent, application time of the microwaves, and the operating temperature	-MAE obtained a TPC of 102.04 mg GAE/g in 10 min, whereas Soxhlet extraction obtained a TPC of 73.54 mg GAE/g in 480 min in *Vernonia amygdalina* -Along with UAE, MAE uses shorter extraction times than PLE and SFE -Exhibited a high performance to extract TPC in lime peel waste than other emerging technologies, only lower than UAE	(Alara et al., 2021, 2018; de la Calle and Costas-Rodríguez, 2017; Huang et al., 2017; Pinela et al., 2016; Rodsamran and Sothornvit, 2019; Yahya et al., 2018)
Pressurized Liquid Extraction (PLE)	-Employs high pressures from 3.3 to 20.3 MPa in combination with high temperatures (between 40 and 200 °C) to facilitate the desorption and solubility of molecules in solvents	-Better use of water as an extraction solvent -Allows a faster rapid extraction and reduces the solvent consumption	-The use of high pressures could compact the raw materials, avoiding the correct contact between matrix and solvent, and decreasing the recovery of phenolics compounds -The interference during the extraction process -The high level of dilution of the extracts, especially when using different numbers of cycles -The need for advanced instrumentation which is an expensive process	-Along with MAE, PLE requires the highest temperatures -PLE enhanced the extraction yield of flavanols from cocoa shells by increasing the temperature and extraction time. However, higher values of both variables degraded the procyanidins B2	(Alara et al., 2021; Okiyama et al., 2018; Osorio-Tobón, 2020; Pimentel-Moral et al., 2018)

Supercritical fluids extraction (SFE)	-Requires the use of supercritical carbon dioxide ($SC\text{-}CO_2$) -Allows the employment of low pressure and temperature, making it a common method in laboratory-scale structures	-Higher extraction capacity -Increased penetration of solvent into samples -Possibility of reusing the CO_2 at the end of the extraction process	-Phenolic compounds are not fully soluble in $SC\text{-}CO_2$, the use of co-solvents being necessary to extract these bioactive compounds -SFE requires the longest extraction times in comparison to other emerging technologies	-Depending on the natural source, extracts with TPC between 1.4 mg GAE/g and 113 mg/g extract have been obtained with this technology -The use of ethanol as co-solvent increased the extraction of TPC in cacao pod husks.	(Alara et al., 2021; De Zordi et al., 2014; Lampakis et al., 2021; Osorio-Tobón, 2020; Valadez-Carmona et al., 2018)

Table 2. Total phenolic compounds (TPC) and antioxidant activity (AOA) obtained from the ultrasound-assisted and conventional extraction

Sample	TPC (mg GAE/g)	AOA (mg TE/g)
Conventional extraction	79.25 ± 9.30^{b}	99.28 ± 13.10^{b}
Ultrasound-assisted extraction	86.08 ± 7.06^{a}	110.81 ± 9.65^{a}

Results are expressed as mean ±standard deviation (n = 3). Different letters in the same column indicate statistically significant differences ($p < 0.05$), according to ANOVA (one-way) and the LSD test.

Table 3. Total phenolic compounds (TPC) and antioxidant activity (AOA) during the optimization of the ultrasound-assisted extraction by the $2^2 + 2$ full factorial Central Composite Face design of experiments at different experimental conditions: Amplitude (x_1), time (x_2)

Treatment	x_1 Amplitude (%)	x_2 Time (min)	TPC (mg GAE/g)	AOA (mg TE/g)
1	10	2	83.98 ± 0.30	110.34 ± 1.84
2	10	8	96.64 ± 0.32	101.06 ± 6.24
3	15	5	92.75 ± 1.06	125.81 ± 4.91
4	23	0.76	88.10 ± 3.91	99.86 ± 0.67
5	23	5	88.35 ± 1.48	92.26 ± 4.08
6	23	5	88.30 ± 1.54	98.83 ± 9.76
7	23	9	86.76 ± 0.47	102.03 ± 1.18
8	35	2	84.93 ± 4.45	113.38 ± 5.51
9	35	8	83.18 ± 1.70	106.29 ±11.98
10	40	5	75.18 ± 2.95	98.26 ± 9.17
Conventional extraction	0	0	79.25 ± 9.3	99.28 ± 13.1

Results are expressed as mean ±standard deviation (n = 3)

Table 4. Regression coefficients and *F*-ratio of the predicted second- order polynomial models for

	TPC		AOA	
	Regression coefficients	*F*-ratio	Regression coefficients	*F*-ratio
Constant	85.2777	-	123.172	-
A:Amplitude	-0.0832305	213.36*	3.49712	43.68*
B:Time	2.26873	55.55*	-3.21046	20.99*
AA	Ns	Ns	-0.108217	27.39*
AB	-0.0661024	31.06*	Ns	Ns

TPC and AOA

*$p < 0.05$, ns: not significant

Table 5. Analysis of variance for the response surface quadratic models of TPC and AOA

	TPC				AOA			
	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Lack-of-fit	13	103.88	7.99	5.05*	13	8121.8	624.75	8.84*
Pure error	2	3.17	1.58	-	2	141.39	70.70	-
Total (corr.)	19	5881.88	-	-	19	18222.5	-	-
R^2	-	0.82	-	-	-	0.55	-	-
Adj-R^2	-	0.77	-	-	-	0.43	-	-

*$p > 0.05$

Table 6. Influence of the extracts from rapeseed ethanol-wash solutes (EWS) on oxidative stability of rapeseed oil measured by the Rancimat® test

Sample	Induction time (h)	Antioxidant activity index (AAI)
RO	9.96 ± 0.20^{c}	1.00
RO+conventional extract	11.57 ± 0.19^{b}	1.18
RO+optimum TPC	12.25 ± 0.56^{b}	1.25
RO+optimum AOA	14.25 ± 0.69^{a}	1.45

RO, Rapeseed oil; RO+optimum TPC, RO+extract with the optimum value of total phenolic compounds; RO+optimum AOA, RO+extract with the optimum value of antioxidant activity.

Data are expressed as the mean ± standard deviation (n = 3). Different letters in the same column indicate statistically significant differences ($p < 0.05$), according to ANOVA (one-way) and the LSD test.

Table 7. Phenolic profile of extracts prepared from rapeseed ethanol-wash solutes (EWS)

Phenolic compound (mg/g DM)	RS	RS-TPC	RS-AOA
Pyrogallol	1.39 ± 0.35[b]	2.56 ± 0.48[a]	ND
Phenylacetic acid	ND	11.93 ± 2.09	ND
Benzoic acid	ND	0.14 ± 0.01[a]	0.15 ± 0.01[a]
Protocatechuic aldehyde	ND	0.20 ± 0.04	ND
Syringaldehyde	ND	0.07 ± 0.01[a]	0.06 ± 0.01[a]
Ferulic acid	ND	ND	0.73 ± 0.01
Protocatechuic acid	ND	ND	0.05 ± 0.01

DM, dried matter; RS, sample conventionally extracted, RS-TPC, sample extracted with the optimum value of total phenolic compounds; RS-AOA, sample extracted with the optimum value of antioxidant activity; ND, not detected.

Data are expressed as the mean ± standard deviation (n = 3). Different letters in the same row indicate statistically significant differences ($p < 0.05$), according to ANOVA (one-way) and the LSD test.

Table 8. Induction time (IT) and antioxidant activity index (AAI) during the optimization of the fortification of rapeseed oil with an extract from rapeseed meal ethanol-wash by the 2^2 + 2 full factorial Central Composite Face design of experiments at different experimental conditions: time of homogenizing (x_1), concentration of extract in oil (x_2)

Runs	x_1 Time (min)	x_2 Concentration of extract (%)	Corresponding concentration of total antioxidant compounds (%)	Induction time (h)	AAI
1	10	30	0.89	13.36 ± 0.08	1.36 ± 0.01
2	3	30	0.89	13.17 ± 0.39	1.32 ± 0.02
3	6.5	18	0.54	11.57 ± 0.16	1.19 ± 0.00
4	3	6	0.18	11.01 ± 0.16	1.13 ± 0.02
5	6.5	35	1.04	16.49 ± 1.33	1.67 ± 0.14
6	10	6	0.18	11.01 ± 0.23	1.13 ± 0.01
7	6.5	1	0.03	10.64 ± 0.22	1.11 ± 0.01
8	1.5	18	0.54	12.66 ± 0.28	1.30 ± 0.02
9	11.4	18	0.54	12.52 ± 0.29	1.28 ± 0.01
10	6.5	18	0.54	13.17 ± 0.18	1.36 ± 0.01
Control	11.4	0	0	9.56 ± 0.05	1.00

Results are expressed as mean ±standard deviation (n = 3)

Table 9. Regression coefficients and *F*-ratio of the predicted second- order polynomial models for Induction time (IT)

	Induction time	
	Regression coefficients	*F*-ratio
Constant	10.431	-
A: Time	0.145	0.03
B: Concentration	0.045	56.46*
AA	-0.017	0.63
AB	0.004	0.40
BB	0.002	0.97

*$p < 0.05$

Table 10. Analysis of variance for the response surface quadratic models of Induction time (IT)

	Induction time			
	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Lack-of-fit	13	9.74	0.75	0.57*
Pure error	2	2.63	1.32	-
Total (corr.)	21	71.33	-	-
R^2	-	0.83	-	-
Adj-R^2	-	0.76	-	-

*$p > 0.05$

Figure legends

Figure 1. Scheme of the ultrasound-assisted (UAE) and conventional extractions

Figure 2. Response surface for ultrasound-assisted extraction (UAE) of a) total phenolic compounds (TPC) and b) antioxidant activity (AOA)

Figure 3. Response surface for the induction time (IT) in function of time of mixing and concentration of extract in the oil

Figure 4. Pareto chart of Induction time (IT)

Figure 1

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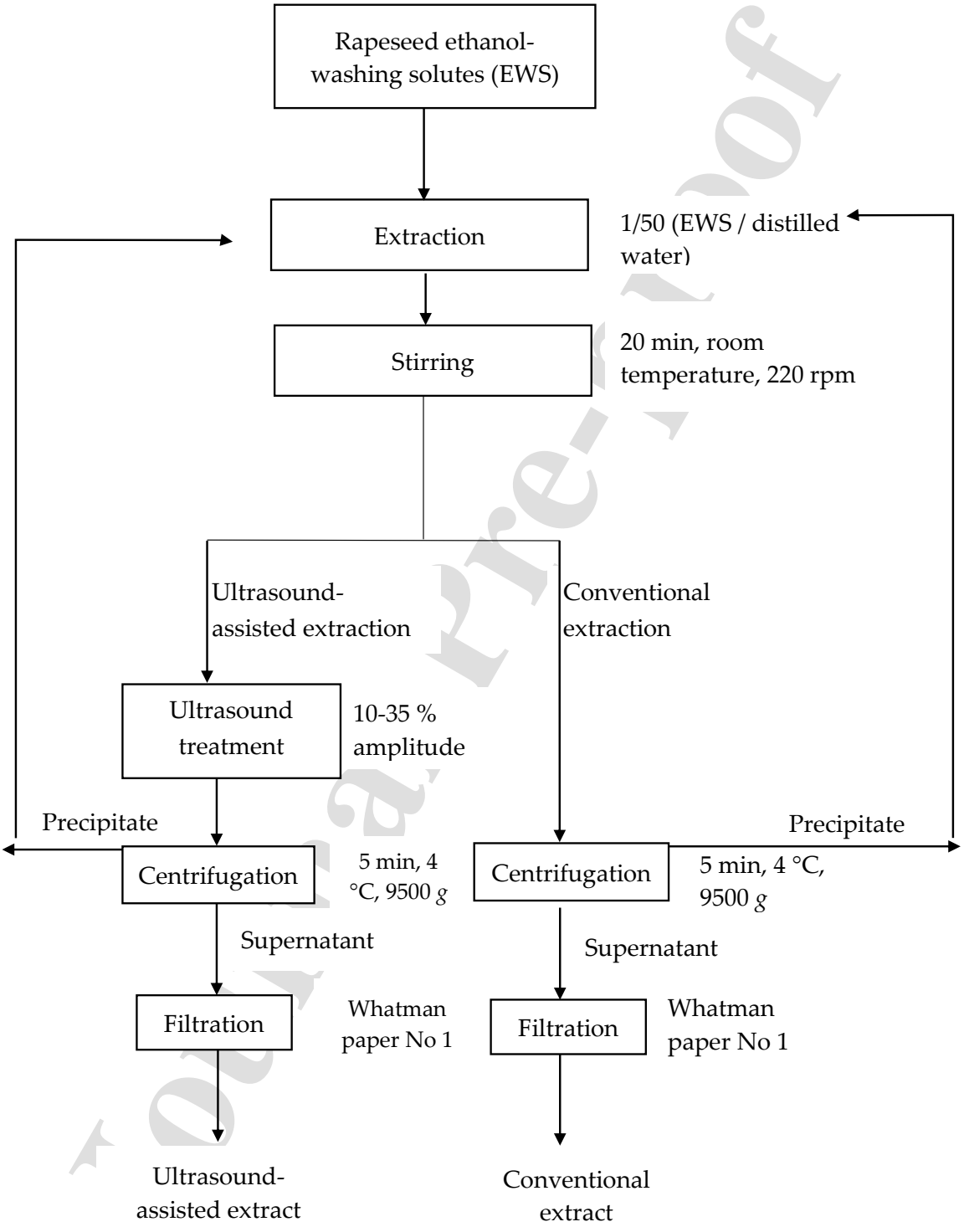

Figure 2

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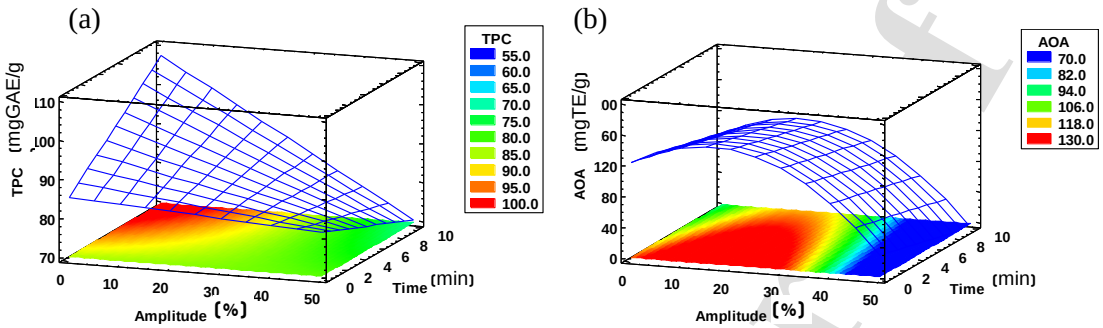
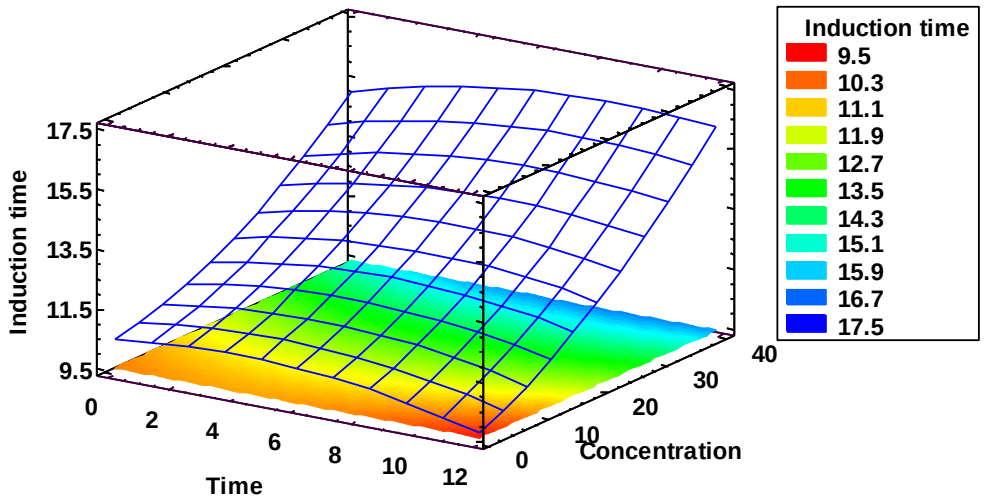
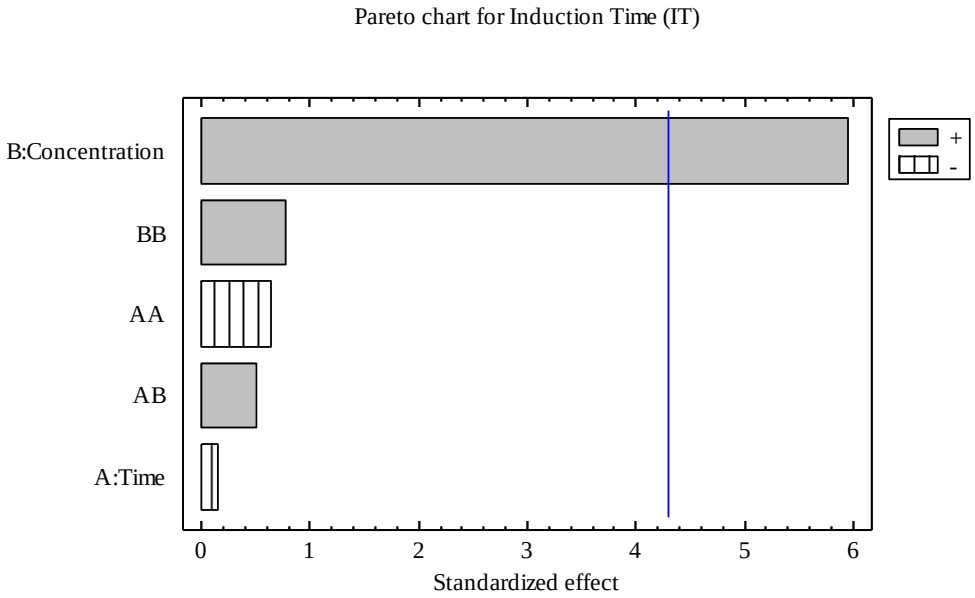
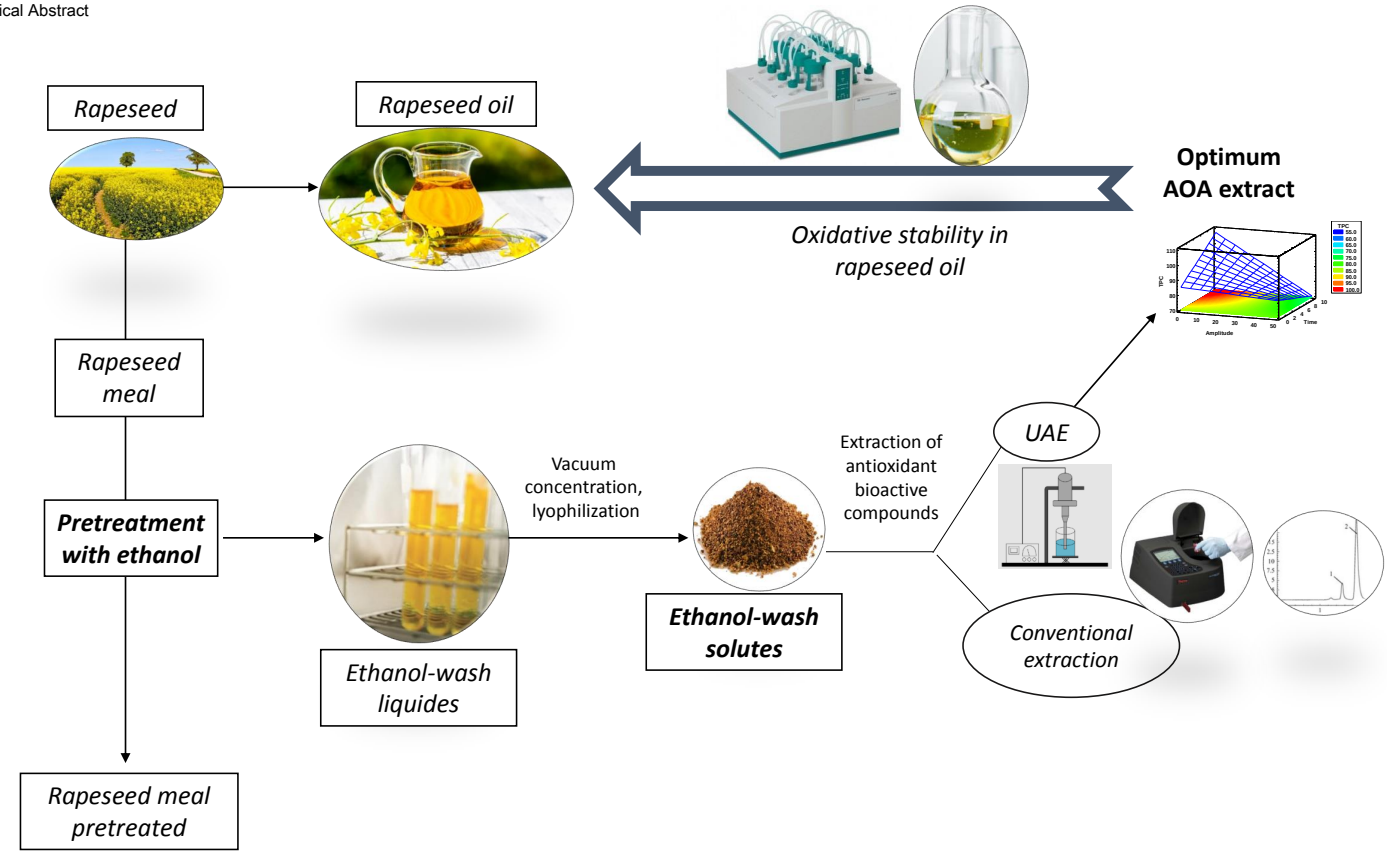






Graphical Abstract



Highlights

- EWS is a waste generated from rapeseed meal when washing it with ethanol.
- An UAE of antioxidant bioactive compounds from EWS was carried out and optimized.
- The optimum TPC and AOA were 99.30 mg GAE/g and 148.99 mg TE/g, respectively
- Fortifying rapeseed oil with the optimum AOA extract delayed the oxidation by 45%.
- The optimum AOA extract from EWS could partially substitute BHT as an antioxidant.

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UNIVERSITÀ DEGLI STUDI DI PADOVA

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This manuscript has not been published, and it is not under consideration for publication elsewhere. In addition, the authors have no conflicts of interest to disclose, and all authors read and approved the manuscript.

Sincerely yours,

Anna Lante (on behalf of my co-authors)

Padova, 28.11.2022

The authors declare no conflict of interest