



## A comparison study between ultrasound-assisted and enzyme-assisted extraction of anthocyanins from blackcurrant (*Ribes nigrum* L.)

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### ABSTRACT

Blackcurrant (*Ribes nigrum* L.) is a fruit rich in vitamins, fatty acids, minerals, essential oils and phenolic compounds, including anthocyanins. In the present work, two anthocyanin extraction methods from blackcurrant samples based on Ultrasound-Assisted Extraction (UAE) and Enzyme-Assisted Extraction (EAE) have been developed. A Plackett–Burman design with seven variables has been preliminary used for both UAE and EAE in order to determine the most influential variables in each methodology. After that, a Box–Behnken design was employed to optimize the extraction methods. The composition of the extraction solvent (% EtOH in water) has been the most influential variable for both UAE and EAE. The optimal extraction times have been 5 min for UAE and 10 min for EAE. No differences have been observed in anthocyanin extraction with both methodologies. Both methods have been applied to blackcurrant-derived products and proven their suitability for quality control analysis.

### Introduction

Red and black fruits are highly appreciated by a large number of consumers around the world for their intense flavour and colour, so that they have been incorporated into a large number of products such as juices, smoothies, jams, or even candies (R. M. Brennan et al., 2014; Lim, 2012). However, their pleasant taste and attractive appearance are not the only reasons for their popularity. Public awareness of the chemical composition of food, the use of additives, colorants, or even adulterants, and their harmful effect on health has notably increased and, as a consequence, consumers' preference for natural and health-promoting foods as regular components of their diets has equally increased. For this

reason, the demand for certain fruits such as grapes, blackcurrants, pomegranates, guavas, plums, blueberries or açai has significantly increased because of the antioxidant properties associated to them (Mohammadi-Moghaddam & Firoozzare, 2021; Popović et al., 2021). Such antioxidant properties are mainly attributable to their high anthocyanin content.

Anthocyanins are secondary metabolites responsible for the red, blue, black and purple colours of different flowers, fruits, and plant tissues (Gharehaghajlou et al., 2021; Shi et al., 2021). Their significantly beneficial biologic and pharmacologic effects as anti-inflammatory, antiviral and antioxidant have been known at least from the 16th century (Castañeda-Ovando et al., 2009; Rodríguez-Rodríguez et al., 2020;

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Sánchez et al., 2020; Zhang et al., 2019). This antioxidant activity has a decisive role in the control of the overproduction of the reactive oxygen and nitrogen species involved in the pathogenesis of numerous chronic diseases, such as cardiovascular diseases, diabetes, or cancers, and consequently they can be used as a form of preventive treatment (Kowalski & Gonzalez de Mejia, 2021; Mazewski et al., 2018).

Blackcurrant (*Ribes nigrum* L.) is a shrub from the order of the Saxifragaceae, that belongs to the gooseberry family (*Grossulariaceae*) (Lim, 2012). It is mainly cultivated throughout Northern Europe, Asia, North America, in the mountainous areas of South America and North-West Africa (R. Brennan, 2008). This fruit has been chosen by many producers, not only for its intense and characteristic flavour, so highly appreciated by many consumers, but also for its high precocity, productivity, and profitability, that ensures high availability levels (Panfilova et al., 2021). Blackcurrant is rich in vitamins, fatty acids, minerals, essential oils, anthocyanins, and phenolic compounds (Krzepiňko et al., 2018). In fact, based on their high bioactive compounds content, blackcurrant fruits have exhibited decisive health-promoting, anti-cancer, antibacterial, and anti-inflammatory properties (Flores & Ruiz del Castillo, 2016) that have made of them a very attractive element in food, cosmetics, pharmacology and medicinal fields.

Multiple techniques have been employed for the extraction of anthocyanins from blackcurrant fruits. Novel methods have been suggested as an alternative to the traditional ones, so that the detrimental effects from high temperatures or long extraction times on the anthocyanins would be avoided and the final extracts would maintain the sensorial attributes of fresh blackcurrants (Aneja et al., 2014).

Ultrasound-Assisted Extraction (UAE) is a technique that benefits from the mechanical effect caused by the phenomenon of cavitation that results from the implementation of ultrasounds (Bruno Romanini et al., 2021; Liu et al., 2021). This combination enhances the transfer of mass from the sample into the solvent and consequently, the extraction of the compounds at low temperature and in shorter extraction times, thus avoiding the degradation of the anthocyanins (Chen et al., 2021). In addition, UAE is an easy method to implement, since it does not require any complex maintenance or expertise. It also uses less solvent than other traditional techniques, which makes it more cost-effective (Cavalaro et al., 2019; El-Shamy & Farag, 2021; Machado et al., 2017). Furthermore, the yields attained through this technique are considerably higher than those achieved by other conventional extraction methods, and may even double the amount of compounds obtained in the extracts (Majid & Silva, 2021). In fact, UAE has already proven its high efficiency for the extraction of phenolic compounds and anthocyanins from natural matrixes such as purple potato, blackberry, açai, lavender, purple onion, etc. (Aliño-González et al., 2020; Carrera et al., 2021; González-de-Peredo et al., 2021).

Enzyme-Assisted Extraction (EAE) is based on the capacity of certain enzymes to degrade or disrupt the cell walls to allow the releasing of the compounds (Akyüz & Ersus, 2021; Meini et al., 2019). Certain enzymes, such as cellulase,  $\alpha$ -amylase,  $\beta$ -glucosidase, protease, xylanase, or pectinase, have been already employed for the extraction of bioactive compounds from plants (Shen et al., 2021). Among all these enzymes, pectinases are the most widely used enzymes in the extraction of natural compounds in plant matrices due to their broad substrate specificity with high stability under extreme conditions (Abdullah et al., 2021; Zohdi & Amid, 2013). EAE exhibits important advantages regarding anthocyanins extraction, since it is a simple process that does not generally require chemical solvents, while low power consumption, time and temperature are required to achieve significant extraction yields (Domínguez-Rodríguez et al., 2021). EAE has been employed for the extraction of phenolic compounds and anthocyanins from multiple matrices such as red dragon fruit, mulberry, monguba, or *Akebia trifoliata* flowers (Phan Van et al., 2020; Zhang et al., 2020).

In the present research work, anthocyanins yields from blackcurrant samples obtained by UAE and by EAE have been compared. For that purpose, the two extraction methods have been first optimized and then

they have been applied to the extraction of bioactive compounds from different blackcurrant samples. Given the multiples applications that the compounds of interest would have in a wide variety of industries, it should be kept in mind that certain environmental aspects are of the utmost importance. In addition, the substantial benefits that UAE and EAE bring about, make them particularly attractive as quality control methods to be employed by different industries or government institutions that intend to ensure the quality and anthocyanins content that can be found in a growing number of products obtained from blackcurrant.

## Materials and methods

### Samples

For the optimization of UAE and EAE, the blackcurrant material was acquired from Good Nutritions (BöhlIggelheim, Germany). The samples consisted in a lyophilized and powdered mixture of different blackcurrant varieties.

### Chemical and solvents

The solvent used for the optimization of the extraction procedures was either methanol or ethanol of HPLC purity acquired from Fisher Scientific (Loughborough, UK) and Milli-Q water obtained by means of a Milli-Q water purification system (Millipore, Bedford, MA, USA). The liquid-liquid mixture at different percentages of the extraction solvent were prepared and then the pH was adjusted using HCl 1 M and NaOH 0.5 M solutions (Panreac, Barcelona, Spain).

For the analysis and quantification of the anthocyanins in the extracts, the following solvents were employed: methanol of HPLC purity (Fisher Scientific, Loughborough, UK) and formic acid of HPLC grade (Panreac, Barcelona, Spain). In addition, cyanidin chloride (95% purity, Sigma-Aldrich Chemical Co., St. Louis, MO, USA), was employed as the reference standard for the quantification of the anthocyanins. The enzyme used for the present study was a pectinase from *Aspergillus niger* (P4716-25KU, Sigma-Aldrich Chemical Co., St. Louis, MO, USA).

### Ultrasound-assisted extraction

#### Ultrasound-assisted extraction equipment

The extraction was performed using a Sonopuls HD 2070.2 processor (20 Hz, 70 W, BANDELIN electronic GmbH & Co KG, Heinrichstrabe, Berlin, Germany), which allows the control of the cycle, the amplitude, and the working time. The probe was fitted into an adjustable double vessel coupled to a thermostatic bath for temperature control (FRIGITERM-10, J.P. Selecta, S.A., Barcelona, Spain).

#### Ultrasound-assisted extraction optimization

A preliminary study on the influence from a number of UAE-related factors on anthocyanin extraction was performed. For that, a Plackett-Burman design was employed, which is a sift design characterized by requiring a short number of experiments that allow a good evaluation of the variables considered within a broad range, while it is assumed that factor interactions are negligible. This design is often suitable for the early experimental phases. Seven variables were selected and two extreme values of each variable were evaluated as follows: extraction time (5 and 30 min), %MeOH in the extraction solvent (25 and 75%), temperature (10 and 60 °C), amplitude (20 and 60% of the maximum amplitude), extraction cycles (0.5 and 1 s<sup>-1</sup>), pH of the solvent (2 and 8) and sample-to-solvent ratio (0.1 and 0.2 g/15 mL). These values were assigned based on the research team's previous experience on similar matrices. The solvent volume was invariably 15 mL for all the runs.

The design was generated from a total of 12 experiments that were performed at random. The extracts obtained were centrifuged twice for 5 min at 1702g. The supernatants were placed into 25 mL volumetric

flasks which were made up to the mark with the same solvent as the one used for the extraction. The extracts were filtered through 0.20  $\mu\text{m}$  nylon syringe filters (Membrane Solutions, Dallas, TX, USA). After that, they were analysed under UHPLC–UV–Vis conditions. The area of the identified anthocyanins was measured and normalized according to the amount of sample weighted. The sum of these normalized areas was taken as the response variable. A summary of the experimental conditions can be found in Table S1.

After that, once the most influential variables had been selected, a Box–Behnken design with response surface methodology (BBD–RSM) was carried out. This kind of design is characterized by the three levels that are assigned to each factor: (–1) as a lowest level, (0) as the intermediate level, and (1) as a the highest level. This means that the axial points are not included, so that a more spherical arrangement of the design points is obtained. Consequently, a smaller number of experiments are required and any extreme conditions are excluded between the experiments. This is of great importance, since any experimental method that may pose an excessive economic or power consumption demand or that might lead to the degradation of the anthocyanins should be discarded.

In this case, the five most influential variables were included in the design and three levels were assigned to each one of them: %MeOH (50,

75, and 100%), temperature (5, 27.5, and 50 °C), amplitude (10, 30, and 50% of the maximum amplitude), pH (2, 4.5, and 8), and sample-to-solvent ratio (0.1, 0.15 and 0.2 g/15 mL). The volume of solvent was setup at a constant 15 mL, the extraction time was established at 5 min and the cycle at 0.9 s<sup>–1</sup>. The selected ranges were based on the research team's previous experience on similar matrices (Aliño-González, Espada-Bellido, et al., 2020; Aliño-González, Jarillo, et al., 2020). A total of 46 experiments with 6 repetitions at the centre point constituted the whole design. All of them were randomly performed. The extracts obtained were centrifuged twice for 5 min at 1702g. The supernatants were placed into 25 mL volumetric flasks and made up to the mark with the same solvent used for the extraction. The extracts were then filtered using 0.20  $\mu\text{m}$  nylon syringe filters (Membrane Solutions, Dallas, TX, USA). Finally, they were analysed under UHPLC–UV–Vis conditions and the sum of the normalized areas were calculated as previously explained to be used as the response variable. A summary of the experimental conditions can be seen in Table 1.

#### Enzyme-assisted extraction

##### Enzyme-assisted extraction equipment

For the EAE, the corresponding grams of lyophilized and powdered

**Table 1**

Conditions and results of the Box–Behnken design used to optimize the UAE procedure for the extraction of anthocyanins from blackcurrant samples.

Experiment	% MeOH	Temperature (°C)	Amplitude (W)	pH	Ratio	Relative area of anthocyanins (measured)	Relative area of anthocyanins (predicted)	Relative error in the prediction (%)
1	75	27.5	50	8	0.15	28,650,700	28,826,300	0.61
2	75	5.0	30	5	0.10	30,183,400	29,815,400	1.23
3	75	27.5	30	8	0.20	30,193,400	28,208,900	7.04
4	75	27.5	30	2	0.10	26,293,400	28,264,000	6.97
5	75	5.0	30	5	0.20	29,607,000	27,427,400	7.95
6	75	27.5	10	5	0.10	29,475,200	28,070,700	5.00
7	50	27.5	30	5	0.20	25,583,400	26,778,800	4.46
8	100	27.5	50	5	0.15	23,922,700	26,592,700	10.04
9	75	50.0	10	5	0.15	30,274,800	28,971,500	4.50
10	100	27.5	30	5	0.10	26,695,900	26,665,900	0.11
11	50	5.0	30	5	0.15	27,699,000	28,005,600	1.09
12	100	27.5	30	8	0.15	28,327,500	25,981,500	9.03
13	75	5.0	50	5	0.15	28,104,600	28,654,200	1.92
14	75	27.5	50	5	0.20	26,588,400	26,949,900	1.34
15	100	27.5	30	5	0.20	25,221,200	25,207,000	0.06
16	75	50.0	30	5	0.10	28,913,900	28,985,100	0.25
17	75	50.0	30	2	0.15	27,057,800	27,027,800	0.11
18	50	27.5	10	5	0.15	29,414,800	27,826,400	5.71
19	50	27.5	50	5	0.15	23,935,600	25,680,700	6.80
20	75	27.5	10	5	0.20	24,589,800	28,004,200	12.19
21	100	27.5	30	2	0.15	27,145,700	24,644,100	10.15
22	75	5.0	30	8	0.15	26,542,200	27,776,100	4.44
23	100	5.0	30	5	0.15	23,782,500	25,316,200	6.06
24	75	27.5	50	5	0.10	33,702,000	29,244,500	15.24
25	100	27.5	10	5	0.15	24,991,000	24,327,400	2.73
26	75	27.5	30	2	0.20	25,503,300	26,450,600	3.58
27	75	27.5	50	2	0.15	25,544,800	26,120,800	2.21
28	75	50.0	30	8	0.15	26,575,300	29,721,900	10.59
29	75	27.5	30	8	0.10	27,717,800	28,756,600	3.61
30	50	27.5	30	2	0.15	27,708,800	26,149,600	5.96
31	50	27.5	30	8	0.15	28,466,600	27,063,000	5.19
32	75	50.0	50	5	0.15	29,693,100	28,072,800	5.77
33	50	27.5	30	5	0.10	26,501,500	27,681,100	4.26
34	75	27.5	10	8	0.15	27,047,200	27,186,400	0.51
35	75	5.0	10	5	0.15	26,769,300	27,635,900	3.14
36	100	50.0	30	5	0.15	25,737,500	27,089,200	4.99
37	75	27.5	10	2	0.15	27,101,600	27,641,200	1.95
38	50	50.0	30	5	0.15	26,862,200	26,986,800	0.46
39	75	5.0	30	2	0.15	30,162,100	28,219,400	6.88
40	75	50.0	30	5	0.20	30,752,300	29,011,900	6.00
41	75	27.5	30	5	0.15	30,042,800	27,468,800	9.37
42	75	27.5	30	5	0.15	25,243,500	27,468,800	8.10
43	75	27.5	30	5	0.15	23,903,200	27,468,800	12.98
44	75	27.5	30	5	0.15	26,993,400	27,468,800	1.73
45	75	27.5	30	5	0.15	28,640,400	27,468,800	4.27
46	75	27.5	30	5	0.15	26,989,400	27,468,800	1.75

samples were mixed with the corresponding amount of solvent and placed into an Erlenmeyer flask. After that, different amounts of enzyme were added to the mixture and placed in an temperature-controlled orbital shaker incubator (Nahita 640/1, 580 W, I.C.T., S.L., Lardero, La Rioja, Spain). The mixture was shaken and heated. The enzyme extraction time was another factor to also be optimized.

#### Enzyme-assisted extraction optimization

First of all, a Plackett–Burman design was built in order to detect the most influential variables in the EAE. Seven variables were preliminary selected and two extreme values were evaluated: extraction time (10 and 60 min), extraction temperature (40 and 60 °C), agitation (50 and 200 rpm), percentage of ethanol in water in the extraction solvent (20 and 60%), pH of the solvent (4 and 6), enzyme amount (100 and 1000 units per gram of sample), and the ratio or relation sample:solvent (0.1:15 and 0.2:15 g:mL). These variables and the range evaluated had been selected according to bibliographic reports (Phan Van et al., 2020; Rezende et al., 2021).

A total of 12 randomly performed experiments were run to generate the design. As for UAE, the extracts were subjected to the same procedure and filtered using 0.20 µm nylon syringe filters. Later, the extracts were analyzed by UHPLC–UV–Vis and the area of the identified anthocyanins were measured and normalized according to the amount of sample weighted. A summary of the experimental conditions has been presented in Table S2.

Once the most influential variables had been selected, a BBD–RSM design was generated. The variables selected and their corresponding ranges were: %ethanol in solvent (10, 35, and 60%), pH of the solvent (4, 5, and 6), temperature (30, 45, and 60 °C), and units of enzyme per gram of sample (100, 550, and 1000 U/g). Consistent 0.2 g samples were added to 15 mL of solvent, agitated at 100 rpm and subjected to extraction for 20 min. The values of the ranges studied were selected according to the research team's previous experience and the bibliography that had been examined. The resulting design comprised 27 experiments with three repetitions at the centre point (Table 2), all of them had been randomly performed. The extracts were subjected to the same process as the one described for UAE and filtered using 0.20 µm nylon

syringe filters. Finally, the extracts were analysed under UHPLC–UV–Vis conditions and the sum of the normalized areas were calculated to be used as the response variable.

#### Identification of anthocyanins by UHPLC–PDA–QToF–MS

First of all, the anthocyanins present in blackcurrant were identified using an ultra–high–performance liquid chromatography system coupled to a photodiode array detector and to a quadrupole time-of–flight mass spectrometer (UHPLC–PDA–QToF–MS) model Xevo G2 (Waters Corp., Milford, MA, USA).

A reverse–phase C18 analytical column (100 Å, 2.1 mm, 1.7 µm, Acquity UPLC BEH C18, Waters) was employed for the analyses. The Phase A used was water with 2% of formic acid, while phase B was 100% MeOH. The flow rate applied was 0.4 mL/min with a chromatographic gradient (%B, time) as follows: 5% B, 0 min; 20% B, 3.30 min; 30% B, 3.86 min; 40% B, 5.05 min; 55% B, 5.35 min; 60% B, 5.64 min; 95% B, 5.94 min; 95% B, 7.50 min. This implies a 12–minute total analysis time including the 4 min allowed to return to the initial conditions. With respect to the mass analyses, a positive ionization method was applied. The desolvation gas and the source temperatures were 500 °C and 150 °C respectively. The capillary cone was set at 700 V, and the cone voltage was 20 V. Finally, the desolvation gas and the cone gas flow were set at 700 L/h and 10 L/h respectively. The trap collision energy was 4 eV. Full–scan mode in a 100–1200 *m/z* range was employed for the identification of the anthocyanins. The mass chromatograms of the identified anthocyanins can be seen in Fig. S1. The following major anthocyanins [M] + were identified: delphinidin 3–O–glucoside (*m/z* 465), delphinidin 3–O–rutinoside (*m/z* 611), cyanidin 3–O–glucoside (*m/z* 449), cyanidin 3–O–rutinoside (*m/z* 595), petunidin 3–O–rutinoside (*m/z* 625), pelargonidin 3–O–rutinoside (*m/z* 579), peonidin 3–O–rutinoside (*m/z* 509). The identified anthocyanins were in agreement with those described by other authors (Nour et al., 2013).

#### Separation and quantification of the anthocyanins by UHPLC–UV–Vis

The separation and quantification of the anthocyanins from

**Table 2**

Conditions and resulting Box–Behnken design used to optimize the EAE procedure for the extraction of anthocyanins from blackcurrant samples.

Experiment	% Ethanol	pH	Temperature (°C)	Enzyme U / g sample	Relative area of anthocyanins (measured)	Relative area of anthocyanins (predicted)	Relative error in the prediction (%)
1	10	4	45	108	158,658,876	171,098,000	7.27
2	60	4	45	108	110,490,629	118,975,000	7.13
3	10	6	45	108	137,277,073	141,796,000	3.19
4	60	6	45	108	129,792,680	130,357,000	0.43
5	35	5	30	24	150,684,398	164,092,000	8.17
6	35	5	60	24	140,464,383	153,649,000	8.58
7	35	5	30	240	153,236,000	153,015,000	0.14
8	35	5	60	240	142,140,174	141,777,000	0.26
9	35	5	45	108	154,396,502	157,782,000	2.15
10	10	5	45	24	160,779,605	162,797,000	1.24
11	60	5	45	24	128,011,328	122,319,000	4.65
12	10	5	45	240	137,224,584	140,141,000	2.08
13	60	5	45	240	124,016,650	122,026,000	1.63
14	35	4	30	108	176,258,440	168,370,000	4.69
15	35	6	30	108	159,359,918	156,120,000	2.08
16	35	4	60	108	152,462,078	154,328,000	1.21
17	35	6	60	108	142,144,635	148,658,000	4.38
18	35	5	45	108	157,311,039	157,782,000	0.30
19	35	4	45	24	159,002,689	145,545,000	9.25
20	35	6	45	24	152,767,023	143,308,000	6.60
21	35	4	45	240	144,157,374	142,714,000	1.01
22	35	6	45	240	122,086,724	123,189,000	0.89
23	10	5	30	108	179,771,488	173,611,000	3.55
24	60	5	30	108	143,040,124	147,143,000	2.79
25	10	5	60	108	183,903,811	168,172,000	9.35
26	60	5	60	108	136,545,049	131,077,000	4.17
27	35	5	45	108	161,638,688	157,782,000	2.44

blackcurrant samples was conducted by means of an Elite UHPLC LaChrom System (Hitachi, Tokyo, Japan). This equipment is fitted with an L-2200U autosampler, an L2300 column oven, as well as two L-2160U pumps. A reverse-phase C18 column was used (2.1 × 50 mm and 2.6 μm, Phenomenex, Kinetex, CoreShell Technology, Torrance, CA, USA), and the column oven was set up at 50 °C constant temperature. The solvent A was water containing 5% formic acid, and the solvent B was pure methanol. The solvents were finally filtered using 0.22 μm filters (RephiLe Bioscience, Ltd., Shanghai, China) and degassed by means of an ultrasonic bath (Elma S300, Elmasonic, Singen, Germany) to remove impurities and bubbles. The flow rate applied was 0.7 mL/min and the injection volume was 15 μL. The chromatographic gradient (%B, time) was as follows: 2% B, 0.00 min; 2% B, 1.50 min; 15% B, 3.30 min; 15% B, 4.80 min; 35% B, 5.40 min; 100% B, 6 min. This system was coupled to a UV-Vis detector L-2420U, which was set at 520 nm for quantification purposes. This method allowed to separate in less than seven minutes the seven major anthocyanins that were successfully identified (Fig. S2).

Cyanidin chloride, with a coefficient of regression of  $R^2 = 0.9999$  and a calibration curve defined as  $y = 300,568.88x - 28,462.43$  was the reference standard used. The limits of detection (LOD) and quantification (LOQ) were 0.198 mg L<sup>-1</sup> and 0.662 mg L<sup>-1</sup>, respectively. Finally, the normal distribution of the residuals was evaluated based on Shapiro-Wilk tests. The W value obtained was 0.8514 (very close to 1), and the *p*-value was 0.803 (above 0.05), which confirms hypothesis H<sub>0</sub>. Assuming similar absorbance levels of the anthocyanins in the study and considering the individual molecular weight of each anthocyanin, the cyanidin chloride curve was employed to quantify the anthocyanin content in the blackcurrant extracts.

#### Statistical software

Statgraphic Centurion (version XVII) (Statgraphics Technologies, Inc., The Plains, VA, USA) and Minitab (version X) (Minitab LLC, State College, PA, USA) were the statistical software applications employed to generate the Plackett-Burman and BBD-RSM designs and for the later evaluation of the responses that had been obtained.

## Results and discussion

### Ultrasound-assisted extraction

#### Optimizing the UAE conditions

First of all, a Plackett-Burman test was performed with the aim of identifying the variables with the most relevant influence on the extraction process. Seven variables (temperature, %MeOH in the solvent, extraction time, amplitude, cycle, pH, and ratio) within the ranges that have been already indicated were preliminary selected. A total of 12 extractions were carried out under their corresponding individual conditions (Table S1) on the lyophilized blackcurrant samples. All the extracts were analyzed by HPLC-UV-Vis and the total anthocyanins were quantified. The results revealed that none of the variables studied were significantly influential on the concentration of total anthocyanins content in the extracts, since their *p*-values were greater than 0.05 in all the cases. Furthermore, two variables exhibited *p*-values clearly above 0.5; namely, extraction time (*p*-value: 0.7898), and cycle (*p*-value 0.7776), which clearly indicated that they hardly affected the content of anthocyanins in the blackcurrant extracts.

Once the five most influential variables had been determined, a BBD-RSM design was generated using such variables, namely % MeOH, temperature, amplitude, pH, and ratio. 46 randomly performed extractions were completed to generate the design. The volume of solvent remained invariable at 15 mL, the cycle was also consistently 0.9 s<sup>-1</sup> and the total extraction time was 5 min in every case. The total area obtained under such conditions was quantified and the measured and predicted values were correlated (Table 1). The prediction average error was

4.96%, ranging from 0.06% up to 15.24%. In addition, the model obtained exhibited an R-squared statistic of 0.6. A lack-of-fit analysis was performed to determine the linearity of the model and the result was 0.07 ( $F = 1.69$ ), which means that the developed model was linear and that the differences between the predicted and the real values were not relevant. Then, a *t*-test was performed by Minitab at 95% confidence level to calculate the *p*-values corresponding to each of the variables. The variables that presented *p*-values lower than 0.05 were considered as significantly influential.

None of the variables exhibited *p*-values below 0.05, which suppose that any of them were significantly influential. However, when the Pareto chart was graphically represented (Fig. 1) it could be observed that the square interaction of the percentage of MeOH, the percentage of MeOH, the quadratic interaction of the temperature, the ratio, the pH and the interaction of the temperature and the amplitude, with *p*-values lower than 0.5, were influential on the extraction of anthocyanins from blackcurrant samples.

The coefficients of the second-order polynomial equation to calculate the area of the anthocyanins extracted were obtained from the analysis of the BBD-RSM design, which resulted in the Equation 1:

$$Y = 3.38 \cdot 10^7 + 225007.0X_1 - 278538.0X_2 - 126076.0X_3 - 853664.0X_4 - 7.65 \cdot 10^7 X_5 - 2276.9X_1^2 + 1240.8X_1X_2 + 2205.5X_1X_3 + 1413.4X_1X_4 - 111326.0X_1X_5 + 1587.6X_2^2 - 1065.0X_2X_3 + 11620.0X_2X_4 + 536633.0X_2X_5 + 152.7X_3^2 + 12168.0X_3X_4 - 557039.0X_3X_5 - 9578.0X_4^2 + 2.91 \cdot 10^6 X_4X_5 + 2.14 \cdot 10^8 X_5^2$$

In this equation, X<sub>1</sub> represents the %MeOH in the solvent, X<sub>2</sub> the extraction temperature, X<sub>3</sub> the amplitude, X<sub>4</sub> the pH and X<sub>5</sub> the sample-to-solvent ratio. The optimal conditions for the UAE were: 0.1 g of sample per 15 mL of solvent; 65% of MeOH in H<sub>2</sub>O; pH 4.97; 5 °C temperature and 50% amplitude. These conditions were in agreement with those previously established by other studies on the extraction of anthocyanins from similar matrices (Aliño-González et al., 2020; González-de-Peredo et al., 2021).

#### Optimizing the extraction time

Once the optimum conditions had been established, the optimum extraction time was to be determined. For that aim, several extractions were performed using different extraction times, between 2 and 25 min, under the previously established optimum conditions. All the analysis were performed in triplicate. The relative area of the anthocyanins extracted from each run has been illustrated in Fig. 2A. As can be observed, the maximum recovery was achieved when the extraction time used was 5 min, with a significant difference when compared to the rest of the extraction times, which means that this was the extraction time to be selected as optimal. Longer extraction times, between 10 and 25 min, exhibited lower relative area values, which is most probably attributable to the degradation of the anthocyanins due to mechanical or chemical processes (Prakash Maran et al., 2013).

#### Repeatability and intermediate precision

The repeatability and intermediate precision of the developed method was still to be determined in order to evaluate its accuracy and reliability to detect and quantify the contents of anthocyanins in the extracts. For repeatability evaluation purposes, nine extractions were completed on the same day under the established optimum conditions (including 5 min extraction time). On the other hand, for the evaluation of its intermediate precision, nine extractions were completed on three consecutive days, which made a total of 27 extractions ( $n = 9 + 9 + 9$ ). The Residual Relative Standard Deviation (RSD) was employed to evaluate both aspects. The RSD of the method's repeatability was 3.71%, whereas the RSD corresponding to its intermediate precision was 4.53%. Both of them were below 5%, which would confirm a high repeatability and intermediate precision level of the optimized UAE method for the extraction of anthocyanins from blackcurrant samples.

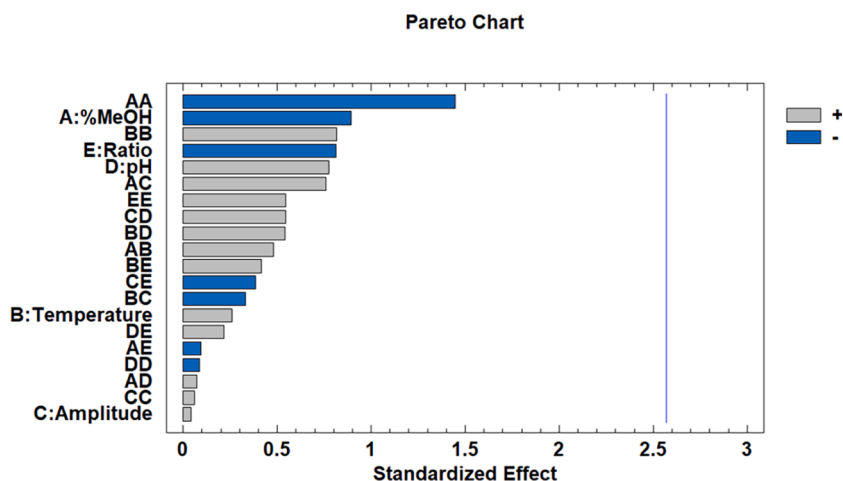


Fig. 1. Pareto chart of the BBD-RSM design corresponding to the extraction of anthocyanins from blackcurrant samples. A: %MeOH in the solvent; B: extraction temperature; C: amplitude; D: pH; E: sample-to-solvent ratio.

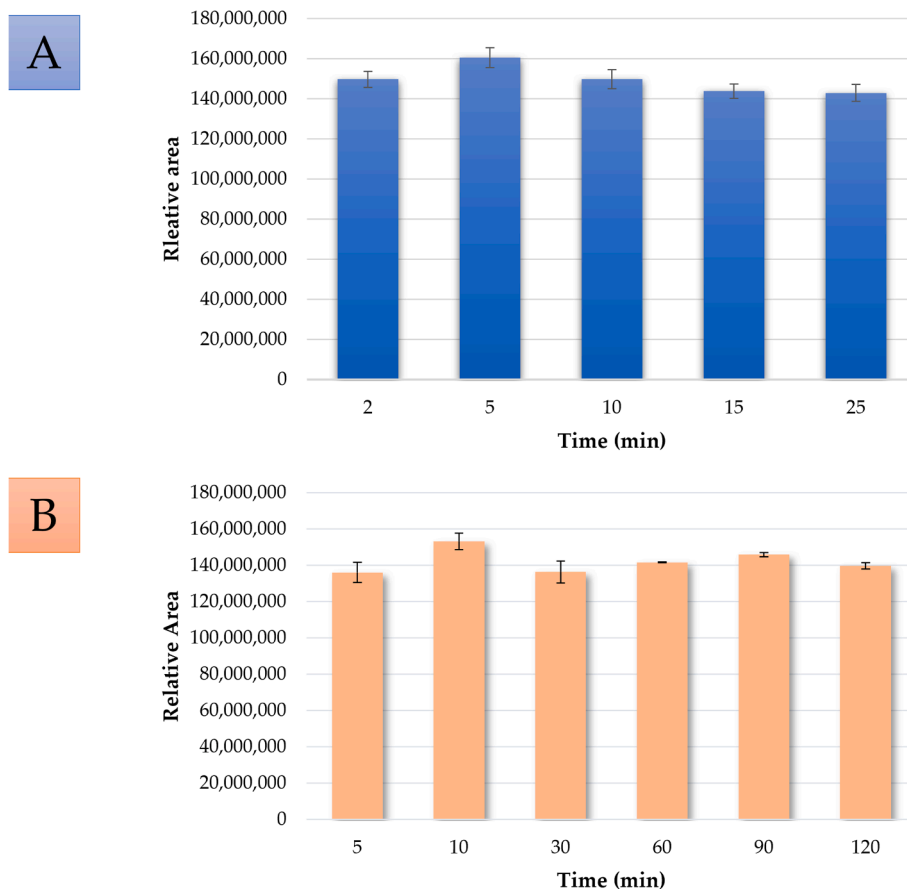


Fig. 2. Average anthocyanin recoveries using different extraction times ( $n = 3$ ) by (A) Ultrasound-Assisted Extraction or by (B) Enzyme-Assisted Extraction.

Enzyme-assisted extraction

Optimizing the EAE conditions

A Plackett-Burman design was generated in order to determine the most influential variables on the extraction of anthocyanins from blackcurrant samples. Seven variables were preliminarily considered for their evaluation (extraction time, %ethanol in the solvent, extraction temperature, pH, agitation, sample-to-solvent ratio, and units of enzyme per gram of sample). A total of 12 extractions were performed on the lyophilized blackcurrant samples. The experiments were randomly

carried out and a summary of the conditions can be seen in Table S2. The extracts were then filtered and analysed by UHPLC-UV-Vis.

The relative total area was quantified for each run and the results were statistically analysed. It was revealed that, like in the previous case, none of the variables was significantly influential. However, some variables showed  $p$ -values lower than 0.5, which would indicate their actual influence on the extractions' outcome: Units of enzyme ( $p$ -value: 0.3943), % ethanol in the solvent ( $p$ -value: 0.4314), extraction temperature ( $p$ -value: 0.3149), and pH of the solvent ( $p$ -value: 0.4456). These variables were selected to generate the BBD-RSM design.

The BBD–RSM design based on the four previously mentioned variables was generated after a total of 27 randomly performed extractions with three central points. Each sample was invariably formed by 0.2 g of lyophilized plant material in 15 mL of solvent, agitated at 100 rpm and subjected to extraction for 20 min. The total area of the anthocyanins was quantified and the measured and predicted values were correlated (Table 2). The average predictive error was 3.69%, ranging from 0.14% up to 9.35%. The regression model exhibited an R-squared statistic of 0.82 and a lack-of-fit analysis of 0.35 ( $F = 1.65$ ), which indicates that the predicted and the actual measured values did not differ significantly.

Following the BBD-RSM design, a *t*-test at 95% confidence was conducted to determine the influence of the variables and their interactions on the extraction of anthocyanin from blackcurrant. The results have been graphically represented in a Pareto chart (Fig. 3). As can be observed, only the percentage of ethanol in the solvent (*p*-value: 0.0007) had a significant influence on the extraction of anthocyanins.

The following second-order polynomial equation (Equation 2) was obtained to calculate the area of the anthocyanins extracted:

$$Y = 1.29 \cdot 10^8 - 1.50 \cdot 10^6 X_1 + 5.74 \cdot 10^7 X_2 - 3.35 \cdot 10^6 X_3 + 3.2 \cdot 10^5 X_4 - 15275 X_1^2 + 406839 X_1 X_2 - 7085 X_1 X_3 + 2071 X_1 X_4 - 7.68 \cdot 10^6 X_2^2 + 109685 X_2 X_3 - 40021 X_2 X_4 + 30069 X_3^2 - 123 X_3 X_4 - 915 X_4^2$$

In this equation,  $X_1$  represents the %EtOH in the solvent,  $X_2$  the pH,  $X_3$  the extraction temperature, and  $X_4$  the units of enzyme per gram of sample. The optimal conditions for the EAEs were as follows: 10% of EtOH in H<sub>2</sub>O; pH 4.1; 30 °C temperature; 50% amplitude and 91.0 units of enzyme per gram of sample. These conditions were in agreement with those reported by other authors regarding their studies on enzyme-assisted extractions from similar matrices (Akyüz & Ersus, 2021; Domínguez-Rodríguez et al., 2021; Shen et al., 2021).

#### Optimizing the extraction time

As in the UAE optimization, the optimum extraction time for EAEs was to be determined. Several extractions were carried out using different extraction times (between 5 and 120 min), using the previously established optimum conditions. All the extractions were conducted in triplicate. The extracts were analyzed and the relative area of the anthocyanins was quantified (Fig. 2B). It was observed that the maximum relative area was achieved when 10 min extraction time was used. Longer extraction times resulted in lesser yields of total anthocyanins.

#### Repeatability and intermediate precision

The repeatability and intermediate precision of the developed EAE method was also evaluated. Similarly to the procedure employed to determine UAE precision, for the repeatability evaluation of the EAE

method, nine runs were completed on the same day under the previously determined optimum conditions. On the other hand, for the intermediate precision test, nine extractions were performed on each of three consecutive days (27 extractions). The RSD obtained for repeatability was 2.46% and the RSD of the intermediate precision was 3.39%. Since both values were below 5%, the high precision of the optimized extraction method had been confirmed.

#### Comparison between anthocyanin extractions by UAE and by EAE

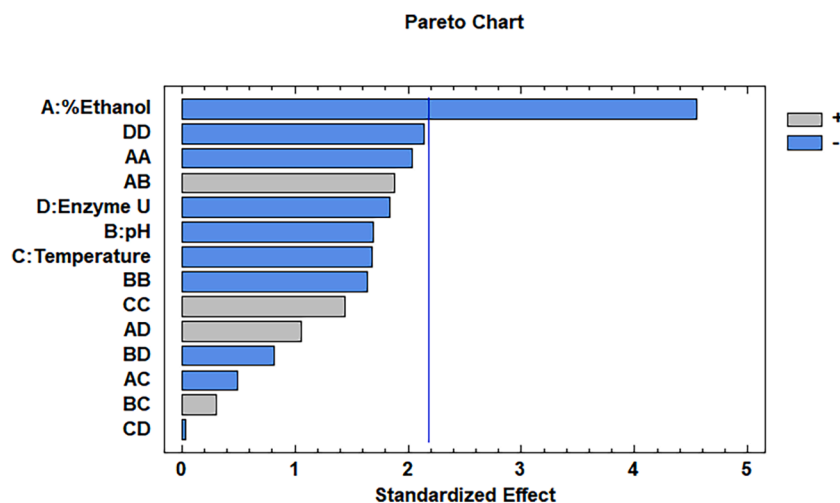
Once the two extraction methods (EAE and UAE) had been fully developed and optimized, they were applied to the same type of samples (frozen-dried blackcurrant). For this purpose, three extractions using each one of the two methods were performed on the same day and applying the same filtration procedure to all the extracts before analyzing them by UHPLC-PDA. The results obtained for both individual and total anthocyanins, using both extraction methods, are shown in Table 3.

No significant differences between the amount of total anthocyanins extracted by UAE and by EAE can be observed in Table 3. It can also be seen that the most abundant anthocyanins in blackcurrant were cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside, accounting for 90% of the total anthocyanins present in blackcurrant. The next most abundant anthocyanins are delphinidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside, accounting for 6 and 5% of the total anthocyanins present in the

**Table 3**

Individual and total anthocyanins yields obtained through EAE and UAE ( $n = 3$ ).

Anthocyanin	mg anthocyanin / g sample (EAE)	mg anthocyanin / g sample (UAE)
Delphinidin	2.12 ± 0.09	2.24 ± 0.14
3- <i>O</i> -glucoside		
Delphinidin	1.23 ± 0.07	1.33 ± 0.09
3- <i>O</i> -rutinoside		
Cyanidin	9.51 ± 0.49	9.07 ± 0.68
3- <i>O</i> -glucoside		
Cyanidin	8.34 ± 0.40	8.26 ± 0.40
3- <i>O</i> -rutinoside		
Petunidin	0.11 ± 0.01	0.33 ± 0.01
3- <i>O</i> -rutinoside		
Pelargonidin	0.24 ± 0.02	0.46 ± 0.02
3- <i>O</i> -rutinoside		
Peonidin	0.10 ± 0.01	0.28 ± 0.01
3- <i>O</i> -rutinoside		
Total	21.64 ± 1.08	21.99 ± 1.20



**Fig. 3.** Pareto chart of the BBD–RSM design corresponding to the extraction of anthocyanins from blackcurrant by EAE. A: %EtOH in the solvent; B: pH; C: temperature; D: units of enzyme per gram of sample.

currant. Finally, petunidin 3-O-rutinoside, pelargonidin 3-O-rutinoside and peonidin 3-O-rutinoside were identified as minority anthocyanins in blackcurrant. It can, therefore, be concluded that both methods are suitable for the extraction of anthocyanins from blackcurrant and to determine the quality of the final extracts in terms of anthocyanin content.

## Conclusion

Over the present research work two extraction techniques based, on the one hand, on the assistance by ultrasounds and, on the other, on the use of enzymes for the extraction and quantification of the anthocyanins in blackcurrant samples have been successfully developed and optimized. Both methodologies have been confirmed to exhibit high precision and repeatability characteristics, with RSDs below 5%. In addition, their applicability to the analysis of lyophilized blackcurrant samples has been demonstrated. Both UAE and EAE methods have display the same efficiency regarding the extraction of the anthocyanins that can be found in blackcurrant.

It is important to highlight the multiple advantages associated to UAE and EAE, because of their short extraction times, their trouble-free operating procedures and their environmentally friendly features, among others. This is, therefore, a development of great importance with regard to the extraction of anthocyanins from blackcurrant, that opens the door to its implementation in a multitude of fields so that the antioxidant, anti-inflammatory or antibacterial properties of these compounds can be properly be exploited for the benefit of all. In addition, the shorter times required and the greater performance attained by these extraction methods also opens the door to their application as extraction as well as quantification procedures in the control analyses that should guarantee the quality of the products derived from blackcurrant.

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## CRedit authorship contribution statement

**María José Aliño González:** Data curation, Formal analysis, Investigation, Writing – original draft. **Ceferino Carrera:** Data curation, Formal analysis, Methodology, Investigation, Writing – original draft. **Gerardo F. Barbero:** Conceptualization, Investigation, Software, Methodology, Supervision, Writing – review & editing. **Miguel Palma:** Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2021.100192>.

## References

- Abdullah, S., Pradhan, R. C., Pradhan, D., & Mishra, S. (2021). Modeling and optimization of pectinase-assisted low-temperature extraction of cashew apple juice using artificial neural network coupled with genetic algorithm. *Food Chemistry*, 339, 127862. <https://doi.org/10.1016/j.foodchem.2020.127862>
- Akyüz, A., & Ersus, S. (2021). Optimization of enzyme assisted extraction of protein from the sugar beet (*Beta vulgaris* L.) leaves for alternative plant protein concentrate production. *Food Chemistry*, 335, 127673. <https://doi.org/10.1016/J.FOODCHEM.2020.127673>
- Aliño-González, M. J., Espada-Bellido, E., Ferreiro-González, M., Carrera, C., Palma, M., Ayuso, J., Álvarez, J. Á., & Barbero, G. F. (2020). Extraction of anthocyanins and total phenolic compounds from Açai (*Euterpe oleracea* Mart.) using an experimental design methodology. Part 2: Ultrasound-assisted extraction. *Agronomy*, 10(3), Article 326. 10.3390/agronomy10030326.
- Aliño-González, M. J., Jarillo, J. A., Carrera, C., Ferreiro-González, M., Álvarez, J. Á., Palma, M., Ayuso, J., Barbero, G. F., & Espada-Bellido, E. (2020). Optimization of a Novel Method Based on Ultrasound-Assisted Extraction for the Quantification of Anthocyanins and Total Phenolic Compounds in Blueberry Samples (*Vaccinium corymbosum* L.). *Foods*, 9(12), Article 1763. 10.3390/foods9121763.
- Aneja, K. R., Dhiman, R., Aggarwal, N. K., & Aneja, A. (2014). Emerging preservation techniques for controlling spoilage and pathogenic microorganisms in fruit juices. *International Journal of Microbiology*, 2014, 1–14. <https://doi.org/10.1155/2014/758942>
- Brennan, R. (2008). Currants and Gooseberries. In J. F. Hancock (Ed.), *Temperate Fruit Crop Breeding* (1st ed., Vol. 1). Springer.
- Brennan, R. M., Caligari, P. D. S., Clark, J. R., Brás de Oliveira, P. N., Finn, C. E., Hancock, J. F., Jarret, D., Lobos, G. A., Raffle, S., & Simpson, D. (2014). Berry Crops. In G. R. Dixon & D. E. Aldous (Eds.), *Horticulture: Plants for People and Places* (2nd ed., Vol. 1, pp. 301–325). Springer.
- Bruno Romanini, E., Misturini Rodrigues, L., Finger, A., Perez Cantuaria Chierito, T., Regina da Silva Scapim, M., & Scaramal Madrona, G. (2021). Ultrasound assisted extraction of bioactive compounds from BRS Violet grape pomace followed by alginate-Ca<sup>2+</sup> encapsulation. *Food Chemistry*, 338, 128101. <https://doi.org/10.1016/j.foodchem.2020.128101>
- Carrera, C., Aliño-González, M. J., Valaityte, M., Ferreiro-González, M., Barbero, G. F., & Palma, M. (2021). A Novel Ultrasound-Assisted Extraction Method for the Analysis of Anthocyanins in Potatoes (*Solanum tuberosum* L.). *Antioxidants*, 10(9), Article 1375. 10.3390/ANTIOX10091375.
- Castañeda-Ovando, A., Pacheco-Hernández, M. d. L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry*, 113(4), 859–871. <https://doi.org/10.1016/j.foodchem.2008.09.001>
- Cavalaro, R. I., Cruz, R. G. da, Dupont, S., de Moura Bell, J. M. L. N., & Vieira, T. M. F. de S. (2019). In vitro and in vivo antioxidant properties of bioactive compounds from green propolis obtained by ultrasound-assisted extraction. *Food Chemistry: X*, 4, Article 100054. 10.1016/J.FOCHX.2019.100054.
- Chen, Zhi-Ling, Wang, Chun, Ma, Haile, Ma, Yanhong, & Yan, Jing-Kun (2021). Physicochemical and functional characteristics of polysaccharides from okra extracted by using ultrasound at different frequencies. *Food Chemistry*, 361, 130138. <https://doi.org/10.1016/j.foodchem.2021.130138>
- Domínguez-Rodríguez, Gloria, Marina, María Luisa, & Plaza, Merichel (2021). Enzyme-assisted extraction of bioactive non-extractable polyphenols from sweet cherry (*Prunus avium* L.) pomace. *Food Chemistry*, 339, 128086. <https://doi.org/10.1016/j.foodchem.2020.128086>
- El-Shamy, Sherine, & Farag, Mohamed A. (2021). Novel trends in extraction and optimization methods of bioactives recovery from pomegranate fruit biowastes: Valorization purposes for industrial applications. *Food Chemistry*, 365, 130465. <https://doi.org/10.1016/j.foodchem.2021.130465>
- Flores, G., & Ruiz del Castillo, M. L. (2016). Accumulation of anthocyanins and flavonols in black currants (*Ribes nigrum* L.) by pre-harvest methyl jasmonate treatments. *Journal of the Science of Food and Agriculture*, 96(12), 4026–4031. <https://doi.org/10.1002/jsfa.7595>
- Ghareaghajlou, Nazila, Hallaj-Nezhadi, Somayeh, & Ghasempour, Zahra (2021). Red cabbage anthocyanins: Stability, extraction, biological activities and applications in food systems. *Food Chemistry*, 365, 130482. <https://doi.org/10.1016/j.foodchem.2021.130482>
- González-de-Peredo, A. V., Vázquez-Espinosa, M., Espada-Bellido, E., Carrera, C., Ferreiro-González, M., Barbero, G. F., & Palma, M. (2021). Flavonol Composition and Antioxidant Activity of Onions (*Allium cepa* L.) Based on the Development of New Analytical Ultrasound-Assisted Extraction Methods. *Antioxidants*, 10(2), Article 273. 10.3390/antiox10020273.
- Kowalski, Rebecca, & Gonzalez de Mejia, Elvira (2021). Phenolic composition, antioxidant capacity and physical characterization of ten blackcurrant (*Ribes nigrum*) cultivars, their juices, and the inhibition of type 2 diabetes and inflammation biochemical markers. *Food Chemistry*, 359, 129889. <https://doi.org/10.1016/j.foodchem.2021.129889>
- Krzepiłko A., Prażak R., Skwaryło-Bednarz B, & Świącilo A. (2018). Buds, leaves, and seeds of blackcurrant - Source of bioactive substances with pro-health properties. *Food Science Technology. Quality*, 25(2), 24–33. 10.15193/ZNTJ/2018/115/230.
- Lim, T. K. (2012). *Ribes nigrum*. In Springer (Ed.), *Edible Medicinal and Non-Medicinal Plants* (1st ed., pp. 30–44). Springer.
- Liu, Y.-Y., Sun, W.-H., Li, B.-Z., Shang, N., Wang, Y., Lv, W.-Q., ... Wang, L.-J. (2021). Value-added application of *Platyodon grandiflorus* (Jacq.) A.DC. roots (PGR) by ultrasound-assisted extraction (UAE) process to improve physicochemical quality,



- structural characteristics and functional properties. *Food Chemistry*, 363, 130354. <https://doi.org/10.1016/J.FOODCHEM.2021.130354>
- Machado, A. P. D. F., Pereira, A. L. D., Barbero, G. F., & Martínez, J. (2017). Recovery of anthocyanins from residues of *Rubus fruticosus*, *Vaccinium myrtillus* and *Eugenia brasiliensis* by ultrasound assisted extraction, pressurized liquid extraction and their combination. *Food Chemistry*, 231, 1–10. <https://doi.org/10.1016/J.FOODCHEM.2017.03.060>
- Majid, H., & Silva, F. V. M. (2021). Kanuka bush leaves for Alzheimer's disease: Improved inhibition of  $\beta$ -secretase enzyme, antioxidant capacity and yield of extracts by ultrasound assisted extraction. *Food and Bioprocess Processing*, 128, 109–120. <https://doi.org/10.1016/J.FBP.2021.04.018>
- Mazewski, C., Liang, K., & Gonzalez de Mejia, E. (2018). Comparison of the effect of chemical composition of anthocyanin-rich plant extracts on colon cancer cell proliferation and their potential mechanism of action using in vitro, in silico, and biochemical assays. *Food Chemistry*, 242, 378–388. <https://doi.org/10.1016/J.FOODCHEM.2017.09.086>
- Meini, M. R., Cabezudo, I., Boschetti, C. E., & Romanini, D. (2019). Recovery of phenolic antioxidants from Syrah grape pomace through the optimization of an enzymatic extraction process. *Food Chemistry*, 283, 257–264. <https://doi.org/10.1016/J.FOODCHEM.2019.01.037>
- Mohammadi-Moghaddam, T., & Firoozzare, A. (2021). Investigating the effect of sensory properties of black plum peel marmalade on consumers acceptance by Discriminant Analysis. *Food Chemistry: X*, 11, 100126. <https://doi.org/10.1016/J.FOCHX.2021.100126>
- Nour, V., Stampar, F., Veberic, R., & Jakopic, J. (2013). Anthocyanins profile, total phenolics and antioxidant activity of black currant ethanolic extracts as influenced by genotype and ethanol concentration. *Food Chemistry*, 141(2), 961–966. <https://doi.org/10.1016/J.FOODCHEM.2013.03.105>
- Panfilova, O., Tsoy, M., Golyaeva, O., Knyazev, S., & Karpukhin, M. (2021). Agrometeorological and morpho-physiological studies of the response of red currant to abiotic stresses. *Agronomy*, 11(8), Article 1522. 10.3390/agronomy11081522.
- Phan Van, M., Tran Duc, D., Thi Thanh, H. D., & Chi, H. T. (2020). Comparison of ultrasound assisted extraction and enzyme assisted extraction of betacyanin from red dragon fruit peel. *E3S Web of Conferences*, 187, Article 04004. 10.1051/E3SCONF/202018704004.
- Popović, Boris M., Blagojević, Bojana, Kucharska, Alicja Z., Agić, Dejan, Magazin, Nenad, Milović, Maja, & Serra, Ana Teresa (2021). Exploring fruits from genus *Prunus* as a source of potential pharmaceutical agents – In vitro and in silico study. *Food Chemistry*, 358, 129812. <https://doi.org/10.1016/j.foodchem.2021.129812>
- Prakash Maran, J., Manikandan, S., Thirugnanasambandham, K., Vigna Nivetha, C., & Dinesh, R. (2013). Box-Behnken design based statistical modeling for ultrasound-assisted extraction of corn silk polysaccharide. *Carbohydrate Polymers*, 92(1), 604–611. <https://doi.org/10.1016/j.carbpol.2012.09.020>
- Rezende, Yara Rafaella Ribeiro Santos, Nogueira, Juliete Pedreira, Silva, Tafs Oliveira Matos, Barros, Romy Gleyse Chagas, Oliveira, Christean Santos de, Cunha, Grazielle Costa, ... Narain, Narendra (2021). Enzymatic and ultrasonic-assisted pretreatment in the extraction of bioactive compounds from Monguba (*Pachira aquatic* Aubl) leaf, bark and seed. *Food Research International*, 140, 109869. <https://doi.org/10.1016/j.foodres.2020.109869>
- Rodríguez-Rodríguez, E., Sánchez-Prieto, M., & Olmedilla-Alonso, B. (2020). Assessment of carotenoid concentrations in red peppers (*Capsicum annuum*) under domestic refrigeration for three weeks as determined by HPLC-DAD. *Food Chemistry: X*, 6, 100092. <https://doi.org/10.1016/J.FOCHX.2020.100092>
- Sánchez, B. A. O., Celestino, S. M. C., de Abreu Gloria, M. B., Celestino, I. C., Lozada, M. I. O., Júnior, S. D. A., de Alencar, E. R., & de Lacerda de Oliveira, L. (2020). Pasteurization of passion fruit *Passiflora setacea* pulp to optimize bioactive compounds retention. *Food Chemistry: X*, 6, Article 100084. 10.1016/J.FOCHX.2020.100084.
- Shen, D., Kou, X., Wu, C., Fan, G., Li, T., Dou, J., ... Zhu, J. (2021). Cocktail enzyme-assisted alkaline extraction and identification of jujube peel pigments. *Food Chemistry*, 357, 129747. <https://doi.org/10.1016/J.FOODCHEM.2021.129747>
- Shi, Jiang, Simal-Gandara, Jesus, Mei, Jufen, Ma, Wanjun, Peng, Qunhua, Shi, Yali, ... Lv, Haipeng (2021). Insight into the pigmented anthocyanins and the major potential co-pigmented flavonoids in purple-coloured leaf teas. *Food Chemistry*, 363, 130278. <https://doi.org/10.1016/j.foodchem.2021.130278>
- Zhang, Lixia, Fan, Gongjian, Khan, Muhammad Ammar, Yan, Zheng, & Beta, Trust (2020). Ultrasonic-assisted enzymatic extraction and identification of anthocyanin components from mulberry wine residues. *Food Chemistry*, 323, 126714. <https://doi.org/10.1016/j.foodchem.2020.126714>
- Zhang, L., Wang, L., Zeng, X., Chen, R., Yang, S., & Pan, S. (2019). Comparative transcriptome analysis reveals fruit discoloration mechanisms in postharvest strawberries in response to high ambient temperature. *Food Chemistry: X*, 2, 100025. <https://doi.org/10.1016/J.FOCHX.2019.100025>
- Zohdi, N. K., & Amid, M. (2013). Optimization of extraction of novel pectinase enzyme discovered in red pitaya (*Hylocereus polyrhizus*) peel. *Molecules*, 18(11), 14366–14380. <https://doi.org/10.3390/molecules181114366>