Selection of Conditions of Ultrasound-Assisted, Three-Step Extraction of Ellagitannins from Selected Berry Fruit of the *Rosaceae* Family Using the Response Surface Methodology

Milczarek Agnieszka¹ · Sójka Michał¹ · Klewicki Robert¹

Received: 16 September 2019 / Accepted: 30 April 2020 / Published online: 2 June 2020
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Abstract

The study examined the influence of the method of grinding the fruit—homogenisation and grinding using a cryogenic mill—on the extraction efficiency of the ellagitannins (ETs). Two methods of extraction support were compared: sonication and shaking. In the second stage, selection of the extraction with the best conditions was carried out using the response surface methodology (RSM). Ellagitannins were extracted in different variants of acetone concentration (40, 60, and 80%), duration of sonication (5, 10, and 15 min), and temperature (20, 35, and 50 °C). The extracts obtained were subjected to quantitative analysis by high-performance liquid chromatography with a diode-array detector (HPLC–DAD) and identification using a mass spectrometer. Grinding is an important stage in the extraction of ellagitannins from berries. Extracts from fruit shredded with a cryogenic mill were characterised by an average of 20% lower content of ellagitannins than extracts from homogenised fruit. Studies using RSM showed that the most important factor affecting the amount of extracted ellagitannins from all tested fruits is the concentration of acetone. The highest ETs concentration was obtained using 80% acetone solution (in the tested range 40–80%). In the case of raspberries, strawberries, and wild strawberries, under the conditions used, the temperature and duration of sonication did not significantly affect the extraction efficiency. An interaction between extraction time and solvent concentration was observed for blackberries, indicating optimal conditions for this fruit as 15 min and an acetone concentration of 80%, respectively.

Keywords Ellagitannins · Extraction · Homogenisation · *Rosaceae* · RSM

Introduction

In recent years, interest has been focused on ellagitannins (ETs), a group of compounds belonging to polyphenols (Rao et al. 2010; Feldman et al. 2003). ETs are monomeric and oligomeric hydrolysable tannins with a complex structure, whose mass ranges from 500 to 3000 Da (Lekha and Lonsane 1997; Rao and Snyder 2010; Feldman et al. 2003; Niemetz and Gross 2005). One characteristic of ETs is the ester bond between the hexahydroxydiphenyl acid (HHDP) molecule and the saccharide, most commonly glucose (Lei et al. 2001). Hydrolysable tannins occur naturally in nature (Rao and Snyder 2010; Feldman et al. 2003). Plants of the *Rosaceae* family contain significant amounts of polyphenols

(including ETs) (Hakkinen & Torronen, 2000). Due to the wide consumption, availability, and high economic importance of berries, they are the main source of ETs in the human diet (Rao and Snyder 2010; Perez-Jimenez et al. 2011).

The fruits of the *Rosaceae* family, which are characterised by a high content of ETs, comprise raspberry, blackberry, wild strawberry, and strawberry (Hager et al. 2008; Gasperotti et al. 2010; Kahkonen et al. 2012; Del Rio et al. 2010; Boyles and Wrolstad 1993; Wu and Prior 2005). In the fruit of raspberries and blackberries, characteristic ETs, which constitute up to 80% of their total content, are lambertianin C and sanguin H-6 (Gasperotti et al. 2010; Beekwilder et al. 2005); while in strawberry and wild strawberry, this is agrimoniin (about 20–40% of total ETs) (Vrhovsek et al. 2012) (Fig. 1).

ETs have an anti-inflammatory effect, inhibit the growth of *Helicobacter pylori*, and reduce gastric ulcers (Enrico Sangiovanni et al. 2013). In addition, they show strong antioxidant properties, which is attributed to the large number of hydroxyl groups present in their structure (Kahkonen et al. 2012; Dias et al. 2015; Heinonen et al. 1998; Oszmiański and Wojdyło 2009). From the plum tree (*Spondias mombin*),

✉ Milczarek Agnieszka
agnieszka.milczarek@p.lodz.pl

¹ Institute of Food Technology and Analysis, Lodz University of Technology, ul. Stefanowskiego 4/10, 90-924 Lodz, Poland

Fig. 1 The structure of the main ETs and ellagic acid of the Rosaceae fruit plants. (1) Sanguin H-6. (2) Lambertianin C. (3) Agrimoniin. (4) Ellagic acid

an ET was isolated that exhibits antiviral activity against two viruses—*Coxsackie* and *Herpes simplex* (Corthout et al. 1991). It was also shown that the berries are anticancerous, thanks to their ability to stimulate apoptosis of colon cancer cells (Larrosa et al. 2010; Dai and Mumper 2010; Seeram et al. 2006) and inhibit cervical cancer cell proliferation (Ross et al. 2007). In addition, ETs have chelating properties, scavenge free radicals (thereby reducing oxidative stress), and they prevent chronic inflammation (Larrosa et al. 2006).

A large proportion of ellagitannins is closely related to the plant cell wall. This is mainly due to the ability of ETs to bind to basic compounds, high molecular weight compounds, proteins, metal ions, or pigment compounds (Okuda et al. 1992). Therefore, it is often impossible to make an accurate ETs assessment both quantitatively and qualitatively. It is important to correctly indicate the concentration of ellagitannins in

fruits. Determining the exact ellagitannin profile will allow further research into their health-promoting effects. According to the literature, ETs were determined mainly on the basis of the amount of ellagic acid released during acid hydrolysis (Wada and Ou 2002; Siriwoharn and Wrolstad 2004; Klimczak and Król 2010).

Extraction is the first step on the path to quantitative and qualitative characterisation of the fruit profile of ellagitannins. Effective extraction of ETs from plant material is influenced by a number of factors, including temperature of extraction, use of supporting processes (e.g. sonication, shaking), and the degree of fragmentation of raw material (Galvan d'Alessandro et al. 2012). Extraction is supported by the use of ultrasound (Galvan d'Alessandro et al. 2012; Chandrapala et al. 2013). This technique is widely used in the extraction of polyphenolic compounds from plant material (Vilku et al. 2007;

Baqueiro-Pena and Guerrero-Beltran 2017). Sonication causes mechanical disintegration of the cell wall (Guerrero et al. 2001; Bashi et al. 2012; Vilku et al. 2007). As a result, the contact area of the plant material with the solvent increases, which may promote increased extraction efficiency (Ma et al. 2008; Galvan d'Alessandro et al. 2012; Chandrapala et al. 2013). Analytical methods using ultrasound are environmentally friendly and are not very expensive (Khan et al. 2010).

For effective extraction, it is also important to select the right concentration of the extractant (Medina-Torres et al. 2017). The pH of the solvent has a significant influence on the extraction of polyphenols. It is advisable to use acidified solvents due to the denaturation of plant cell walls and higher polyphenol stability at low pH (Salminen 2003).

The RSM method is a hybrid of mathematics and statistics; it is an extremely precise tool for determining the relationship between the parameters studied and for studying the interactions that occur between them (Bezerra et al. 2008). The advantages of RSM and the Box-Benken tool include the fact that this method determines a reduced number of experiments to ascertain the interaction between many variables (García-Marquez et al. 2012; Bezerra et al. 2008). Simultaneously, this method is less labor-intensive and time-consuming, and the results obtained are presented in a clear, transparent form. This method is increasingly used to determine dependencies in food (Lee et al. 2006).

To date, the selection of ETs extraction conditions from *Rosaceae* berries assisted with ultrasound has not been presented with the simultaneous use of the homogenisation process. Therefore, the purpose of the work was to indicate the significance of the initial treatment of the raw material as the first stage of preparing the fruit for extraction. An equally important aim was to determine the optimal parameters (time, temperature, acetone concentration) of ETs extraction from selected berries of the *Rosaceae* family.

Materials and Methods

Fruits

Four kinds of berry fruits of the *Rosaceae* family were selected for the study—raspberry (*Rubus idaeus* L.), garden blackberry (*Rubus fruticosus* L.), strawberry (*Fragaria grandiflora*), and wild strawberry (*Fragaria vesca*). These fruits are characterised by a varied, high content of ETs. Frozen fruit came from the Cajdex warehouse (Łódź, Poland). The fruit species have been identified by the supplier. Until the beginning of the tests, they were stored in tightly sealed polyethylene bags in a freezer at -20°C .

Chemicals

All reagents used for ETs extraction were of high purity. Solvents used for chromatography were of HPLC grade. Ultra-clean water was obtained with a Hydrolab-HLP5 demineraliser (Straszyn, Poland). Acetone (99.8% purity) was purchased from Chempur (Piekary Śląskie, Poland), acetonitrile (LC-MS grade) and phosphoric acid (85% purity) from J.T. Baker (Deventer, The Netherlands), and formic acid from Sigma-Aldrich Chemie (Steinheim, Germany). Ellagic acid (> 95% purity) was purchased from Extrasynthese (Genay, France).

Ellagitannin standards were used, which were obtained and purified (Sójka et al. 2016) at the Institute of Food Technology and Analysis of Lodz University of Technology. The standards were characterised by HPLC (210 nm). The purity of the standards was higher than 90%.

The Impact of the Grinding Process on the Extraction of Raspberry ETs

Frozen raspberry fruit (ca. 50 g) in liquid nitrogen (ca. 100 mL) were ground with an IKA Basic cryogenic grinder (Staufen, Germany). Two gram portions of ground fruit were placed into 7-mL polyethylene tubes, followed by a three-stage extraction process using a 60% aqueous acetone solution acidified with 0.1% formic acid. The extraction consisted of pouring into the weighed portion of fruit 3 mL of acidified acetone, sonication in an IS-4 ultrasonic bath with 950 W power (Olsztyn, Poland), or shaking in an IKA TSS 2 laboratory shaker at 150 RPM (Staufen, Germany). Subsequently, the samples were centrifuged for 15 min at 14000g using an MPW-260R centrifuge (Warsaw, Poland). The obtained extract was decanted from the precipitate. The residue was subjected to the same extraction twice. The obtained extracts after three stages were dropped into one volumetric flask and filled up with an extractant to a volume of 10 mL.

Extraction with the additional step of grinding was carried out in an analogous manner, with the difference that after the first sinking of the solvent from the sludge, the fruit was subjected to a 1-min homogenisation process at the fourth power level out of six available (using an IKA T10 basic homogeniser with a power of 125 W (Staufen, Germany)).

List of Variants:

1. Sonication-assisted extraction (15 min)—SON
2. Sonication-assisted extraction (15 min) with homogenisation of the sample (1 min)—SON/HOM
3. Extraction with shaking (15 min)—SH
4. Extraction with shaking (15 min) with homogenisation of the sample (1 min)—SH/HOM

Each variant was made in triplicate. The extracts obtained in this way were diluted 1:1 with mobile phase A and centrifuged at 12000g in an MPW-260R centrifuge (Warsaw, Poland). The supernatant was placed in a chromatography vial and subjected to HPLC analysis.

Selection of ETs Extraction Conditions with Response Surface Methodology

In order to select conditions for the most effective extraction, variant number 2 (extraction supported by sonication with homogenisation of the sample) was selected.

Liquid fruit samples frozen in liquid nitrogen were ground in an IKA Basic cryogenic mill (Staufen, Germany). The fruit was subjected to three-stage extraction with acetone assisted with sonication using an IS-4 ultrasonic bath (Olsztyn, Poland) (in the same way as in point 2.3). The parameters that were chosen are the concentration of aqueous acidified (0.1% formic acid) extractant solution (acetone 40, 60, and 80%), temperature (20, 35, and 50 °C), and duration of sonication (5, 10, and 15 min).

To select the best conditions for three-stage extraction of ETs, the RSM method was used with Statsoft Statistica (v. 7.0.61.0 EN). Independent variables, i.e. acetone concentration, temperature, and duration of sonication, were designated as X₁, X₂, and X₃, respectively (Table 1). The dependent value—the answer—was the sum of the ellagitannins, i.e. the combined content of ellagitannins and ellagic acid [mg/100 g fresh weight (f.w.)]. The RSM test was designed on three levels using the Experimental Design Tool. The parameters were adjusted with the help of the Box-Benken design tool. The program matched 15 different experiments, of which

the first 12 related to the vertices of the studied area, while the last three included central values of independent variables. The experiments were performed three times, and the presented results are their average value.

The second-order polynomial model was used to analyse ET extracts. The analysis of the surface area of the response of independent factors (X₁, X₂, X₃) on the efficiency of extraction measured with their concentration [mg/100 g f.w.] was determined by the equation:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j$$

where β_0 , β_i , β_{ii} , β_{ij} —regression coefficients, X₁–X₃—independent variables (temperature, time, acetone concentration).

The results were based on a statistical analysis of variance. Each parameter was tested, determining the probability at 0.05. The measurement precision for the whole experiment was also determined based on repeatable centre points. The CV (coefficient of variance) was determined from the formula:

$$CV = \frac{SD}{\bar{X}} \times 100\%$$

where SD is the standard deviation, and $\bar{X}$ is the average value for repeatable centre points.

Analysis of Ellagitannins Content by HPLC

Quantitative analysis was performed using a Smartline Knauer liquid chromatograph (Berlin, Germany). The set consisted of a degasser (Manager 5000), two pumps (P1000), a PDA detector (2800), an autosampler (3950), and a thermostat. Gemini C18 110 A columns with dimensions of 250 nm × 4.6 mm, 5 μm (Phenomenex, Torrance, CA) were used for the separation of the ETs. The flow rate was 1.25 mL/min and the injection volume was 20 μL. The column temperature was 35 °C. A PDA detector with a wavelength of 250 nm was used. The distribution proceeded in a gradient system with the following phases: phase A—0.05% aqueous phosphoric acid and phase B—aqueous solution of 83% acetonitrile acidified with 0.05% phosphoric acid solution. The proportion of phases in the gradient was as follows: 0–5 min - 5% phase B, 95% phase A; 5–10 min - 5–15% phase B, 95 - 85% phase A; 10–35 min - 15–40% phase B, 85 - 60% phase A; 35–40 min - 40–73% phase B, 60 - 27% phase A; 40–44 min - 73% phase B, 27% phase A; 44–46 min - 73 - 5% phase B, 27–95% phase A; and 46–54 min - 5% phase B, 95% phase A.

Standard curves were constructed for ellagic acid, lambertianin C, sanguin H-6, and agrimoniin (in the range of 0.5–300, 0.5–225, 0.5–200, and 0.5–100 mg/L, respectively). The concentration of lambertianin D was calculated on the basis of the lambertianin C standard curve, the concentration

Table 1 Combinations of parameters - Box-Benken design

Acetone concentration [%] X ₁ ^a	Time [min] X ₂	Temperature [°C] X ₃
40 (– 1) ^b	5 (– 1)	35 (0)
80 (1)	5 (– 1)	35 (0)
40 (– 1)	15 (1)	35 (0)
80 (1)	15 (1)	35 (0)
40 (– 1)	10 (0)	20 (– 1)
80 (1)	10 (0)	20 (– 1)
40 (– 1)	10 (0)	50 (1)
80 (1)	10 (0)	50 (1)
60 (0)	5 (– 1)	20 (– 1)
60 (0)	15 (1)	20 (– 1)
60 (0)	5 (– 1)	50 (1)
60 (0)	15 (1)	50 (1)
60 (0)	10 (0)	35(0)
60 (0)	10 (0)	35 (0)
60 (0)	10 (0)	35 (0)

^a Levels of factors, ^b Ranges of factors

of sanguiin H-10 and unidentified ETs on the basis of the sanguiin H-6 curve, and the concentration of derivatives and ellagic conjugates on the basis of the ellagic acid curve. The main validation parameters, i.e. LOD (limit of detection) and LOQ (limit of quantification), were determined on the basis of standard curve equations. LOD was the b factor. LOQ was calculated by multiplying the LOD three times (Table 2).

Data were collected using ClarityChrom v. 3.0.5.505 (Berlin, Germany).

Identification of ETs

The ETs were identified in accordance with the methodology described by Sójka et al. (2016). Samples for LC-MS analysis were extracted under medium conditions, i.e. 60% acidified acetone solution, temperature 35 °C, and duration of sonication 10 min. Extraction was carried out three times.

The separation of ETs was carried out using a HPLC chromatograph (Dionex Ultimate 3000) coupled to a DAD detector and a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

For the chromatographic separation, Luna C18 100 Å column with dimensions 250 mm × 4.6 mm, 5 µm (Phenomenex, Torrance, CA) was used. The column was thermostatically regulated at 35 °C. The injection volume was 20 µL, and the flow rate was set to 1 mL/min. The following mobile phases were used: A—1% aqueous formic acid solution; B—80% aqueous acetonitrile solution. The determination was made in a concentration gradient with the following characteristics of the phase participation: 0–6.5 min - 5% phase B, 95% phase A; 6.5–12.5 min - 5 - 15% phase B, 95 - 85% phase A; 12.5–44 min - 15 - 45% phase B, 85 - 55% phase A; 44–45 min - 45–75% phase B, 55 - 25% phase A; 45–50 min - 75% phase B, 25% phase A; 50–52 min - 75 - 5% phase B, 25–95% phase A; 52–65.5 min - 5% phase B, 95% phase A.

The mass spectrometer was equipped with a heated electrospray ion source operating in a negative mode. Mass spectrometer parameters were set as follows: capillary temperature—400 °C, ionic sputtering voltage—4 kV, and evaporator temperature—500 °C. The sheath and auxiliary gas flows were 75 and 20 units, respectively. The detector worked in Full MS mode and in full scan mode MS/dd-MS²

(MS with data-dependent tandem mass spectrometry). In the MS full scan mode, the mass range was set to 200–2000. In the MS/dd-MS² scanning mode, the product's ionic spectra were obtained. For this purpose, the collision energy needed to generate the MS² spectra was set to 20.

The results were collected using Xcalibur (Thermo) software.

Results and Discussion

Identification of ETs from Selected Berry Fruit of the Rosaceae Family

Table 3 shows the identification of the main ETs present in the tested fruits. According to the literature, in the case of raspberry and blackberry, the dominant ETs are lambertianin C and sanguiin H-6 (peaks 6 and 7) (Fig. 2) (Gasperotti et al. 2010; Mullen et al. 2002). In both fruits, there was also a conjugate of ellagic acid with pentose (peak 8) (Gasperotti et al. 2010). The difference in mass (433–301) indicates the possibility of pentose (Gasperotti et al. 2010). In addition, lambertianin D (peak 4) also appeared in the blackberry (Tanaka et al. 1993). A double-charged main ion at m/z 1401 with a simultaneous triple ion m/z 934.07 was identified as lambertianin C (Gasperotti et al. 2010; Sójka et al. 2016). The fragmentation ion m/z 1567.15 derived from the detachment of the rest of HHDP (1869–302). Further fragmentation indicates the detachment of further HHDP residues or glucosyl residues (Mullen et al. 2002). Numerous reports confirm that sanguiin H-6 and lambertianin C are characteristic ellagitannins for raspberry and blackberry (Tanaka et al. 1993; Mullen et al. 2002; Sójka et al. 2016; Hager et al. 2008).

Agrimoniin was identified in strawberry and wild strawberry (peak 9) (Vrhovsek et al. 2012). As unknown, ET was also identified in wild strawberry (peak 1). This compound has already been indicated as an unknown ellagic acid trimer derivative (Bubba et al. 2012).

Lambertianin C and sanguiin H-6 have also been detected in strawberry (Mullen et al. 2002). However, in the polyphenolic profile of the fruits studied in this work (strawberry, wild strawberry), there were no mass peaks corresponding to these

Table 2 Analytical parameters used for quantitative analysis of ellagitannins

ET	Linear range [mg/L]	R ²	LODa [mg/L]	LOQ ^b [mg/L]	System
LC ^c	0.5–225	0.999	0.4	1.3	Smartline Knauer liquid chromatograph, column: Gemini C18 110A (250 nm × 4.6 nm, 5 µm), λ = 250 nm, t = 35 °C
SH-6 ^d	0.5–200	0.999	0.3	1.0	
EA ^e	0.5–300	0.998	0.2	0.7	
AGR ^f	0.5–100	0.998	0.4	1.3	

^a Limit of detection, ^b Limit of quantification, ^c Lambertianin C, ^d Sanguiin H-6, ^e Ellagic acid, ^f Agrimoniin

Table 3 Identification of the ETs in selected berries

Peak	RT [min]	MS data	MS/MS value	MS/MS data	Compound	References	Fruit		
							S ^a	W ^b	R ^c B ^d
1	13.12	[947.05] ⁻¹	947	[902.04] ⁻¹ [901.04] ⁻¹ [883.03] ⁻¹ [871.03] ⁻¹ [599.03] ⁻¹ [301.00] ⁻¹	Unknown ellagic acid trimer derivative	Bubba et al. 2012			+
2	16.91	[1567.15] ⁻¹ [783.07] ⁻²	783	[1235.08] ⁻¹ [936.09] ⁻¹ [633.07] ⁻¹ [469.01] ⁻¹ [301.00] ⁻¹	Sanguin H-10 isomer	Gasperotti et al. 2010			+
3	17.20	[577.14] ⁻¹	577	[451.11] ⁻¹ [425.07] ⁻¹ [407.08] ⁻¹ [301.07] ⁻¹ [289.07] ⁻¹ [125.02] ⁻¹	ET un	-			+
4	21.70	[1245.77] ⁻³ [934.07] ⁻²	1245	[1869.14] ⁻¹ [1567.14] ⁻¹ [1235.07] ⁻¹ [935.08] ⁻¹ [633.08] ⁻¹ [314.98] ⁻¹ [301.00] ⁻¹	Lambertianin D	Tanaka et al. 1993			+
5	22.00	[1401.01] ⁻² [934.07] ⁻³	1401	[1869.15] ⁻¹ [1567.15] ⁻¹ [1235.07] ⁻¹ [935.08] ⁻¹ [633.08] ⁻¹ [301.00] ⁻¹	Lambertianin C iso	Gasperotti et al. 2010			+
6	22.23	[1401] ⁻² [934.07] ⁻³	1401	[1869.15] ⁻¹ [1567.15] ⁻¹ [1401.61] ⁻² [1235.07] ⁻¹ [935.08] ⁻¹ [897.05] ⁻¹ [613.05] ⁻¹ [301.00] ⁻¹	Lambertianin C	Mullen et al. 2002			+
7	23.38	[934.08] ⁻² [1870.16] ⁻¹	934	[1567.15] ⁻¹ [1235.08] ⁻¹ [963.14] ⁻¹ [935.08] ⁻¹ [897.05] ⁻¹ [633.08] ⁻¹ [301.00] ⁻¹	Sanguin H-6	Gasperotti et al. 2010			+
8	25.32	[433.04] ⁻¹	433	[301.00] ⁻¹	Ellagic acid pentose conjugate	Gasperotti et al. 2010			+
9	26.11	[934.08] ⁻²	934	[1085.08] ⁻¹ [936.09] ⁻¹ [935.08] ⁻¹ [897.05] ⁻¹ [783.07] ⁻¹ [633.08] ⁻¹ [613.05] ⁻¹ [301.00] ⁻¹	Agrymonin	Vrhovsek et al. 2012		+	+
10	27.38	[301.00] ⁻¹	301	-	Ellagic acid	Gasperotti et al. 2010		+	+
11	28.40	[514.33] ⁻¹	514	[514.33] ⁻¹ [447.06] ⁻¹ [301.04] ⁻¹ [227.38] ⁻¹ [61.99] ⁻¹	ET un	-			+
12	28.43	[477.07] ⁻¹	477	[345.98] ⁻¹ [301.04] ⁻¹	Methyl ellagic acid hexoside	Bubba et al. 2012		+	+

^a Strawberry, ^b Wild strawberry, ^c Raspberry, ^d Blackberry

Fig. 2 Chromatogram of ellagitannins in selected berry fruits (Smartline Knauer liquid chromatograph, column: Gemini C18 110A, 250 nm × 4.6 nm, 5 μ m, λ = 250 nm, t = 35 °C)

compounds. The presence of sanguin H-6 in strawberry may result from the lack of unambiguous identification of the structure of agrimoniin (Vrhovsek et al. 2012).

The spectra of sanguin H-6 and agrimoniin are very similar to each other because these compounds are composed of the same monomers. In the case of sanguin H-6, in addition to m/z 934.08 (characteristic for both compounds), there is also a peak at m/z 1870.16. The literature reports that sometimes in the spectrum of agrimoniin, it is also possible to observe this peak (Vrhovsek et al. 2012). For correct identification, an accurate analysis of the ion fragmentation is indicated. The low intensity ion at m/z 1567.15 occurs in a typical mass decay for sanguin H-6 (Gasperotti et al. 2010). The weight of 1567.15 comes from the loss of one HHDP residue followed by the transfer of 2H to glucose. The HHDP unit is then transformed to ellagic acid, giving a mass m/z 301.00, which is characteristic for the ETs (Gasperotti et al. 2010). Agrimoniin is characterised by a different profile of mass decay (Vrhovsek et al. 2012). The literature distinguishes between agrimoniin and sanguin H-6 on the basis of a different retention times (26.11 min for agrimoniin). Agrimoniin elutes at a later time during the gradient due to the fact that its polarity is lower. This compound was identified in strawberry and wild strawberry as the primary ellagitannin (peak 8) (Vrhovsek et al. 2012). The occurrence of agrimoniin in strawberry and wild strawberry was confirmed earlier, but its structure was clearly defined in 2012 (Vrhovsek et al. 2012). The occurrence of three isomers of agrimoniin in strawberry leaves (Okuda and Ito 2011) was also indicated.

Sanguin H-10 isomer (peak 2) was also recognised in raspberry, for which the retention time was 16.91 min. In the mass spectrum, the dominant peak was m/z 783.07. Such a mass distribution is also found for bis-HHDP-glucose; however, the characteristic for sanguin H-10 is a double-charged fragment m/z 783.07, which gives a mass of 1557.15 (Tanaka et al. 1993).

The ellagic acid was identified in all the tested fruits of the *Rosaceae* family, which is consistent with the literature (Mullen et al. 2002; Hager et al. 2008; Sójka et al. 2016; Bubba et al. 2012).

The Impact of the Grinding Process on ETs Extraction

The values of total ETs concentrations for different extraction methods were determined based on one-way ANOVA analysis and post-hoc Duncan tests. Raspberry was chosen for the study of the impact of the grinding process on the extraction of ETs because it is a fruit with a well-understood composition of ETs (both quantitative and qualitative) (Sójka et al. 2016). The homogenisation process significantly supports the extraction of ETs from raspberry fruit (Fig. 3). The total content of ETs from fruit further minced with a homogeniser increases on average by more than 20%. During the extraction of fruit chopped only in a cryogenic mill, 145 mg of ellagitannins were extracted from 100 g of fruit, while additional

Fig. 3 Comparison of the influence of extraction aids and homogenisation methods on the content of ETs in raspberry fruit, ^ahomogenisation + sonication, ^bhomogenisation + shaking, ^csonication, ^dshaking, ^esanguin H-6 ^flambertianin C, ^gtotal content of ETs with ellagic acid and conjugates; the results marked with the same roman letter do not differ statistically ($p < 0.05$)

Fig. 4 Photographs of fruit after grinding in liquid nitrogen (LN) and after additional homogenisation (LN + HOM); R raspberry, B blackberry, S strawberry, WS wild stawberry

homogenisation increased this to 190 mg from 100 g of fruit. This is largely due to the fact that the homogeniser mechanically crushes the seeds and degrades the cell wall (Popko 1981). According to a study by Klimczak, 18% of the ellagic acid released during the hydrolysis is in the strawberry seeds, 38% in the pulp, and 44% in the juice (Klimczak et al. 2011). The milling of raspberries only in a cryogenic mill is therefore not a sufficient process to effectively isolate the ETs from the plant matrix. The particle size depending on the grinding method is shown in Fig. 4.

Fragmentation of plant tissue increases the contact area between plant material and solvent (Popko 1981; Galvan d'Alessandro et al. 2012). This is confirmed by research on the extraction of polyphenols from blueberries; the highest efficiency of phenolic compound extraction is obtained for particles with diameter smaller than 0.5 mm (Galvan d'Alessandro et al. 2012). The results obtained by Galvan indicate that a 4-h extraction at 50 °C (without sonication) of finely divided particles (< 0.5 mm) allows results similar to those obtained using ultrasound-assisted extraction for half-cut berries to be obtained (Galvan d'Alessandro et al. 2012). Galvan d'Alessandro et al. (2012) also emphasises the importance of supporting sonication extraction due to its simultaneous shortening of the whole process; 15 min of sonication allowed a higher process efficiency to be obtained compared to 1 h of extraction without support.

The studies also compared the process of shaking and sonication as the main elements supporting extraction of ellagitannins from raspberry fruit. In both cases, the values of extracted ETs range from 145 to 148 mg/100 g f.w., which are comparable values. A higher concentration of extracted ETs was observed for sonication plus homogenisation. Therefore, the sonication and homogenisation processes were tested.

Results of Ellagitannins Extraction Analysis

Further studies for all the analysed fruits were carried out using an additional homogenisation process, due to the higher

extraction efficiency of the ellagitannins. Extraction was supported by the sonication process. The values of ellagitannin concentrations for individual factors were determined based on one-way ANOVA analysis and post-hoc Duncan tests.

Effect of Solvent Concentration

The literature suggests that using acetone concentrations of 60–70% can achieve a much higher polyphenol content in the extract compared to lower or higher acetone concentrations (Salminen 2003). So far, it has been established that the most efficient solvent used to extract ellagitannins is a 70% acidified acetone solution (Salminen 2003; Klimczak and Król 2010). The two main ellagitannins, lambertianin C and sanguin H-6, exhibit stability in acidic solutions (pH 2 and 4) (Sójka et al. 2019). Therefore, the tests were carried out in three different concentrations of acidified acetone, i.e. 40, 60, and 80%.

Studies carried out as part of the work clearly indicate that for the effective extraction of the ETs from the tested fruits of the *Rosaceae* family, the best concentration of acetone in the examined scope was 80%. For blackberry, wild strawberry, and strawberry, extracts with the highest concentration of ellagitannins were obtained using an acidified 80% acetone solution (Fig. 5). When using 80% acetone, 120, 55, and 270 mg of ETs were extracted from 100 g of wild strawberry, strawberry, and blackberry, respectively. In the case of raspberry, the total sum of ETs was 245, 290, and 285 mg/100 g, respectively, using acetone concentrations of 40, 60, and 80%, respectively.

Lower ET values were obtained using lower concentrations of acetone (40 and 60%): 110 mg/100 g for wild strawberry and 45 mg/100 g for strawberry. Similar results were obtained by da Silva Pinto et al. (2008) who also extracted the most tannins hydrolysable from strawberry using 80% acetone. A similar relationship was observed in the presented studies, e.g. for blackberry—the acetone with the highest concentration extracted 270 mg ETs per 100 g of fruit, while in the lowest, extracted 250 mg per 100 g.

Fig. 5 The effect of extraction conditions on the amount of ETs extracted, the results marked with the same roman letter do not differ statistically ($p < 0.05$)

Fig. 6 Plane response plots—dependencies between tested parameters and sum of ETs from blackberry fruit

Too low a concentration of acetone means higher water content, which is not conducive to extraction efficiency (Salminen 2003). By the addition of water with high polarity, the polarity of moderately polar solvents gradually changes. This in turn affects the ability to extract polyphenols (Chirinos et al. 2007; Zhang et al. 2007; Liu et al. 2000). A higher proportion of water favors the extraction of other compounds, such as sugars or proteins (Chirinos et al. 2007).

The Influence of Temperature

ETs are polyphenols that degrade much faster at temperatures of 60–80 °C (Sójka et al. 2019; Chira and Teissedre 2013); therefore, for the present research, the temperature range of 20–50 °C was tested. In addition, the use of temperatures greater than 50 °C may involve the risk of acetone evaporation (the boiling point of acetone is 56 °C), which changes the efficiency of extraction (Garcia-Marquez et al. 2012). The presented studies confirm this relationship; for selected fruits of the *Rosaceae* family (i.e. blackberry, raspberry, strawberry, wild strawberry), the highest ETs concentration in the extracts was recorded when conducting the process at 20 °C (Fig. 5). In the case of raspberry and strawberry, the increase in temperature did not cause an unambiguous increase in the ellagitannins—the values of the extracted ETs in each of the tested temperatures were similar and amounted, respectively, to 270 and 45 mg/100 g f.w. The difference is observed in the

case of blackberry and wild strawberry, where the increase in temperature caused a decrease in ETs concentration. In the case of wild strawberry, the value of ETs at 20 °C was 125 mg/100 g, and at higher temperatures—110 mg/100 g, whereas analysing the extracts from blackberry at 20 °C and higher, respectively, 265 and 250 mg/100 g.

The literature indicates that increasing the temperature (> 25 °C) of extraction may favor the degradation of phenolic moieties and hydroxyl groups in certain polyphenol groups. Interestingly, even those polyphenols that are associated with the fruit matrix can undergo degradation (Akowuah et al. 2009; Davidov-Pardo et al. 2011). On the other hand, this degradation depends largely on the structure of the extracted compounds. In the literature, it was pointed out that the gentle heating of the material to be extracted may favor the change in the properties of plant cells and hence the increased release of polyphenols from fruits (Akowuah et al. 2009).

Duration of Sonication

Sonication contributes to increasing the area of contact of the plant tissue with the solvent. This is due to the formation of bubbles inside the plant cells. Implosion of the bubbles—cavitation—generates a local increase in temperature and pressure (Japon-Lujan et al. 2006). In research, Klimczak and Król (2010) compiled the length of extraction time with the amount of ellagic acid released during hydrolysis. To obtain the greatest

Fig. 7 Plane response plots—dependencies between tested parameters and sum of ETs from raspberry fruit

Table 4 Quantification ETs [mg/100 g f.w.] in extracts from selected berries and precision of the whole experiments

Acetone Concentration [%]	Time [min]	Temperature [°C]	Raspberry			Blackberry			Strawberry			Wild strawberry		
			ET ^a	EAC ^b	ET + EAC ^c	ET	EAC	ET + EAC	ET	EAC	ET + EAC	ET	EAC	ET + EAC
–1 [*]	–1	0	235.3 ± 3.3 ^{**}	6.1 ± 0.4	241.3 ± 3.7	266.0 ± 8.7	7.1 ± 0.5	273.0 ± 9.2	40.8 ± 8.5	4.3 ± 0.5	45.0 ± 8.9	93.1 ± 7.6	14.0 ± 0.8	107.1 ± 8.4
1	–1	0	284.2 ± 2.2	7.8 ± 0.3	292.0 ± 2.5	241.0 ± 3.6	5.6 ± 0.2	246.6 ± 3.8	54.4 ± 8.1	5.6 ± 0.4	60.0 ± 8.5	96.9 ± 5.4	15.4 ± 0.5	112.2 ± 5.9
–1	1	0	251.0 ± 4.8	7.4 ± 0.8	258.4 ± 5.5	242.4 ± 4.8	7.8 ± 0.6	250.1 ± 5.4	41.8 ± 4.9	4.7 ± 0.5	46.5 ± 5.3	90.1 ± 2.0	13.5 ± 0.6	103.7 ± 2.6
1	1	0	270.8 ± 5.5	8.5 ± 0.4	279.3 ± 5.8	278.9 ± 7.6	7.1 ± 0.4	286.0 ± 7.9	48.5 ± 4.3	5.0 ± 0.2	53.5 ± 4.5	91.3 ± 8.4	16.2 ± 0.5	107.5 ± 8.8
–1	0	–1	217.5 ± 8.6	5.7 ± 0.4	223.2 ± 9.0	250.3 ± 8.6	6.0 ± 0.3	256.3 ± 8.8	42.5 ± 3.2	4.6 ± 0.2	47.1 ± 3.4	102.8 ± 5.4	13.6 ± 0.6	116.5 ± 6.0
1	0	–1	261.9 ± 6.1	8.2 ± 0.6	270.0 ± 6.6	283.3 ± 6.2	6.5 ± 0.4	289.8 ± 6.6	42.4 ± 1.8	4.7 ± 0.4	47.1 ± 2.2	122.9 ± 4.6	17.0 ± 0.4	139.8 ± 4.9
–1	0	1	231.3 ± 3.2	7.4 ± 0.6	238.7 ± 3.8	233.7 ± 8.0	5.4 ± 0.4	239.0 ± 8.4	42.5 ± 2.9	5.0 ± 0.2	47.5 ± 3.1	91.9 ± 2.8	13.0 ± 0.6	104.9 ± 3.3
1	0	1	273.8 ± 6.0	9.6 ± 0.8	283.3 ± 6.8	241.5 ± 9.2	6.0 ± 0.5	265.0 ± 9.7	44.4 ± 2.8	5.4 ± 0.2	49.7 ± 3.0	93.4 ± 8.4	14.5 ± 0.5	107.9 ± 8.9
0	–1	–1	283.0 ± 5.3	7.1 ± 0.5	290.1 ± 5.8	239.3 ± 5.6	4.6 ± 0.2	243.8 ± 5.8	43.5 ± 3.5	5.4 ± 0.4	48.8 ± 3.9	107.8 ± 8.9	13.6 ± 0.8	121.3 ± 9.7
0	1	–1	262.3 ± 3.5	7.1 ± 0.3	269.4 ± 3.8	266.4 ± 2.6	5.8 ± 0.4	272.3 ± 2.9	42.0 ± 1.9	4.1 ± 0.3	46.1 ± 2.1	109.6 ± 5.7	12.6 ± 0.4	122.2 ± 6.0
0	–1	1	246.3 ± 12.8	8.4 ± 0.5	254.7 ± 13.2	247.6 ± 3.9	5.4 ± 0.4	253.0 ± 4.3	44.0 ± 2.7	4.9 ± 0.3	49.0 ± 3.0	99.9 ± 6.7	13.1 ± 0.6	113.0 ± 7.3
0	1	1	259.0 ± 3.8	8.9 ± 0.7	267.8 ± 3.4	251.9 ± 7.8	5.6 ± 0.5	257.5 ± 8.2	44.4 ± 2.0	5.4 ± 0.5	49.8 ± 2.5	94.4 ± 7.8	12.9 ± 0.5	107.2 ± 8.2
0	0	0	266.9 ± 22.8	8.4 ± 0.7	275.4 ± 23.5	235.2 ± 12.8	5.5 ± 0.4	240.7 ± 13.1	43.1 ± 4.4	5.4 ± 0.4	48.5 ± 4.8	92.0 ± 8.7	12.0 ± 0.8	104.0 ± 9.4
0	0	0	250.1 ± 7.0	6.4 ± 0.7	256.4 ± 7.6	241.0 ± 8.9	5.1 ± 0.4	246.0 ± 9.3	41.7 ± 2.2	5.2 ± 0.3	46.9 ± 2.5	88.7 ± 7.3	13.1 ± 0.7	101.8 ± 8.0
0	0	0	272.0 ± 15.9	7.3 ± 0.9	279.3 ± 16.8	242.5 ± 9.8	4.5 ± 0.3	247.5 ± 10.2	41.5 ± 2.3	5.0 ± 0.5	46.5 ± 2.8	85.7 ± 6.2	13.4 ± 0.4	99.1 ± 6.6
CV ^{***} [%]			5.7	10.0	5.8	4.4	7.3	4.4	7.0	7.7	7.1	8.3	5.0	7.9

^{*} Level “–1” referred to the lowest parameters values; Level “0”, to medium parameters and level “1”, to the highest parameters; ^{**} Values are means ± standard deviation; $n = 3$; ^{***} Coefficient of variation - precision was also determined based on repeatable centre points; ^a Total content of ellagittannins, ^b Total content of ellagic acid with conjugates, ^c Total content of ETs with ellagic acid and conjugates

Table 5 Analysis of variance results of quadratic model

Fruit	Factor	Acetone concentration (1)	Time (2)	Temperature (3)	1*2	1*3	2*3	Lack of fit	Pure error	Total SS
<i>Rubus idaeus</i> L.	SS ^a	3321.1	183.1	176.2	222.0	1.2	285.6	608.2	300.2	5486.2
	df ^b	1	1	1	1	1	1	3	2	14
	MS ^c	3321.2	183.1	176.2	222.0	1.2	285.6	202.7	150.1	
	F ^d	22.1	1.2	1.2	1.5	0.0	1.9	1.4	0.5	
	p ^e	0.04	0.39	0.39	0.35	0.94	0.30	0.45		
<i>Rubus fruticosus</i>	SS	1175.1	470.0	387.0	970.3	14.1	144.0	513.1	25.5	3609.7
	df	2	2	2	1	1	1	3	2	14
	MS	587.6	235.0	193.3	970.3	14.1	144.0	171.0	12.8	
	F	46.0	18.4	15.1	76.0	1.1	11.3	13.4		
	p	0.02	0.05	0.06	0.01	0.40	0.08	0.07		
<i>Fragaria vesca</i>	SS	154.9	21.1	557.8	0.4	103.0	11.2	90.1	12.1	1513.2
	df	1	1	1	1	1	1	3	2	14
	MS	154.9	21.1	557.8	0.4	103.0	11.2	30.0	6.0	
	F	25.7	3.5	92.6	0.1	17.1	1.9	5.0		
	p	0.04	0.20	0.01	0.81	0.05	0.31	0.17		
<i>Fragaria grandiflora</i>	SS	83.7	24.9	10.7	16.0	1.2	3.1	50.3	2.2	192.9
	df	2	2	2	1	1	1	3	2	14
	MS	41.9	12.4	5.4	16.0	1.2	3.1	16.8	1.1	
	F	37.4	11.1	4.8	14.3	1.1	2.7	15.0		
	p	0.03	0.08	0.17	0.06	0.41	0.24	0.06		

^a Sum of squares, ^b Degrees of freedom, ^c Mean square, ^d F value, ^e p value

efficiency of strawberry extraction, a three-stage extraction of the ellagitannins was used, supported by 15-min sonication (Klimczak and Król 2010). Taking into account the literature data, as well as the time and energy consumption of running the three-stage extraction, the duration of the process was set at 5–15 min.

For three out of the four tested fruits, the ETs concentration was comparable after a 5-min and 15-min sonication. For raspberries, wild strawberries, and strawberries, it was 270, 110, and 50 mg/100 g f.w. (5 min) and 260, 110, and 50 mg/100 g (15 min), respectively. The use of extended duration of sonication under the used extraction conditions has no analytical justification.

The extended duration of sonication was only important for blackberry, but the extraction should be carried out for 15 min to increase efficiency (within the tested range of 5–15 min). During the 5-min three-step extraction from blackberry fruit, 255 mg of ETs per 100 g of fruit was extracted, while at 15 min, this was 270 mg/100 g. This may be due to the presence

of a large amount of pigment compounds in brambles, which have the ability to bind to the ETs (Hager et al. 2008). The extended time of ETs extraction from blackberry can also be associated with the characteristic profile of this fruit, which includes a predominance of the high-molecular-weight compound lambertianin C (Hager et al. 2008; Sójka et al. 2016).

Duration of sonication is a factor influencing labor consumption and cost of extraction. Therefore, it was important to determine the optimal time for the most effective isolation of ETs from plant material.

Interactions

The values of ETs concentrations for individual factors and interactions occurring between them were determined based on an analysis of variance (Tables 4 and 5).

Charts 1–4 (Figs. 6, 7, 8, and 9) show the interactions of factors affecting the efficiency of ETs extraction from the tested fruits. In the case of raspberry, the highest ETs concentration was

Fig. 8 Plane response plots—dependencies between tested parameters and sum of ETs from strawberry fruit

Fig. 9 Plane response plots—dependencies between tested parameters and sum of ETs from wild strawberry fruit

recorded in the highest acetone concentration tested (80%), regardless of the duration of sonication and extraction temperature. The dependence of time and temperature shows that the highest efficiency was achieved using lower temperatures and shorter duration of sonication. Analysis of the interaction of duration and temperature of extraction of ETs from the strawberry fruit indicates that, regardless of the temperature, in a short time the highest efficiency of ETs separation was achieved. The extraction of ETs from wild strawberry looked similar, but raising the extraction temperature produced a lower concentration of the ellagitannins.

The content of ellagitannins with ellagic acid differed in the tested fruits depending on the extraction parameters used. The Pareto diagrams (Fig. 10) and the analysis of the ANOVA variance confirm that acetone concentration was crucial for all fruits ($p < 0.05$ for raspberries, strawberries, blackberries, and wild strawberries). It was the main and most important factor affecting the efficiency of ETs extraction. Statistical analysis showed that in the case of ET extraction from three out of the four tested fruits, regardless of the length of process

and temperature, the concentration of acidified acetone solution had the greatest influence.

For three out of the four tested fruits, the use of extended duration of sonication at lower acetone concentrations was not more effective compared to the use of higher concentration and shorter duration of sonication. The quantities of ellagitannins extracted during the three-stage 5-min sonication were similar to the effects of 15-min sonication. The obtained results are consistent with literature reports, according to which a longer extraction time did not contribute to more efficient isolation of polyphenols from plant tissue (da Silva Pinto et al. 2008). In addition, a shorter duration of sonication reduces the risk of tannin degradation (Mane et al. 2007).

In the case of blackberry, the interaction of acetone with time can be observed. For the longest duration of sonication (15 min) and with the highest concentration of acetone in the studied range (80%), the highest concentration of ellagitannins was reported to be 286 mg/100 g. Perhaps this is due to the fact that the blackberry contains a large number of anthocyanins that bind the ellagitannins, thus hindering their

Fig. 10 Pareto diagram showing the influence of individual parameters or their interaction on the extraction process of ETs from fruits: **a** raspberry, **b** strawberry, **c** blackberry, and **d** wild strawberry

Table 6 Regression coefficient R^2 and experimental and predictable values for the selected model for three-step extraction of ETs in conditions enabling the best extraction of ETs (in the examined range)

Fruit	Acetone concentration [%]	Temperature [°C]	Time [min]	R^2	Predicted value ETs [mg/100 g f.w.]	Observed value
Raspberry	80	20	5	0.834	299.6	290.0
Blackberry	80	20	15	0.851	259.6	262.6
Strawberry	80	20	5	0.728	56.1	58.3
Wild strawberry	80	20	5	0.933	134.0	130.3

extraction (Hager et al. 2008). The temperature turned out to be a negligible factor ($p > 0.05$).

Selection of the Best Conditions for Extraction of ETs

Based on the response surface methodology and the analysis of variance, the best three-stage ETs extraction model supported by sonication (with the initial homogenisation step) from selected fruit of the *Rosaceae* family (in the examined parameters) was determined (Table 6). In order to check the correctness of the model, the extraction was carried out under optimal conditions, and the experimental results were compared with the predicted results.

The obtained sum values of ETs in the selected model for individual fruits are practically the same, which confirms the reliability of the selected models. The high regression coefficient (0.7–0.9) confirms that the variability of the ellagitannin concentration is well described by the presented model.

The paper also indicates the maximum values of the main ellagitannins, characteristic for the tested fruits. According to literature data, the profile of the fruits of the *Rosaceae* family is shaped in the following way: in raspberry and blackberry, the highest concentrations are sanguin H-6 and lambertianin C, while in the wild strawberry and strawberry—agrimoniin (29 and 23 mg/100 g f.w., respectively) (Hager et al. 2008; Bubba et al. 2012; Aaby et al. 2005; Sójka et al. 2016; Klimczak and Król 2010). In wild strawberry, unidentified ellagitannins were also noted, which accounted for more than 50% of the total ETs (60 mg/100 g of fresh weight). In studies carried out to date, it was suggested that this may be an unknown ellagic acid trimer derivative (Bubba et al. 2012).

Conclusions

Extraction of ETs from the fruits matrix is an important element in their correctly identification and quantification. The research clearly showed that homogenisation of fruits is an important element, which allows over 20% more ETs to be obtained and significantly shortens the extraction time. The crushing of the test fruits using a cryogenic mill is therefore not an extensive pre-treatment process.

The extraction can be assisted both by shaking and sonication, resulting in similar concentration of ETs in the extract. The difference in ETs extraction efficiency appeared in the combined techniques: homogenisation plus sonication and homogenisation plus shaking. Higher concentration of extracted ETs was observed for sonication plus homogenisation. Using the RSM, an appropriate ETs extraction model was determined for the four fruits of the *Rosaceae* family. In all cases, the most important factor was the concentration of acetone, while for the blackberry, there was an interaction with the duration of sonication. For raspberry, wild strawberry, and strawberry, the extraction parameters (temperature, duration of sonication, acetone concentration) were set at 20 °C, 5 min, and 80%, respectively. In the case of blackberry, the duration of sonication was changed, which in the best model in tested parameters was 15 min.

Selection of appropriate extraction conditions for ETs allows their quick and effective separation from the tested fruits. Using this method, the optimum efficiency of the extraction system can be developed. This allows the ETs extraction efficiency to be increased, as well as reducing the time and costs of the entire process.

Compliance with Ethical Standards

Conflict of Interest Agnieszka Milczarek declares that she has no conflict of interest. Michał Sójka declares that he has no conflict of interest. Robert Klewicki declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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